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Biodegradation of Organic Pollutants in the Rhizosphere

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I. Introduction

It has been almost one hundred years since Lorenz Hiltner, a professor of agronomy at the Technical College of Munich in Germany, recognized the presence of a distinctive plant root environment and coined the term ''rhizosphere'' (Hiltner, 1904). Since then the significance of this diffuse zone with regard to nutrient cycling, soil structure, disease prevalence, and soil fertility in general has been intensively researched and endlessly debated (Pinton *et al.*, 2001; Weller *et al.*, 2002; Persello-Cartieaux *et al.*, 2003). However, it is only with the development and widespread adoption of molecular and microscopic innovations (Ramos *et al.*, 2000; Bringhurst *et al.*, 2001; Duineveld *et al.*, 2001; Sorensen *et al.*, 2001; Kowalchuk *et al.*, 2002) that biologists have begun to understand this complex environment. It is anticipated that the rational manipulation of the rhizosphere and the many benefits that follow will become real possibilities in the next 5 to 10

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years. One of the major areas for research is the use of microbes and plants for the remediation of polluted soils (Paszczynski and Crawford, 2000; Germida *et al.*, 2002). Here, we critically review the literature concerning the enhanced biodegradation of organic pollutants in the rhizosphere. We put particular emphasis on understanding the mechanisms and the use of this knowledge in the design of rhizoremediation strategies.

II. The Rhizosphere Environment and Microbial Ecology

A. The Rhizosphere Effect

Most studies have reported greater microbial numbers and activities in the rhizosphere in comparison to the nonrhizosphere or bulk soil (i.e., that soil remote from and not influenced by the plant). For example, the presence of an 8-week-old rhizosphere of maize increased the culturable bacterial numbers from circa 6.3 to circa 7.5 log cfu g^{-1} soil and invertase activities at the root–soil interface were *ca.* 2.3 times those in nonplanted soil (Buyer *et al.*, 2002). For barley, rhizosphere-to-soil (R/S) ratios of 3.9 have been reported for microbial activity as determined by measures of leucine incorporation (Soderberg and Baath, 1998). Soil sampled from grassland sites and supporting a wide range of species showed R/S ratios for microbial biomass carbon (determined by fumigation extraction) and fungal biomass (determined by ergosterol concentration) of 3.5 and 3.9, respectively (Joergensen, 2000). Rhizosphere-to-soil values for microbial biomass nitrogen of 2.4 have been recorded for a 15-day-old ryegrass rhizosphere (de Neergaard and Magid, 2001). The reasons for the increase in microbial numbers and activity are many but paramount among them is the significant amount of carbon that leaks from or is actively secreted by plant roots. The soil microflora react to the composition and concentration of these varied rhizodeposits (Table I) and the potential for both general and specific microbial response is great.

The rhizosphere environment is both temporally and spatially dynamic. The likelihood of plant–microbial interaction increases with proximity to the plant root surface and extends even into the root epidermis and the cortical cells of the plant. Thus, a number of cross-sectional subrhizosphere zones or environments can be experimentally or microscopically defined and these are depicted in Fig. 1. The influence of the plant (i.e., to the outer edge of the ectorhizosphere) may extend from 1.5 mm (Toal *et al.*, 2000) to as much as 9 mm (Kandeler *et al.*, 2002) away from the root surface, depending on the parameter

TABLE I

Organic Chemicals Identified in Rhizodeposits of Higher Plants

Rhizodeposit	Mechanism of deposition	Components and compounds
A. Root exudates		
Diffusates	Passively leaked from intact cells	Sugars: glucose, fructose, galactose, maltose, ribose, xylose, rhamnose, arabinose, raffinose, deoxyribose, oligosaccharides
		Amino acids: α-alanine, β-alanine, asparagines, aspartate, cystein, cystine, glutamate, glycine, isoleucine, leucine, methionine, serine, threonine, proline, valine, tryptophan, ornithine, histidine, arginine, homoserine, phenylalanine, γ-aminobutyric acid, α-aminoadipic acid
		Organic acids: citric, oxalic, malic, fumaric, succinic, acetic, butyric, valeric, glycolic, piscidic, formic, aconitic, lactic, pyruvic, glutaric, malonic, aldonic, erythronic, tetronic
		Vitamins: biotin, thiamine, niacin, pantothenate, riboflavin
Excretions	Actively deposited waste products of metabolism	Ethylene
Secretions	Actively deposited to facilitate external processes (e.g., nutrient acquisition)	Enzymes: acid/alkaline phosphatase, invertase, amylase, protease
		Mucilage: polysaccharides and polygalacturonic acid
		Siderophores: 3-epihydroxymugineic acid
		Allelochemicals: various phenolic compounds
		Microbial chemoattractants: flavonoids
B. Root debris	Passively deposited	Sloughed-off root cap cells, cell contents, and, eventually, dead roots

Adapted from Goodwin and Mercer (1983), Crowley (2001), Uren (2001), Dakora and Phillips (2002).

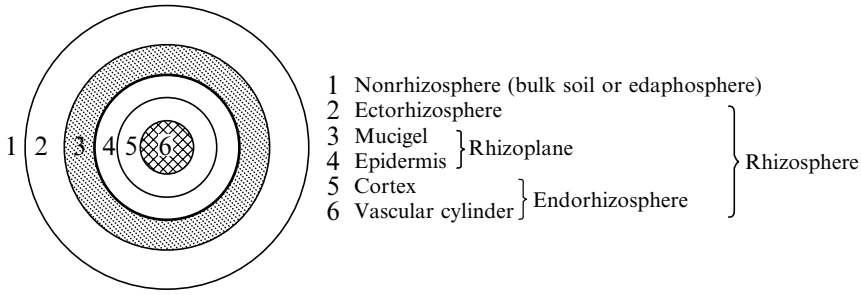

FIG. 1. Diagramatic cross section of a root showing the many rhizosphere zones. The nonrhizosphere, ectorhizosphere, rhizoplane, and endorhizosphere are often defined experimentally. For example, Marilley *et al.* (1998) sampled the various zones as follows. A soil core (6 cm in diameter) enclosing *Lolium perenne* roots was obtained. The nonrhizosphere was defined as the soil remaining after the root system had been picked out from the core. The ectorhizosphere was defined as the soil adhering to the roots after gentle shaking. The rhizoplane and endorhizosphere fractions were obtained after pounding washed (soil-free) roots in the presence of quartz sand.

measured. No doubt, the roots of well-established trees have an even more extensive influence on the microbiology of the surrounding soil. It has been calculated that for densely rooted soil, such as ryegrass pasture, half the maximum distance between roots would be 1.4 mm (assuming even root distribution) and, therefore, the rhizosphere of one root would overlap with that of its nearest neighbor. In these circumstances, the rhizosphere would be a continuum (Toal *et al.*, 2000).

The concentration and composition of rhizodeposition also varies in root long section. For example, diffusate ''hotspots'' have been identified that include root apices of primary and lateral roots and the area behind the root cap (Curl and Truelove, 1986), whereas mucilage secretion and sloughing off of root cap cells is more pronounced at the root tip (Bowen and Rovira, 1991). Rhizodeposition patterns also change with plant age with greatest exudate concentrations in the spermosphere (the immediate environment of the germinating seed) and the young rhizosphere (Gransee and Wittenmayer, 2000). The composition of rhizodeposits continues to change over the life of the plant, culminating in the death of the root and the creation of the root residuesphere (the environment associated with dead and decaying roots).

The probability is that most of the microbes that thrive in the rhizosphere are nutritionally versatile, opportunistic, and fast growing and are able to rapidly respond to the release of soluble carbon sources. More mature roots or areas distant from the growing tips may be

dominated by slow-growing organisms capable of utilizing more complex substrates. The enrichment of native microbes already located where the developing root grows will be further enhanced by: (1) chemotaxis (i.e., more distantly located bacteria and fungi moving into the rhizosphere in response to chemicals released by roots); (2) an ability of some strains to migrate and adhere to root surfaces (the rhizoplane) and thereby to be in a location to utilize soluble exudates, proliferate rapidly, and promote plant growth in a number of ways (Burdman *et al.*, 2000); and (3) directed development of certain species in microbial communities with mutualistic benefits for all the components (e.g., the sequential breakdown of otherwise unavailable complex substrates and the horizontal transfer of catabolic plasmids).

B. Microbial Ecology Methods

It can be predicted that rhizosphere microbial community composition will be highly variable because of the spatial and temporal variability of rhizodeposition. Understanding the dynamics of soil communities has been limited because only a small fraction of soil microorganisms are accessible for study using traditional cultivation-based techniques (Liesack *et al.*, 1997; Janssen *et al.*, 2002). In recent years, with the advent of cultivation-independent nucleic acid–based methods, in particular, fingerprinting techniques based on PCR- or RT-PCR-amplified ribosomal DNA or RNA (e.g., denaturing or temperature gradient gel electrophoresis (DGGE or TGGE), terminal restriction fragment length polymorphism (t-RFLP), and single-strand conformational polymorphism (SSCP) analysis) have made the problems associated with the description of microbial community structure and dynamics in the rhizosphere more tractable. In general, molecular studies have demonstrated reduced diversity in the rhizosphere as compared to bulk soil (Marilley *et al.*, 1998; Duineveld *et al.*, 2001; Gomes *et al.*, 2001; Kowalchuk *et al.*, 2002) with the rhizosphere effect particularly pronounced for young plants (Duineveld *et al.*, 2001; Gomes *et al.*, 2001). Microbial succession, according to plant age and growth stage (Smalla *et al.*, 2001; Baudoin *et al.*, 2002; Marschner *et al.*, 2002; Thirup *et al.*, 2003) and variation in community composition, according to plant root zone (e.g., primary root tips versus sites of lateral root emergence), has also been demonstrated (Yang and Crowley, 2000). Kowalchuk *et al.* (2002) compared the influence of hound's tongue and spear thistle on soil microbial community composition using PCR-DGGE of 16S rDNA fragments. They concluded that the rhizosphere selects for a specific subset of genotypes from the bulk soil and that this effect is

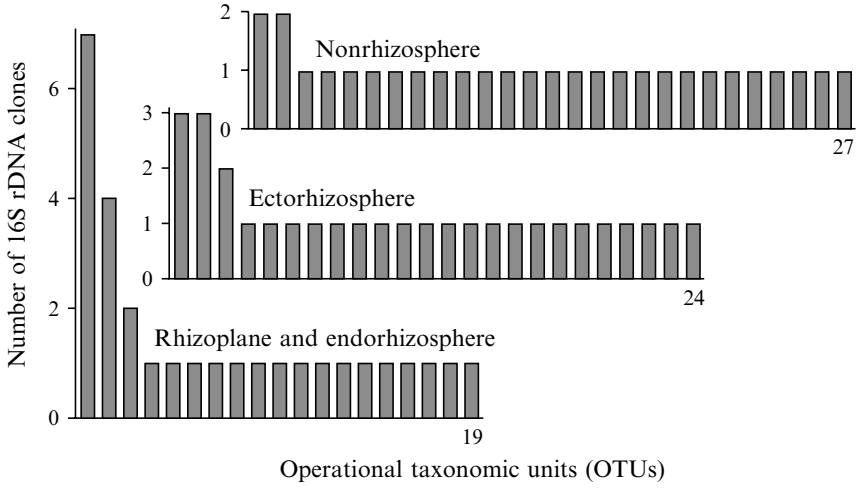

FIG. 2. Microbial community diversity patterns based on operational taxonomic unit (OTU) abundance of bacterial 16S rDNA clones from *Lolium perenne* rhizosphere zones. 16S rDNA gene fragments were amplified using universal bacterial primers from genomic DNA extracted the various rhizosphere zones. A clone library was constructed from the resulting amplicons, 16S rDNA fragments were re-amplified, subjected to restriction analysis and clones with identical patterns classified as an OTU. The distribution of clones among the OTUs is shown (in most abundant to least abundant OTU order). Reproduced with permission from Marilley *et al.* (1998).

plant species specific and highly reproducible. Similarly, Normander and Prosser (2000) conclude that bacteria colonizing the rhizoplane of barley originate from the surrounding soil as opposed to those arriving with the seed. Marilley and Aragno (1999) reported that plant roots of white clover and ryegrass are selective towards the γ-*Proteobacteria*, to the detriment of gram-positive bacteria and the Holophaga–Acidobacterium group. What role bulk soil physico-chemical properties play in determining the background microbial population which is the source of the rhizosphere community is not yet clear (Kowalchuk *et al.*, 2002). Figure 2 shows an example of the dominance pattern of operational taxonomic units (based on the restriction pattern of 16S rDNA cloned fragments) for the nonrhizosphere soil, rhizosphere, and rhizoplane/endorhizosphere of ryegrass. The reasons for the reduction in diversity in the rhizosphere have not been thoroughly investigated. However, it can be speculated that the reduced heterogeneity of carbon resources (i.e., simple sugars in exudates) or the greater potential for cell–cell interaction (and therefore competition) in the rhizosphere is responsible for the observed dominance structure (Zhou *et al.*,

2002). Despite the reduction of microbial diversity in the rhizosphere, cloning and sequencing of amplified 16S rDNA or rRNA (or 18S rRNA for fungi; Smit *et al.*, 1999) fragments has revealed that plant roots support genotypes from a broad phylogenetic range. These include members of the α-, β-, γ-, and δ-*Proteobacteria* and the class Actinobacteria (Chelius and Triplett, 2001; Chow *et al.*, 2002), high-G+C gram-positive bacteria (Smalla *et al.*, 2001), and uncultured *Archaea* (*Crenarchaea* and *Euryarchaea*) (Simon *et al.*, 2000; Chelius and Triplett, 2001).

III. Fate of Organic Pollutants in the Rhizosphere

Organic pollutants may enter the rhizosphere as a consequence of many different processes and at a wide range of concentrations. The pollutant may be present in the soil prior to the introduction of a plant and the establishment of its rhizosphere. This occurs in agriculture when a pesticide is applied either before the seed is sown or the crop emerges or at an industrial site where chemicals have entered the bare soil over a period of time. Conversely, the pollutant is added to a soil that already has an established plant community. This may be due to the conventional agricultural application of, for example, post-emergent pesticides or may result from accidental contamination from a direct spill (acute) or via atmospheric deposition (chronic). Direct spills usually result in the highest pollutant concentrations; for example, total concentrations of 13 different pesticides of up to 19.3 mg g^{-1} have been detected in the topsoil at a pesticide disposal site (Winterlin *et al.*, 1989). On the other hand, roadside plants and soils may take up low concentrations (ng pollutant g^{-1} grass dry weight) of volatile organic compounds generated in exhaust fumes (Binnie *et al.*, 2002). In a recent survey of 191 surface soils sampled away from populated, industrialized, and agrochemical application areas, a mean total polychlorinated biphenyl (PCB) concentration of 5.4 μg g^{-1} was detected (Meijer *et al.*, 2003). Thus, all soils and rhizospheres, even those remote from anthropogenic influence, receive inputs of persistent organic pollutants via long-range atmospheric transport and global distillation.

A. Direct Plant Effects: Uptake and Enzyme Activities

Once in the rhizosphere, the fate of an organic pollutant is governed by many processes, some directly mediated by the plant and others in which the plant has an indirect effect. The indirect effect—principally,

the stimulation of microbial biodegradation—is the main focus of this chapter. But first, we consider how a plant may directly affect organic pollutants.

Plant-mediated attenuation of organic pollutant concentrations in the rhizosphere may be divided into the following four steps: uptake into the transpiration stream, translocation in the plant, transformation in the plant, and transpiration from the plant (volatilization) (Davis *et al.*, 2002; Schroder and Collins, 2002). Once in the above-ground plant tissues, an organic pollutant undergoes oxidation, reduction, and/or hydrolysis and conjugation with glutathione, sugars, or amino acids (Dietz and Schnoor, 2001). Conjugates may be bound to cell wall components or compartmentalized within vacuoles. The enzymatic and biochemical relevance of above-ground plant metabolism to phytoremediation has been reviewed (Macek *et al.*, 2000; Dietz and Schnoor, 2001; Davis *et al.*, 2002; Schroder and Collins, 2002) and is outside the scope of this chapter. However, the plant-mediated processes operating below ground and controlling the rate and extent of pollutant uptake (and therefore removal from the rhizosphere) are relevant and are discussed briefly.

When organic pollutants in the soil water come into contact with plant roots, they may reversibly absorb to root cell wall constituents such as hemicellulose and lignin. Construction of adsorption isotherms in hydroponic solution and comparison of linear adsorption coefficients (termed the root concentration factor, RCF (Briggs *et al.*, 1982)) between pollutants reveals a strong relationship between the hydrophobicity of the pollutant (as represented by log K_{ow}) and its tendency to adsorb to roots (Dietz and Schnoor, 2001). According to this relationship, all organic xenobiotics with a log $K_{ow} > 3$ should be highly adsorbed by roots, resulting in depleted pollutant concentrations in the diffuse ecto rhizosphere and increased concentrations in the endorhizosphere and rhizoplane. Briggs *et al.* (1982) and Burken and Schnoor (1998) further developed the RCF concept to take into account the tendency of plants to remove pollutants from the rhizosphere completely (including the endorhizosphere and rhizoplane). For a range of organic pollutant compounds, these workers determined the transpiration stream concentration factor (TSCF), defined as the pollutant concentration in the transpiration stream divided by the external concentration in solution. Thus a pollutant with a TSCF of 1 will move freely with water into and within the plant, whereas a pollutant with a TSCF of 0 will not be taken up (Aitchinson *et al.*, 2000). Plotting the TSCF against log K_{ow} (Fig. 3) shows the optimum hydrophobicity (Log $K_{ow} \approx 2$) for a chemical to enter the transpiration stream. Very

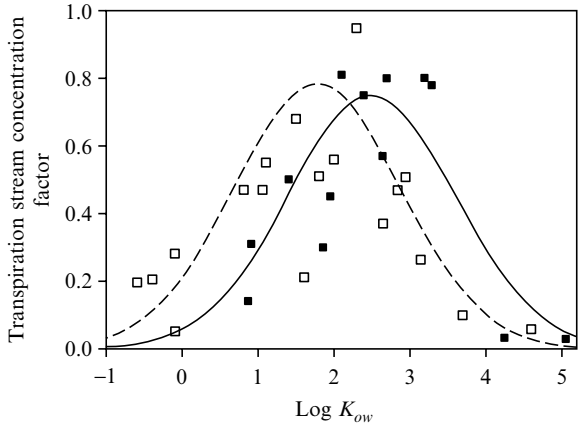

FIG. 3. Vegetative uptake of organic contaminants (transpiration stream concentration factor, TSCF) as a function of hydrophobicity. Briggs *et al.* (1982) experimental data (□) and model (– – –): TSCF = 0.784 exp-[(log K_{ow} − 1.78)^2/2.44]; Burken and Schnoor (1998) experimental data (■) and model (——): TSCF = 0.75 exp-[(log K_{ow} −2.5)^2/2.44]. Reproduced with permission from Aitchinson *et al.* (2000).

hydrophobic (log $K_{ow} > 4$) compounds are not taken up by the plant, presumably because they have such high affinity for root mucigel and membrane lipids that they cannot enter the transpiration stream (Dietz and Schnoor, 2001). On the other hand, in the absence of an active transport protein, hydrophillic (log $K_{ow} < 0$) compounds will not be taken up because they are not sorbed to the root in sufficient quantity to establish a concentration gradient and cannot penetrate root lipid membranes (Dietz and Schnoor, 2001). Log K_{ow} values for some of the pollutant organics discussed in this review are given in Table II.

In the studies described, both the RCF and the TSCF values were determined in a homogeneous hydroponic experiment. However, rhizosphere soil is a multiphase system consisting of four dominant components: colloidal internal and external surfaces, water-soluble humic material, soil solution, and plant roots (Fig. 6). Thus, in the rhizosphere, hydrophobic pollutants also have a high tendency to adsorb to hydrophobic soil surfaces and partition within soluble humic material. In addition, cationic pollutants will be adsorbed to the soil clay fraction. Therefore, to get a truer picture of root–pollutant interactions in the rhizosphere, it is essential to conduct experiments using soil–plant systems.

It should be noted that some pollutants, in particular those containing amino or hydroxyl functional groups, may undergo rapid

TABLE II

RANGE IN LOG K_{ow}S OF SOME ORGANIC POLLUTANTS

Compound	Log K_{ow}
Ethylene glycol	−1.36
Propylene glycol	−0.92
1,4-dioxane	−0.27
Phenol	1.46
Benzene	2.13
Carbofuran (2,3-dihydro-2,3, dimethyl-7-benzofuranylmethylcarbamate)	2.32
3-CBA (3-chlorobenzoate)	2.68
Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine)	2.75
2,4-D (2,4-dichlorophenoxyacetic acid)	2.81
2,4-DCP (2,4-dichlorophenol)	3.06
2,4,5-T (2,4,5-trichlorophenoxyacetic acid)	3.31
Biphenyl	3.98
Anthracene	4.45
Phenanthrene	4.46
Pyrene	4.88
n-Decane	5.01
PCP (pentachlorophenol)	5.12
Chrysene	5.81
Dodecane	6.10
Benzo(a)pyrene	6.13
Arochlor 1248 (polychlorinated biphenyl mix)	6.20
n-Hexadecane	8.25

Data were compiled from the Syracuse Research Corporation Database (*http://esc.syrres.com/interkow/physdemo.htm*) and the U.S. Environmental Protection Agency website (http://www.epa.gov/OGWDW/hfacts.html).

transformation by root-associated or free extracellular enzymes. The outcomes of these are chemically altered and irreversibly bound species (Dietz and Schnoor, 2001). For example, Gramss *et al.* (1999) studied enzymes released from the roots of 12 terrestrial plants and concluded that exudates of certain plant species contain sufficient quantity of peroxidases to contribute to the degradation of natural soil constituents (e.g., humus phenolic groups). Plant (and microbial) peroxidases can also play an important role in the polymerization of

phenolic units and lead to the detoxification or immobilization of pollutants (e.g., the herbicide bentazon (3-isopropyl-1*H*-2,1,3-benzothiadiazine-4(3*H*)-one-2,2-dioxide), chlorinated phenols, trinitrotoluene, and their metabolites) in soil humic material (Kim *et al.*, 1998; Dec and Bollag, 2000; Wang *et al.*, 2002). Thus, the plant can only directly affect pollutant biodegradation in the rhizosphere through the release of root surface–associated or freely diffusible extracellular enzymes. Otherwise, depending on the physico-chemical characteristics of the pollutant (and presumably also the root), the plant may deplete pollutant concentrations in the entire rhizosphere zone either by uptake into the transpiration stream or by concentrating the pollutant in the rhizoplane and endorhizosphere by root sorption. The indirect effect that root sorption has on bioavailability and microbial biodegradation is discussed further in Section IV.C.2.

B. Indirect Plant Effects: Degradation by Rhizosphere Microorganisms

1. *Evidence for the Rhizosphere Effect*

In addition to the direct effects, plants may also indirectly bring about pollutant biodegradation in the rhizosphere. As discussed in Section II, plant roots and their rhizodeposits increase microbial numbers and activity by as much as two orders of magnitude. It is reasonable to assume that this stimulation will enhance biodegradation of organic pollutants in the rhizosphere zone. The first study to test this hypothesis investigated the microbial biodegradation of organophosphate insecticides and was conducted by Hsu and Bartha (1979). The rationale behind the research was as follows: The initial step in the biodegradation of diazinon (*O,O*-diethyl-*O*-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphorothioate) and parathion (*O,O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate) is cometabolic and cometabolism requires the presence of a growth substrate. Plant roots are known to excrete a wide range of potential carbon and energy sources, and, therefore, cometabolic transformation should be favored in the rhizosphere. The authors compared the mineralization rates of diazinon and parathion in the rhizosphere of bush bean with those in nonplanted soils. After 30 days, 12.9 and 17.9% of the added diazinon and parathion had been mineralized, respectively, compared with 5.0 and 7.8% in nonplanted soil. The sterile roots mineralized less than 1.8% of the added ^{14}C, strongly suggesting that the decline in insecticide concentration was not due to metabolism by the plant itself. However, no attempt was made to look for transformation products and it could be argued that the bush bean was responsible for the partial transformation of the insecticides

and that the rhizosphere microorganisms were able to complete the mineralization. Regardless of whether the process was solely a consequence of microbial metabolic activity or due to a sequence involving both plants and microbes, there clearly was a rhizosphere effect.

As shown by the preceding example, it can be difficult to distinguish which process(es) and which of the rhizosphere components make the greater contribution to pollutant biodegradation. This tells us that all plant, microbe, and plant–microbe contributions should be taken into account when making decisions about experimental design and the choice of controls. Of course, the necessary inclusion of nonsterile soil in some of the treatments adds a further level of complexity that challenges experimental design. It is widely accepted that using ^{14}C-labeled compounds is essential in order to gain mass balances in plant–atmosphere–soil microcosm studies (e.g., Fig. 4). However, still greater analytical resolution may be required to understand fully the fate of the chosen pollutant in the experimental system (Siciliano and Germida, 1998b). For example, Ferro *et al.* (1994) investigated the effect of wheatgrass on the fate of pentachlorophenol (PCP). At the end of the 155d experiment, approximately 22% of PCP ^{14}C had been mineralized but 31% of the label remained in the soil and 26% had been taken up by the plant (the remaining was distributed among the volatile organic, evapotranspirational condensate, leachate or unrecovered factors). It is not apparent from this whether the ^{14}C in the plant and soil was parent compound, plant metabolite, or microbial metabolite. Therefore, no definite conclusions could be drawn concerning plant transformation of PCP or whether microbial metabolites of PCP were generated in the rhizosphere.

As pointed out by Cunningham *et al.* (1997), when it comes to pollutant breakdown, plants are at an inherent disadvantage when compared to microorganisms. Plants are autotrophic and, unlike heterotrophic bacteria and fungi, do not require organic carbon as energy substrates. As a result, it is probable that pollutant metabolism by plants will be restricted to a relatively small range of chemical structures. Additionally, plant metabolism usually involves the modification of the pollutant (e.g., hydroxylation, conjugation), whereas metabolism by heterotrophic microorganisms often results in complete mineralization, is linked to growth, and proceeds exponentially (at least *in vitro*). Since the seminal work of Hsu and Bartha (1979), numerous other studies with pesticides and various industrial pollutants have compared degradation in the rhizosphere with that in nonplanted soil and, where rhizosphere-enhanced biodegradation occurred, attributed it to root-stimulated microbial activities. The findings of these studies are summarized in Table III. Since much of the early work has been

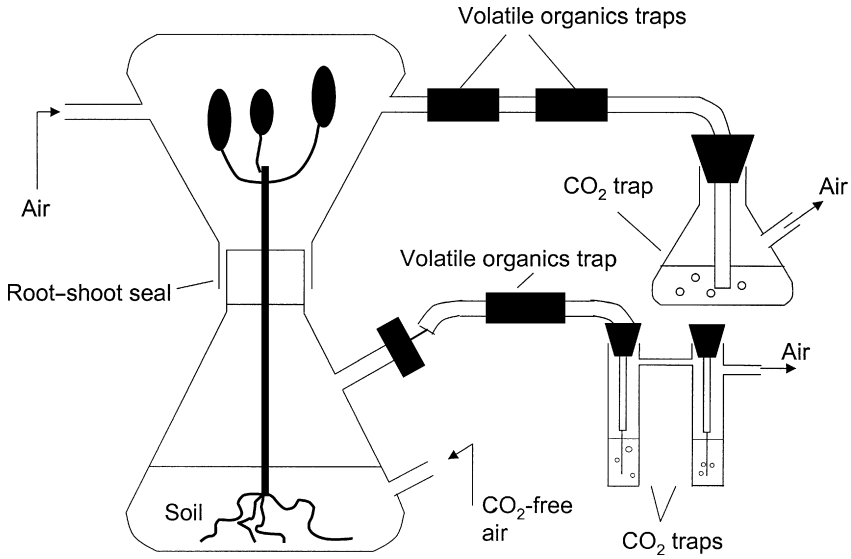

FIG. 4. Example of a microcosm apparatus separating above- and below-ground plant atmospheres to enable continuous monitoring of a ^{14}C-labeled pollutant mineralization and volatilization in intact rhizospheres. CO_2 traps contain 1 M NaOH, organics traps contain activated carbon. Reproduced with permission from Kelley *et al.* (2001).

reviewed previously (Anderson *et al.*, 1993; Anderson *et al.*, 1994; Anderson *et al.*, 1995; Crowley *et al.*, 1997; Siciliano and Germida, 1998b), we report only on those papers published in the last 10 years. As can be seen from Table III, there is overwhelming evidence for a rhizosphere effect: of the 40 studies summarized, 85% report a significant enhancement of biodegradation of a wide range of organic pollutants and involving a large number of plant species.

2. *Methodology*

As well as the diversity of plant species, pollutants, and indeed soils studied, a bewildering assortment of methodologies and experimental designs have been used in order to assess the effect of the rhizosphere on pollutant biodegradation. A number of general observations can be made. First, studies may be categorized according to whether they used an intact or a harvested (destructively sampled) rhizosphere. In intact rhizosphere studies, the plant is present in the experimental system and the fate of the pollutant at any time may be a product of both plant and microbial metabolism. As discussed earlier, it may be

TABLE III

SUMMARY OF RESEARCH REPORTING A RHIZOSPHERE EFFECT ON THE BIODEGRADATION OF ORGANIC POLLUTANTS

Reference	Pollutant(s)	Rhizosphere (I = intact, H = harvested)	Rhizosphere effect
Pesticides and metabolites			
Boyle and Shann, 1995	2,4-D, 2,4,5-T, 2,4-DCP and phenol	[H] Red clover, daisy fleabane, barnyardgrass, fall panicum, early goldenrod, chicory, timothy grass, green foxtail	Mineralization rate and extent increased for 2,4-D and 2,4,5-T in the order non-planted < dicot rhizosphere < monocot rhizosphere. No effect on mineralization of 2,4-DCP and phenol
Boyle and Shann, 1998	2,4,5-T	[H] Timothy grass, red clover, sunflower	Planted soil increased the amount of $^{14}CO_2$ mineralized, but the extent of the increase varied (30 to 163%) with soil type. Percent mineralized increased in the order nonrhizosphere < grass and sunflower < clover
Buyanovsky *et al.*, 1995	Atrazine and carbofuran	[I-field study] Maize	Concentrations in soil 15 days after application 2× higher in nonrhizosphere soil than in planted soil at 0–10 cm depth
Fang *et al.*, 2001	Atrazine	[H] Sudan grass, ryegrass, tall fescue, crested wheatgrass, switchgrass	10–15% mineralization in rhizosphere, 25% mineralization in nonrhizosphere in 120 d
Ferro *et al.*, 1994	PCP	[I] Hycrest crested wheatgrass	22% mineralization in rhizosphere, 6% mineralization in nonrhizosphere in 155 d

Haby and Crowley, 1996	3-CBA	[H–soil–water suspensions] ryegrass	For soil not previously exposed to 3-CBA, degradation in rhizosphere was complete within 8 d, whereas >50% remained after 15 d in nonrhizosphere soil Little difference in biodegradation kinetics for soil previously exposed to 3-CBA
Hoagland *et al.*, 1997	Metolachlor (2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl) acetamide) and alachlor	[I] Maize	50% of the herbicide remaining in nonrhizosphere soil, 13–22% remaining in rhizosphere
Kruger *et al.*, 1997	Atrazine	[H] Summer cypress	Lag time prior to mineralization less for rhizosphere soils than for nonrhizosphere soil
Marchand *et al.*, 2002	Atrazine	[H] Maize	Greater percentage mineralized in soil collected from the rhizosphere of 4-week-old seedlings (61%) compared to nonplanted (48%) soil. Increased rate of mineralization for soil with a prior history of atrazine application
Pai *et al.*, 2001	Mefenoxam ((R)-N-(2,6,dimethylphenyl)-(methoxyacetylamino) propionic acid methyl ester)	[I–potting mix] Common zinnia	78% degraded in rhizosphere, 44% degraded in nonrhizosphere in 21 d
Perkovich *et al.*, 1996	Atrazine	[H] Summer cypress	62% mineralized in rhizosphere, 49% mineralized in nonrhizosphere after 36 d

(*continued*)

TABLE III (*Continued*)

Reference	Pollutant(s)	Rhizosphere (I = intact, H = harvested)	Rhizosphere effect
Piutti *et al.*, 2002	Atrazine	[H] Maize	For soils pretreated with atrazine, mineralization was enhanced (1.45×) in the rhizosphere. No difference for soil with no prior treatment
Reddy and Sethunathan, 1994	*p*-Nitrophenol	[I] Rice	80% degraded in 15 d in (flooded) rhizosphere, 40% degraded in nonrhizosphere
Siciliano and Germida, 1997	2-Chlorobenzoic acid (2-CBA)	[I] Common brome, Dahurian wild rye, intermediate wheatgrass, meadow brome, streambank wheatgrass	Streambank wheatgrass and Dahurian wild rye consistently increased the disappearance of high concentrations ($800\,\mu g\ g^{-1}$) by up to 63%
Zablotowicz *et al.*, 1997	Acifluorfen (5-[2-chloro-4-(trifluoromethyl) phenoxy]-2-nitrobenzoic acid)	[H – soil suspensions] Soybean	Half-lives for parent compound disappearance were 2.5 d in rhizosphere soil and 4.5 d in nonrhizosphere soil
Hydrocarbons			
Banks *et al.*, 1999	Benzo(a)pyrene	[I] Tall fescue	Residual concentrations significantly less in planted soil (56% degraded) compared to nonplanted soil (47% degraded) after 6 months
Binet *et al.*, 2000	3–6 ring PAHs	[I] Ryegrass	81.5% (nonrhizosphere) and 50% (rhizosphere) of freshly added chrysene was extractable after 40 days. Rhizosphere effect less pronounced when the PAHs had been aged in soil for 180 days before planting

Binet *et al.*, 2001	Anthracene	[I] Ryegrass	~33% degraded in rhizosphere, ~11% degraded in nonrhizosphere after 40 d
Chaineau *et al.*, 2000	Fuel oil hydrocarbons (60% saturated C12–C26, 30% aromatic, 10% resins)	[I] Maize	Rhizosphere increased the biodegradation rate of total chloroform-extractable hydrocarbons. GC analysis revealed a higher extent of biodegradation of branched alkanes and naphthenes in the root zone
Fang *et al.*, 2001	Phenanthrene	[H] Sudan grass, ryegrass, tall fescue, crested wheatgrass, switch grass	Mineralization rate of phenanthrene not significantly different between planted and nonplanted soil
Ferro *et al.*, 1994	Phenanthrene	[I] Hycrest crested wheatgrass	No difference in mineralization between planted and nonplanted soil
Ferro *et al.*, 1997	Benzene	[I] Lucerne	Lucerne did not enhance biodegradation
Gunther *et al.*, 1996	C10–C24 N-alkanes, pristane, hexadecane, 3- & 4-ring PAHs	[I] Ryegrass	97% degraded rhizosphere, 82% degraded nonrhizosphere after 22 weeks for aliphatic hydrocarbons. No rhizosphere effect for PAHs
Joner *et al.*, 2001	Anthracene, chrysene, dibenz(a,h)anthracene	[I] Ryegrass and white clover (mixed)	Chrysene and dibenz(a,h)anthracene concentrations lower in planted soil than in nonplanted soil after 8 weeks. PAH loss further enhanced by presence of arbuscular mycorrhiza. No rhizosphere effect for anthracene
Liste and Alexander, 2000a	Pyrene	[I] Oat, lupin, rape, dill, pepper, radish, jack pine, red pine, white pine	All plants tested enhanced degradation (up to 74% degradation in planted soil compared with <40% in unplanted soil)

(*continued*)

TABLE III (*Continued*)

Reference	Pollutant(s)	Rhizosphere (I = intact, H = harvested)	Rhizosphere effect
Miya and Firestone, 2000	Phenanthrene	[I] Slender oat	Biodegradation rate higher in rhizosphere soil (17.5 mg kg^{-1} d^{-1}) compared with nonrhizosphere soil (12.5 mg kg^{-1} d^{-1})
Pichtel and Liskanen, 2001	Diesel range organics (DROs–C10–C36 *n*-alkanes)	[I] mixed grasses (hard fescue, red fescue, ryegrass) and mixed legumes (white clover, green pea)	~89% degradation in rhizosphere, ~52% degradation in nonrhizosphere soil in 150 d
Reilley *et al.*, 1996	Pyrene and anthracene	[I] Tall fescue, lucerne, sudangrass, switchgrass	PAH concentrations were smaller in rhizosphere than in nonrhizosphere soil after 24 weeks (e.g., for anthracene, 1.15 mg kg^{-1} nonrhizosphere, 0.6 mg kg^{-1} lucerne)
Schwab and Banks, 1994	Pyrene	[I] Lucerne, tall fescue, switch grass, sudangrass	2.18% remaining in nonplanted soil versus 0.75–1.48% in planted soil after 4 weeks
Siciliano *et al.*, 2003	Petroleum hydrocarbons (diesel fuel and heavy oil)	[I] Grass or grass and legume mixtures	Rhizosphere soil degraded 38 mg of total petroleum hydrocarbons (TPHs) kg^{-1} $month^{-1}$, nonrhizosphere soil degraded 19 mg TPH kg^{-1} $month^{-1}$
Siciliano *et al.*, 2003	Hexadecane and phenanthrene	[H] Grass or grass and legume mixtures	~7 mg phenanthrene C mineralized g^{-1} soil $week^{-1}$ in nonrhizosphere soil, 13 mg C g^{-1} wk^{-1} mineralized in planted soil; ~10 mg hexadecane C g^{-1} wk^{-1} mineralized in nonrhizosphere, 20 mg C g^{-1} wk^{-1} in rhizosphere
Wetzel *et al.*, 1997	Pyrene and anthracene	[H] Lucerne	No rhizosphere effect

Wiltse *et al.*, 1998	Crude oil petroleum hydrocarbons	[I] Lucerne (20 genotypic clones)	After 12 months, 56 and 46% of total petroleum hydrocarbons degraded in rhizosphere (best-performing genotype) and nonrhizosphere soil, respectively
Yoshitomi and Shann, 2001	Pyrene	[H] Maize	~30% ^{14}C-pyrene mineralized in rhizosphere soil, ~20% in nonrhizosphere in 40 d. Amendment of nonrhizosphere soil with root exudates stimulated mineralization (~36% mineralization in exudate-amended, ~32% in nonamended after 80 d)
Other			
Dzantor and Woolston, 2001	PCBs (arochlor 1248)	[I] Flat pea, reed canarygrass, burr medic	69% of initial PCB recovered in nonrhizosphere soil, 65, 59, and 54% recovered for flat pea, reed canarygrass, and burr medic, respectively, after 100 d. These differences were not significant ($p > 0.05$)
Kelley *et al.*, 2001	1,4-dioxane	[I] Hybrid poplar	Percent dioxane remaining was significantly lower ($p < 0.05$) in planted reactors than in nonplanted controls
Knaebel and Vestal, 1992	Surfactants (dodecyl linear alkylbenzene sulfonate, dodecyl linear alcohol ethoxylate, sodium stearate, dodecyltrimethylammonium chloride)	[I] Soybean and maize	Rhizosphere significantly increased initial first-order rates of mineralization by between 1.1 and 1.9×. The asymptotic amount of $^{14}CO_2$ evolved was not significantly affected

(*continued*)

TABLE III (*Continued*)

Reference	Pollutant(s)	Rhizosphere (I = intact, H = harvested)	Rhizosphere effect
Mehmannavaz *et al.*, 2002	Polychlorinated biphenyls (72 congeners analyzed)	[I] Lucerne	PCB concentrations significantly lower in rhizosphere soil (60.47 mg kg^{-1}) compared to the nonrhizosphere (79.86 mg kg^{-1}) after 270 d
Rice *et al.*, 1997	Ethylene glycol	[H] Tall fescue, ryegrass, Kentucky blue grass, lucerne, birdsfoot trefoil	Enhanced mineralization in rhizosphere soil for all plant species tested
Shupack and Anderson, 2000	Propylene glycol	[H] Lucerne, birdsfoot trefoil, switchgrass, Kentucky bluegrass, ryegrass, sand dropseed, tall fescue	Mineralization rate enhanced in lucerne rhizosphere soil

difficult to untangle the relative contribution of each component. Of course, the plant will also be growing (and therefore transpiring and photosynthesizing) throughout the experiment. Thus, the intact rhizosphere microbial community will receive a continuous input of rhizodeposits and will be bathed in the transpiration stream. In contrast, in harvested rhizosphere experiments, the soil has been removed and the plant is no longer part of the experimental system. Consequently, the rate and extent of microbial degradation will depend on a whole raft of soil antecedent properties (number, activity, and composition of microorganisms present; quality and quantity of rhizodeposition prior to harvest) and the act of harvesting itself (soil disturbance; breakage and subsequent death of roots). Subsequent storage of the soil will also have an impact as the rhizosphere effect may persist for only a short time. As mentioned in Section II, the rhizosphere and its component parts (Fig. 1) are often defined experimentally. For example, some studies define the rhizosphere as the entire volume of soil contained within the pot in which the plant is grown (Marchand *et al.*, 2002); the roots may be either removed or the entire below-ground plant parts (presumably including the rhizoplane and endorhizosphere) incorporated with the soil. In other studies, only the closely adhering soil, harvested by arbitrarily defined root washing, sonication, or brushing, is counted as the rhizosphere (Wieland *et al.*, 2001; Boyle and Shann, 1998). Microscopic examination of soil–root sections may reveal the inaccuracies inherent in many of the soil-harvesting methodologies.

Rhizosphere studies (Table III) can also be categorized according to the method chosen to quantify biodegradation. In general, studies monitor either the disappearance of the parent compound or the evolution of $^{14}CO_2$ from a ^{14}C-labeled pollutant. For the former, usually used for intact rhizosphere studies, the entire plant soil–system is sacrificed on each sampling occasion and a time course therefore depends on setting up and processing a large number of replicates. The pollutant remaining in the soil is extracted using an appropriate solvent, the extract cleaned up and concentrated (if necessary), and the parent compound (and metabolites) determined by gas or liquid chromatography. For the latter type of experiment which uses radiolabeled compounds, often adopted for harvested rhizosphere studies, $^{14}CO_2$ evolution can be monitored continuously (and without destructive sampling) by removal and replacement of CO_2 traps. Quantification of degradation by destructive sampling is clearly more labor-intensive than nondestructive $^{14}CO_2$ trapping. For this reason, pollutant concentrations in planted and nonplanted treatments are usually determined at only

one or two time-points during the experiment (Wiltse *et al.*, 1998; Siciliano and Greer, 2000; Binet *et al.*, 2001); this makes it difficult to assess lag phases and relative biodegradation rates. In contrast, for nondestructive $^{14}CO_2$ trapping, frequent samples can be taken, allowing the construction of comprehensive cumulative mineralization curves (Boyle and Shann, 1995, 1998; Marchand *et al.*, 2002; Piutti *et al.*, 2002). However, the use of ^{14}C-labeled pollutants in mineralization studies is usually restricted to the harvested rhizosphere type of experiment. This is because of problems of photosynthetic re-adsorption of the ^{14}C-label in the closed plant–soil microcosm necessary for quantitative trapping of $^{14}CO_2$. Notwithstanding, a few workers (Hsu and Bartha, 1979; Knaebel and Vestal, 1992; Aitchinson *et al.*, 2000; Kelley *et al.*, 2001) have quantified mineralization in an intact rhizosphere by using microcosms which separate below-ground (soil and root) and above-ground (shoot) atmospheres. An example of such a microcosm apparatus is shown in Fig. 4. Since separation of below- and above-ground atmospheres requires creating a gas-tight seal around the plant shoot, this microcosm design is restricted to larger plant species with robust stems (e.g., soybean, maize, poplar, and bush bean). For more fragile species, Ferro *et al.* (1994) developed a novel high-flow test system without the need for shoot–root atmosphere separation. In this setup, plants are sealed in a glass bell jar and air is drawn through at a rapid flow-rate (1 liter min^{-1}). $^{14}CO_2$ from mineralization of the ^{14}C-pollutant is rapidly diluted by $^{12}CO_2$ in the airstream and negligible amounts of the $^{14}CO_2$ are fixed by photosynthesis.

One final comment should be made regarding the methodology used in rhizosphere biodegradation studies: that is the order in which the plant and pollutant are added. In the intact rhizosphere studies shown in Table III, the plant (as a seed, seedling, or mature plant) is added to soil that either has undergone previous (by minutes to months) contamination or is contaminated at planting. When the sequence is pollutant followed by plant, there is often a rhizoremediation objective.

IV. Mechanisms of Enhanced Biodegradation

Much of the research that reports enhanced pollutant degradation in the rhizosphere can be criticized as descriptive (Cunningham *et al.*, 1997). Increased biodegradation in the presence of a plant root is often attributed to enhanced microbial activity and numbers in the rhizosphere, without any attempt to dissect the interactions between plants and microorganisms and the mechanisms which bring

about increases in contaminant disappearance. A greater mechanistic understanding of plant–microbe interactions in the context of pollutant degradation is important for the rational development and improvement of rhizoremediation strategies. The next section discusses the mechanisms by which plant roots can stimulate microbial biodegradation.

A. Rhizodeposits as Structural Analogs of Organic Pollutants

As discussed in Section II, plant roots release a plethora of chemicals into their root zone. These range from simple sugars and amino acids in exudates to cell structural and cytosolic compounds released through sloughing-off of cells and during root turnover (Table I). It has been noted by many workers (Gilbert and Crowley, 1997; Siciliano and Germida, 1998; Dunning Hotopp and Hausinger, 2001) that some compounds released by rhizodeposition are structurally similar to pollutants or their breakdown intermediates. Such compounds may act as natural analogs of pollutant catabolic pathways. Figure 5 shows examples of structural similarities between pollutants and naturally occurring compounds detected in the rhizosphere. If an analog is released by the plant root, it can influence pollutant biodegradation by acting as: (1) a growth substrate for pollutant-degrading microorganisms; (2) an inducer of the pollutant catabolic pathway; or (3) a substrate for cometabolic degradation of the pollutant.

1. *Growth Substrates*

Rhizodeposit analogs may be used directly as a source of carbon and energy by one or more microbial species that are also capable of degrading the related pollutant. This suggests that a suitable enriched degrader population may exist in the rhizosphere prior to the first appearance of the pollutant. If rhizodeposition was selectively enriching the pristine soil, then the rhizosphere-to-nonrhizosphere ratio (R/S) for pollutant degraders would exceed that for the total heterotrophs. An early example of this comes from the work of Sandmann and Loos (1984), who estimated most probable numbers (MPN) of microorganisms capable of degrading the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in rhizosphere and nonrhizosphere soil and compared R/S values for 2,4-D degraders with those for culturable aerobic heterotrophs. They found that rhizosphere soil sampled from sugar cane with no previous exposure to phenoxyacetic acid herbicides had an enriched 2,4-D degrading population ($R/S_{2,4\text{-}D} = 105$; $R/S_{\text{heterotroph}} = 6$) and suggested that the stimulatory effect was due to the release

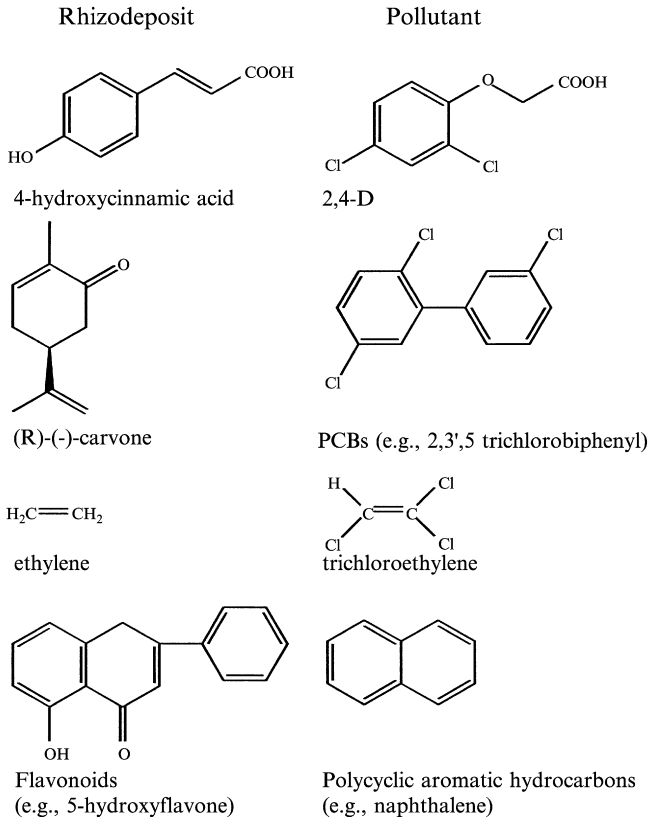

FIG. 5. Structural similarity between putative rhizodeposit compounds and organic pollutants. Compiled from Goodwin and Mercer (1983), Gilbert and Crowley (1997), Siciliano and Germida (1998), and Dunning Hotopp and Hausinger (2001).

of 2,4-D analogs (phenolic compounds) by the sugar cane roots. Subsequently, Dunning Hotopp and Hausinger (2001) postulated that substituted cinnamic acids (Fig. 5) may be natural substrates for 2,4-D degrading enzymes. In a similar example, Fang *et al.* (2001) determined MPNs of phenanthrene degraders and culturable heterotroph counts for soil that was either nonplanted or planted with grass species (tall fescue, ryegrass, sudan grass, and switch grass) and that had no prior history of phenanthrene application. Calculation of R/S values from their data reveals evidence for selective enrichment of phenanthrene degraders in the rhizosphere (R/S_{phen} = 1.6 to 20.0; $R/S_{heterotroph}$ = 1 to 3.2). In the final example, Crowley *et al.* (1996) examined the effect of the bush

bean rhizosphere on the biodegradation of 2,5-dichlorobenzoate (2,5-DCB) by *Pseudomonas fluorescens* strain 2–79 RLD containing plasmid pPB111 encoding genes for 2,5-DCB-1,2-dioxygenase. *P. fluorescens* 2-79 RLD pPB111 inoculated in the rhizosphere degraded 2,5-DCB at a rate greater than in nonplanted soil (time for complete degradation = 2 to 4 d-rhizosphere, 7 to 14 d-nonrhizosphere). The authors noted that there appeared to be a selection pressure for maintenance of plasmid pPB111 in the rhizosphere. This suggests the presence of a 2,5-DCB analog: 14 days after inoculation, 10% of *P. fluorescens* 2-79 RLD cells in the rhizosphere had lost the ability to degrade 2,5-DCB compared to 94% in nonplanted soil.

2. *Inducers*

Crowley *et al.* (1996) suggested that the enhanced biodegradation of 2,5-DCB by *P. fluorescens* 2-79 RLD pPB111 in the bean rhizosphere could be due to the increased induction of 2,5-DCB degrading enzymes by substances in the exudates. Thus, in the second instance, a component of rhizodeposition or an intermediate of rhizodeposit breakdown induces the pollutant degradative pathway. It is well known that expression of many of the bacterial pollutant degradative pathways characterized to date is regulated (McFall *et al.*, 1998; Neilson *et al.*, 1999). For example, expression of the 2,4-D, chlorobenzoate and phenol degradative pathways requires a LysR-type transcriptional activator and the presence of an inducing pathway intermediate, namely, chlorinated or nonchlorinated *cis,cis*-muconate (Filer and Harker, 1997; McFall *et al.*, 1998). Similarly, induction of naphthalene catabolic operons requires the presence of the regulatory protein NahR and the inducer salicylic acid (or derivative), again an intermediate of the pathway (Neilson *et al.*, 1999). It is reasonable to hypothesize that both muconate and salicylate derivatives will be present in the rhizosphere, whether they are produced by the plant directly or whether they are a result of microbial metabolism of aromatic rhizodeposits (De Meyer *et al.*, 1999). *Cis,cis*-muconate is the product of catechol metabolism via the central *ortho*-cleavage pathway (Parsek *et al.*, 1992) and many substituted phenols and aromatic acids which are components of rhizodeposits could be degraded through this route, thus producing substituted-*cis,cis*-muconates. These nonchlorinated muconate analogs could act as inducer molecules for chlorinated pollutant pathways. For example, the LysR-type regulator of chlorocatechol degradation in a *Ralstonia euthropha* can be activated by both chlorinated and nonchlorinated muconate (Ogawa *et al.*, 1999).

3. *Cometabolism*

In the third way in which an analog can influence degradation, a plant exudate acts as a substrate for cometabolic degradation. Cometabolism is defined as the transformation of an organic compound by a microorganism that is unable to use that compound either as a source of energy or an essential nutrient element (Alexander, 1994). An example of a class of organic pollutants that may be transformed cometabolically is the polychlorinated biphenyls (PCBs). PCB biodegradation usually follows the well-characterized biphenyl catabolic pathway (Robinson and Lenn, 1994). However, only mono- and di-chlorinated PCB congeners can be used as a source of carbon for microbial growth, although even then the complete mineralization of these by one bacterial strain is rare. The carbon comes from the least chlorinated ring which is cleaved to produce mono- or di-chlorobenzoate (Robinson and Lenn, 1994; Abraham *et al.*, 2002). The aerobic transformation of more highly chlorinated PCB congeners is by cometabolism in which biphenyl serves as the co-oxidized substrate (co-metabolite) and the inducer of the pathway. For example, Brunner *et al.* (1985) have shown that PCB transformation in soils contaminated with Arochlor 1242 (a mixture of PCBs) can be enhanced by enrichment with the biphenyl analog. The original source of the *bph* genes encoding PCB degradative enzymes has been speculated upon (Abraham *et al.*, 2002) and it is generally thought unlikely that they could have evolved *de novo* in the time since the first manufacture of PCBs in the 1930s. Instead, it has been suggested that plant-derived compounds may be natural substrates or inducers for the *bph* gene-encoded pathway and some evidence has been gathered to support this hypothesis. For example, Fletcher and coworkers (Donnelly *et al.*, 1994; Fletcher *et al.*, 1995; Leigh *et al.*, 2002) have found that several plant compounds (e.g., cinnamic acids, coumarins, flavones, flavanols) and root exudates from mulberry could support the growth of PCB-degrading bacterial isolates. Gilbert and Crowley (1997) discovered that extracts of spearmint could induce PCB biodegradation by *Arthrobacter* sp. strain B1B. The active inducing component of the extract was identified as the monoterpene *l*-carvone (Fig. 5). Although the authors did not look at the production of *l*-carvone in the rhizosphere, they suggested that plants that secrete *l*-carvone (or structurally related monoterpenes) into the rhizosphere may eventually be used to rhizoremediate contaminated soils. However, Master and Mohn (2001) obtained results in contrast to those of Gilbert and Crowley (1997), albeit with a different PCB-degrading bacterium, *Pseudomonas* strain Cam-1. Using *lacZ* as a reporter gene

to quantify the induction of biphenyl dioxygenase, they found that reporter (beta-galactosidase) activity was strongly induced in the presence of biphenyl, but expression levels for cells exposed to terpenoids, soil extracts, and flavenoids were no different to the control (pyruvate-exposed) cells.

B. Genetic Exchange in the Rhizosphere

Many pollutant catabolic genes are located on mobile genetic elements, in particular broad-host-range, self-transmissible plasmids (Top *et al.*, 2002). Thus, horizontal gene transfer via conjugative plasmids probably plays an important role in the adaptation of bacteria to degrade pollutant chemicals in soil and other environments. Indeed, Stuart-Keil *et al.* (1998), in a study of natural gene transfer, provided strong evidence to suggest that a naphthalene catabolic plasmid was involved in the adaptation of a soil microbial community to coal tar contamination. In a another study (van der Meer *et al.*, 1998), *Ralstonia* strain JS705 (isolated from chlorobenzene-contaminated groundwater) was found to have a combination of genes for chlorocatechol degradation and benzene-toluene dioxygenase. This suggests strongly that the chlorobenzene pathway in JS705 arose by horizontal gene transfer and gene recombination between ancestral strains.

For conjugation, in the simplest case, donor and recipient cells form a mating pair and are in intimate contact prior to and during DNA transfer (Dale, 1998). In general, however, soil environments are oligotrophic with low concentrations of organic nutrients that restrict the activity and numbers of microorganisms present. Furthermore, microorganisms in soil may exist in spatial isolation, for example, attached to soil surfaces in thin water films or restricted within aggregates. In fact, species-abundance patterns for unsaturated soils lack a dominance structure, strongly suggesting an absence of prolonged competitive interaction and possibly limited interspecies cell-to-cell contact (Zhou *et al.*, 2002). Thus, processes which depend on cell activity and contact between cells (such as horizontal gene transfer mechanisms) may be restricted in the bulk soil (van Elsas and Bailey, 2002). On the other hand, the rhizosphere has been called a ''hotspot'' for gene transfer activity because nutritional limitations are fewer and the bulk soil contact barriers removed. In addition to the comparatively high levels of nutrients, water flow in the rhizosphere due to transpiration may enhance microbial accumulation and bacteria may be concentrated in biofilms on the rhizoplane, thereby increasing the probability of cell–cell contact (Bianciotto *et al.*, 2001).

Evidence from numerous studies supports the theory that the rhizosphere is indeed a hotspot for gene transfer activity. For example, Schwaner and Kroer (2001) found that the rhizospheres of pea, wheat, and barley were favorable to conjugal plasmid transfer with rate constants up to six orders of magnitude higher than for bulk soil. In addition, the transfer rate depended on the plant species. The authors hypothesized that the difference in transfer ratios between rhizospheres was due to different distribution patterns among cells, presumably with a greater degree of contact between donor and recipient cells in some rhizospheres than others. Sorensen and Jensen (1998) found that the transfer of plasmid RP4 to indigenous bacteria could be detected in the spermosphere and rhizosphere of barley but only in bulk soil if it were amended with nutrients to stimulate microbial proliferation. They suggested that high bacterial activities enhanced plasmid transfer. Experiments by Troxler *et al.* (1997) provide a further example where plasmid transfer occurred in the rhizosphere but was not detectable in nonplanted soil. In this last example, conjugative transfer of chromosomal genes in the rhizosphere of wheat was also demonstrated.

Using inoculated donor and/or recipient cells, many studies have reported the transfer of catabolic conjugative plasmids at detectable frequencies in soil (especially in the selective presence of the target pollutant) and the positive effect this has on pollutant biodegradation rates (De Rore *et al.*, 1994; Top *et al.*, 1998; Dejonghe *et al.*, 2000; de Lipthay *et al.*, 2001). However, to the best of our knowledge, no investigations have been performed to examine transfer rates of pollutant catabolic plasmids in the rhizosphere since studies which explicitly quantify rhizosphere plasmid transfer generally use model plasmids encoding resistance to one or more antibiotics. Crowley *et al.* (1996) interpret enhanced biodegradation of 2,5-dichlorobenzoate (2,5-DCB) in the rhizosphere of bean by *Pseudomonas fluorescens* (pPB111) as a consequence of enhanced transfer of 2,5-DCB catabolic plasmid pPB111 to indigenous recipients. However, comparisons of numbers of donors, recipients, and transconjugants between planted and nonplanted soils were not made. One study (de Lipthay *et al.*, 2001) has examined the transfer of a plasmid carrying the *tfd*A gene (encoding 2,4-D dioxygenase) in the barley ''residuesphere'' (the environment associated with decaying straw) to a phenol-degrading recipient. Interestingly, in the absence of a selective pressure from phenoxyacetic acid, transconjugants appeared after 4 days and were maintained at constant numbers (10^2–10^3 g^{-1} straw dry weight) for the 21-day experiment. No transconjugants were detected in the bulk soil. The suggested

explanation for the difference in numbers of transconjugants between bulk soil and residuesphere is that there was better survival of the recipient strain in the residuesphere. The authors do not speculate on the reasons for the maintenance of foreign DNA in the residuesphere, but perhaps some phenolic breakdown product of lignin selected for plasmid maintenance.

Since the rhizosphere has been shown to be a hotspot for plasmid transfer in general and enhanced pollutant catabolic plasmid transfer has been demonstrated in the residuesphere, it is reasonable to predict that enhanced pollutant catabolic plasmid transfer could also occur in the rhizosphere. If we are to fully understand the mechanisms of enhanced biodegradation in the rhizosphere, future experiments should quantify horizontal gene transfer of pollutant catabolic plasmids in this zone and attempt to understand the conditions that stimulate conjugative transfer.

C. Edaphic Properties and Consequences for Pollutant Biodegradation

In addition to any direct impact on plant growth, rhizosphere microbes influence the chemical and physical properties of the soil. For example, the ''weathering'' of aluminosilicates to form the various clay types is strongly influenced by organic and inorganic acids generated during microbial metabolism (Banfield *et al.*, 1999) and the retention of inorganic ions by highly charged clays may reduce the quantity of soluble plant nutrients. The structure, properties, and composition of humic matter is determined to a large extent by microbial degradative and synthetic activity (Six *et al.*, 2002) and this will impact soil fertility in countless ways. The reciprocal relationship is illustrated further when we consider that soil chemical and physical changes can stimulate or inhibit root activity. Plants, in turn, have a direct impact that can be demonstrated when conducting experiments with sterilized soils. Root hairs stimulate aggregation and root growth generates channels and pores which enhance aeration and facilitate soil water and solute movement. Thus, the rhizosphere environment has different soil physico-chemical properties compared to nonrhizosphere soil and these properties may influence pollutant biodegradation. Two specific examples follow.

1. *Plant-Enhancement of Nutrient Availability*

Plant roots increase the availability of mineral nutrients in their rhizosphere through solubilization by extracellular enzymes (e.g., acid phosphatase; Wasaki *et al.*, 2003) or mobilization (of Fe and P, in

particular) by organic acids, phenolic molecules, and phytosiderophores. Nitrogen availability to microorganisms in the rhizosphere may be enhanced through exudation of nitrogenous macromolecules (e.g., purines and nucleosides). For a detailed recent review of root exudate effects on mineral acquisition, the reader is referred to Dakora and Phillips (2002).

The influence of nutrient availability on pollutant biodegradation is well recognized. According to Alexander (1994), in typical bulk soils, organic carbon is the limiting nutrient for heterotrophic growth. However, introduction of a pollutant that is readily utilizable as a C source may change the situation and render another element as the limiting nutrient. For example, N and P have been shown to be limiting to the biodegradation of oil spills (Roling *et al.*, 2002) and PAHs (Breedveld and Sparrevik, 2000).

As a result of root-mediated processes, N or P may be temporarily more abundant than in bulk soil, but it should be remembered that this is against a backdrop of high concentrations of labile, organic carbon-rich exudates. Thus, microorganisms utilizing a pollutant as a source of carbon in the rhizosphere must compete for N and P with other microorganisms degrading exudate carbon and also with the plant itself (Jensen and Nybroe, 1999; Dakora and Phillips, 2002). It should be remembered that not all pollutants are used as a source of carbon. For example, some microorganisms use the triazine ring of the herbicide atrazine as a source of nitrogen (Mandelbaum *et al.*, 1995) and high concentrations of inorganic nitrogen have been shown to inhibit atrazine mineralization in soil (Abdelhafid *et al.*, 2000). If the rhizosphere is depleted of inorganic nitrogen (through plant uptake) and there is readily utilizable carbon, it can be predicted that atrazine mineralization may be particularly favored. Indeed, the majority of studies examining the influence of the rhizosphere on atrazine mineralization (Table III) report a positive rhizosphere effect, although whether or not this was due to C:N ratio is not discussed.

It is not only competition for macronutrients that can occur in the rhizosphere. For example, Dinkla *et al.* (2001) suggest that the rhizosphere is an iron-limiting environment such that aerobic degradation of hydrocarbons (e.g., toluene) via oxygenases which require iron as a cofactor would be negatively impacted. Therefore, the situation appears to be even more complicated than first supposed. Competition between plants and rhizosphere microorganisms for both macro- and micronutrients and how this, in turn, might affect the degradation of pollutants require elucidation.

2. *Bioavailability in the Rhizosphere—pH, Phytosurfactants, and Enhanced Mass Transfer*

Pollutants in soil tend to exist in an equilibrium between being freely dissolved in the solution phase, in association with water-soluble humic material, and being adsorbed to or partitioned within the three-dimensional soil matrix. As mentioned in Section III.A., the rhizosphere presents an additional sorptive surface, the plant root. Thus, in rhizosphere soils a pollutant may be in dynamic equilibrium between four potential phases (Fig. 6). Bioavailability can be defined as that fraction of a chemical which can interact with a biological system (Robinson and Lenn, 1994), but more complex definitions which consider the dynamics of pollutant availability and the configuration of adsorbed organics are often provided (Burns and Stach, 2002). In the case of the intracellular biodegradation of pollutants by bacteria, the chemical must be available for uptake by the cell, either through passive or active transport. Conventionally, it is believed that cellular uptake requires the pollutant to be freely available in solution (Mihelcic *et al.*, 1993), although other authors suggest that it may be possible for bacteria to take up water-soluble humic material (WSHM)-associated compounds (Shaw *et al.*, 2000) or to directly access sorbed components of molecules (Burns and Stach, 2002). In any scenario, alteration of the partitioning of a pollutant between the root, soil, WSHM, and solution phases (Fig. 6) will have implications for pollutant bioavailability and degradation.

One way in which plant roots can alter pollutant partitioning, and therefore bioavailability, is by mediating changes in the rhizosphere pH. In addition to the production of specific exudates to enhance nutrient mobilization (as mentioned in Section IV.C.1), plants may increase the availability of mineral nutrients for uptake by mediating pH changes in the rhizosphere (Dakora and Phillips, 2002). Briefly, in calcareous soils, plants may exude high concentrations of organic acid anions to lower the rhizosphere pH to that which renders phosphorus and micronutrients (Mn, Fe, Zn) more available. Conversely, some plant species growing in very acid soils can increase their rhizosphere pH by extruding OH^- and HCO_3^- (Dakora and Phillips, 2002). In addition to nutrient limitation conditions, plant-mediated rhizosphere pH changes are also governed by cation/anion balance, influenced by the predominant N source (NO_3^- or NH_4^+) or responses to elemental toxicity. Regardless of the mechanism, plant-induced pH change in the rhizosphere, which may be by as much as two pH units (Brimecombe *et al.*, 2001), will have consequences for pollutant bioavailability. In particular for ionizable compounds, sorption is largely dependent on

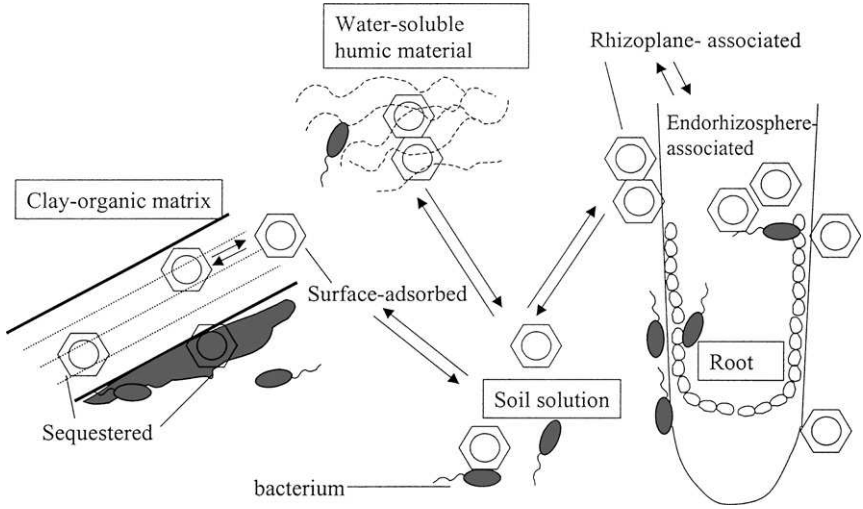

FIG. 6. Pollutant partitioning between four phases (root, clay–organic matrix, water-soluble humic material, and soil solution) in the rhizosphere. Pollutant in the root-phase may be rhizoplane-associated or endorhizosphere-associated whereas pollutant in the clay–organic matrix phase may be sequestered within or adsorbed to the outside surface of the matrix.

the pH-dependent dissociation of the ionizable group: the nondissociated neutral species are more hydrophobic and thus more highly sorbed than their ionized counterparts (Schellenberg *et al.*, 1984; Gundersen *et al.*, 1997). In addition to the pH-dependent ionization of the pollutant, soil humic substances contain many different acidic functional groups. Thus, if the pH is lowered as a result of rhizosphere acidification, humic acids will become protonated and more hydrophobic (Gundersen *et al.*, 1997). This shift may also affect the sorption of nonionizable pollutants.

The second way in which plant roots alter the bioavailability of a pollutant is through the production of phytosurfactants. For example, mucilage from maize, wheat, and lupin has been shown to contain powerful surfactants (Read and Gregory, 1997; Read *et al.*, 2003). Surfactants have also been reported to be released in large quantities from decaying roots (Dormaar, 1969). Surfactants are organic molecules that possess a hydrophilic part and a hydrophobic part and, as a result, tend to concentrate at interfaces (e.g., air–water) and reduce surface tension. It is suggested that surfactants in the mucigel help to maintain root–soil contact during periods of soil drying (Read and Gregory,

1997). An additional property of surfactants is that, above a certain concentration (the critical micelle concentration, CMC), surfactants form micelles with a hydrophilic outside and a hydrophobic shell. By lowering surface tension and the formation of micelles, surfactants can increase the bioavailability of hydrophobic xenobiotics by aiding their dispersion and increasing their apparent concentration in the aqueous phase. The effect of surfactants on pollutant bioavailability has been reviewed by Volkering *et al.* (1998); however, studies with phytogenic surfactants are lacking. One of the few (Soeder *et al.*, 1996) has shown that at concentrations above the CMC, the phytosurfactants soya lecithin and quillaya saponin can completely solubilize phenanthrene and fluoranthene. In shaken batch cultures, the rate of PAH biodegradation by soil bacterial isolates was increased by two-fold in the presence of soya lecithin. The authors conclude that some phytogenic surfactants might improve PAH bioavailability in the rhizosphere.

The soil three-dimensional matrix contains a series of voids connected by narrow, convoluted, and interlocking channels. There is much evidence to show that pollutants may diffuse into soil organic matter and mineral pore networks and become physically segregated from bacterial species capable of biodegrading them (Steinberg *et al.*, 1987; Hatzinger and Alexander, 1995; Pignatello and Xing, 1996; White *et al.*, 1997). This process has been termed sequestration (Alexander, 1995). For a sequestered chemical to become bioavailable, it must diffuse (travel along a concentration gradient) to a soil region containing a bacterium capable of biodegradation. Diffusion is a slow process, dependent on continuous water-filled channels and even then retarded by sorption to nanopore surfaces (Pignatello and Xing, 1996). Nevertheless, all plants exposed to the atmosphere consume water and it has been estimated that a C-3 plant consumes approximately 500 ml of water per gram of net harvestable dry matter, whereas C-4 plants use about 250 ml per gram (Davis *et al.*, 2002). Thus, a significant volume of water flows into and through the rhizosphere to the plant due to the transpiration stream and this would result in the accelerated transport of pollutants dissolved in mobile soil water to the root surface via convection. Convection of pollutants in mobile water toward plant roots would result in a reduction of pollutant concentration in mobile water of soil on the periphery of the rhizosphere and steeper concentration gradients between mobile and nonmobile water regions in nanopores. Establishment of such concentration gradients would lead to enhanced diffusion between mobile and nonmobile water zones and even render previously sequestered pollutants bioavailable (Pignatello and Xing, 1996).

In summary, the following situation can be envisaged. A pollutant in the rhizosphere undergoes transport toward the plant root by mass flow in the transpiration stream. Retardation of transport by sorptive interactions between the pollutant and the soil clays and humates takes place but may be reduced in the rhizosphere due to root-induced pH changes or the production of phytosurfactants. When the pollutant reaches the plant root, if it is hydrophobic it will become concentrated on the rhizoplane and endorhizosphere by partitioning into the mucigel and cell membranes, and by adsorption to cell wall constituents (Section III.A). The presence, concentration, and bioavailability of mucigel-partitioned or plant-cell wall sorbed pollutants has just begun to be investigated using recombinant bacterial biosensors (Casavant *et al.*, 2003).

V. Specific and Nonspecific Interactions between Plant and Microbe

The preceding section outlines mechanisms by which the plant root can enhance microbial biodegradation of pollutants in the rhizosphere. Walton *et al.* (1994) were the first to suggest that enhanced biodegradation in the rhizosphere was not merely a fortuitous coincidence arising from the plant root exuding inducers or cometabolites of a pollutant pathway. On the contrary, they proposed that pollutant degradation results from a specific plant–microbe–toxicant interaction which may be an extension of the plants' defenses against phytotoxic chemicals. Their argument (see also Siciliano and Germida, 1998) is that the protection given by the microbial community to plants against toxicants must have co-evolved to the benefit of both the plant and the microorganism. Whilst the plant benefits from the detoxification of pollutant compounds, the microbial community receives nutritional benefit in the form of rhizodeposition. Walton *et al.* (1994) further suggest that even in the absence of a toxicant, exudation maintains the rhizosphere microbial population. However, if the plant senses a toxic stress, it increases exudation or changes the composition of its exudates to stimulate the appropriate components of the microbial community. In fact, Walton *et al.* (1994) envisage a specific plant–microbe interaction akin to the legume–rhizobia symbiosis whereby plants produce isoflavone signaling molecules in their exudates which induce transcription of nodulation (*nod*) genes (Dakora and Phillips, 2002). It is likely that such signaling is exudate-mediated since exudate quality and quantity can change rapidly in response to plant stress (Siciliano and Germida, 1998).

If such a specific interaction does exist, it begs the question: What was the selective pressure responsible for its co-evolution? It is argued

that the time since the widespread anthropogenic pollution of soil by pollutants is not sufficient for this sort of interaction between plant and microorganism to have evolved. However, a role for allelopathic chemicals or microbial phytotoxins has been postulated (Walton *et al.*, 1994) and the similarity between the structures of these two chemical classes and root exudates has been noted (Siciliano and Germida, 1998).

As pointed out by Siciliano and Germida (1998), if specific interactions determined by the presence of the pollutant are prevalent, then there should be little stimulation of pollutant-degrading microorganisms in its absence. In an attempt to investigate this possibility, Siciliano and coworkers (Siciliano *et al.*, 2001; Siciliano *et al.*, 2003) used probes for genes involved in hydrocarbon degradation (alkane monooxygenase (*alk*B), naphthalene dioxygenase (*ndo*B), catechol-2,3-dioxygenase (*xyl*E), and nitroaromatic degradation (2-nitrotoluene reductase (*ntd*Aa), nitrotoluene monooxygenase (*ntn*M)) in colony and community DNA hybridizations in order to monitor numbers of pollutant degraders in plant–soil–contaminant systems. They hypothesized that the selection pressure exerted by the plant root on rhizosphere populations would be at a maximum in the endorhizosphere and, therefore, if plants do select for catabolic genotypes, then the effect would be most obvious in this zone. Accordingly, they compared genotype prevalence (e.g., probe-positive cells expressed as a percent of the total culturable heterotrophs) between endorhizosphere and bulk soil collected from hydrocarbon- and nitroaromatic-contaminated sites. For the hydrocarbon-contaminated site, *alk*B genes were 10 times more prevalent in the culturable bacteria extracted from the endorhizosphere of mixed grasses and legumes than that from bulk soil. Similarly, *ntd*Aa and *ntn*M genes were 7 to 14 times more prevalent in endophytic bacteria for the nitroaromatic site. The authors argue that their results provide evidence for some as yet unknown, plant-driven process since the enrichment of catabolic endophytes was both contaminant and plant species dependent. For example, *alk*B-positive bacteria were enriched in the endorhizosphere of tall fescue whereas *ndo*B positive bacteria were not. The enrichment in *alk*B-positive bacteria was more pronounced in the endorhizosphere of tall fescue than of rose clover. In addition, numbers of *alk*B- or *ndo*B-positive endophytes of tall fescue were correlated with creosote concentration but not with numbers of *alk*B-positive bacteria in the bulk soil. However, the authors acknowledge that these results can be explained by an alternative process, specifically, that the prevalence of catabolic genotypes in the endorhizosphere may increase due to contaminant flux

through this zone, presumably causing plant-dependent gradients in contaminant concentration and differences in bioavailability.

The previous example illustrates the difficulties in trying to gather experimental evidence to support the existence of a specific plant–microbe–toxicant interaction. Whether or not the plant produced a specific compound in response to soil contamination that enhanced degrader numbers or degrader numbers were increased as a result of direct enrichment and in response to the presence of the contaminant cannot be determined. It follows that enumeration of higher numbers of pollutant degraders in the rhizosphere of plants growing in contaminated soil compared to noncontaminated soil cannot demostrate a specific effect. Even if enrichment in degrader numbers after soil contamination is more pronounced in the rhizosphere than in bulk soil, this can also be explained by nonspecific mechanisms such as local differences in pollutant or mineral nutrient availability.

The alternative to the operation of a specific plant–microbe–toxicant interaction is, of course, that the interaction is nonspecific. A nonspecific interaction would be indicated when plants secrete an inducing chemical or cometabolite or maintain a high population of competent catabolic microorganisms regardless of whether or not there is a xenobiotic present in the rhizosphere. Studies demonstrating enhanced pollutant biodegradation using a harvested rhizosphere (see Table 3) are supportive of a nonspecific interaction; after all, the plant is no longer part of the experimental system. So, the rate and extent of biodegradation in the harvested rhizosphere will depend on antecedent properties and not on any direct microbial response to the presence of the plant (section III.B.2). Despite this evidence, it remains a tantalizing possibility that enhanced degradation in the rhizosphere may be due to a combination of specific signals and nonspecific interactions.

Perhaps the best way to unravel the specific/nonspecific interaction problem is first to elucidate the mechanism of enhanced biodegradation, then to ascertain whether the mechanism occurs as a result of a specific interaction. For example, the initial step would be to demonstrate that a plant is able to bring about enhanced degradation in the rhizosphere because it produces a compound that induces a xenobiotic catabolic pathway (as discussed in section IV.A.2). The second step would be to compare the composition of root exudates collected from plants grown in contaminated and noncontaminated soil and look for increased amounts of the inducer in the exudates from the contaminated soil. With advances in our understanding of the regulation of pollutant pathways and the tools to quantify gene expression (e.g., transcriptionally fused reporters; Neilson *et al.*, 1999), coupled

with the recent application of analytical techniques to environmental chemistry (e.g., LC–MS), such investigations will become more possible.

VI. Stimulating and Initiating Rhizosphere Activity

Combinations of plants and microorganisms have been tested in laboratory experiments for their capacity either to accelerate or initiate the degradation of chemicals in soil. As previously indicated (Section III), the individual and combined impacts of plants and microbes on soil pollutants are numerous and are often indirect and difficult to disentangle. Nonetheless, many attempts have been made to manipulate plant–microbe interactions in the rhizosphere with the object of enhancing pollutant breakdown and the ultimate aim of cleaning up contaminated land. Two general approaches have been adopted: (1) rhizostimulation—the establishment of plants in contaminated soil in an attempt to create a root environment that will stimulate the existing microorganisms to degrade the target pollutant(s); and (2) rhizoaugmentation—the addition of known pollutant-degrading microorganisms to soil with the intention that they become associated with the rhizospheres of either native or introduced plants.

A. Rhizostimulation

Rhizostimulation assumes that appropriate microorganisms already exist in the soil but are not able to degrade the target pollutant at the required rate. This limitation may be a consequence of suboptimal pH, limited availability of inorganic nutrients, absence of organic cosubstrates, compaction and anaerobiosis, and so on. Established bioremediation strategies recognize these constraints and include plowing, irrigation, fertilization, manuring, and liming (Burns *et al.*, 1996; Ka *et al.*, 2001; Margesin and Schinner, 2001; Li *et al.*, 2002; Chaineau *et al.*, 2003); some or all of these will stimulate the indigenous microflora (McGhee and Burns, 1995; Ka *et al.*, 2001; Margesin and Schinner, 2001) and accelerate degradation. From time to time, the limiting intrinsic factor may be more subtle and one such possibility concerns iron deficiency. Dinkla *et al.* (2001), using a transconjugant rhizosphere *Pseudomonas putida* WCS358 containing the TOL plasmid, showed that iron limitation decreased the activities of some of the iron-containing oxidases involved in toluene degradation. On the other hand, the availability of citrate and other plant root exudates may enhance hydrocarbon degraders in iron-deficient soils (Dinkla and

Janssen, 2003). In rhizostimulation (in contrast to conventional biostimulation of the bulk soil), either the native plant species are exploited or new ones are sown which are suited to the soil type and climate and are resistant to the pollutants in question. Where sufficient knowledge exists regarding the pollutant degradative pathway or the constraint to its biodegradation in a particular soil, plant species may be screened for their ability to exude particularly high concentrations of stimulatory compounds (cometabolites, phytosurfactants, inducers of catabolic enzymes, degradative enzymes). Confidence in the potential for the manipulation of microbial communities with plants is increased by the long-established relationship between root exudates and biological control (Chernin and Chet, 2002).

Liste and Alexander (1999) screened a number of plant species grown in quartz sand for their capacity to stimulate phenanthrene degradation in soil. They reported that sunflower and soybean roots released phenols and that this property might be important in stimulating PAH degraders. Liste and Alexander (2000b) subsequently demonstrated that developing roots facilitate the transport of hydrophobic PAHs into the rhizosphere, resulting in a temporary increase in concentration prior to degradation. Thus, plant transpiration depletes the bulk soil of pollutants and increases the concentration of inducers and pollutant substrates in the degradative rhizosphere. Pyrene degradation in the rhizosphere was examined using nine different plant species and all reduced concentrations (by as much as 74%) in comparison with the unplanted soil (Liste and Alexander, 2000a). Some pine tree species even stimulated the breakdown of the Soxhlet extracted fraction which is generally considered less bioavailable than the butanol-extractable component.

A few notable field studies are based on the biostimulation approach. One example, Siciliano *et al.* (2003), stated that the mineralization of hexadecane and phenanthrene was greater in planted than in nonplanted soil and that the level of appropriate catabolic genes was higher. Interestingly, tall fescue increased naphthalene mineralization but rose clover had the opposite effect. In a second example, Robinson *et al.* (2003) reported on a major study which measured the ability of tall fescue to remediate a soil contaminated with creosote. After 36 months, fescue grass increased the degradation of the three-ringed (acenaphthene and fluorene) and four-ringed (fluoranthene, pyrene, and crysene) PAHs in comparison to the nonvegetated plots. Rhizosphere degradation of acenapthene, fluoranthene, and pyrene was corroborated using laboratory microcosms and a doubling of pyrene and crysene breakdown took place in rhizosphere soils. The degradation was

confirmed as a rhizosphere effect since no PAHs or their metabolites were detected in the plant.

There are numerous abiotic factors that may reduce degradation in soil (see preceding), including aeration, compaction, pH, and hydration, and these may be ameliorated prior to sowing or overcome during sowing and the subsequent growth of the plants. Thus, the processes of planting and irrigation will, in themselves, lead to a reduction in the constraints to microbial activity. Subsequently, penetrating roots will aerate the soil and bring about pH changes that will solubilize some inorganic nutrients (Dakora and Phillips, 2002). These effects are in addition to the principal contribution made by the plant roots as a result of the release of a variety of carbon sources that will stimulate microbial activities.

B. Rhizoaugmentation

Rhizoaugmentation involves introducing microbes into the soil with the aim of their becoming associated with the rhizosphere of either native plants or introduced plant species. The bacteria and fungi have been shown previously, in laboratory experiments and carefully thought-out microcosms (Burns, 1988; Angle *et al.*, 1996; Schmidt and Scow, 1996), to be capable of catabolizing or transforming the target compound(s) in the presence of soil. However, additional qualities are required of a successful inoculant, most obviously, that it is competitive with the native microflora and survives and proliferates to become a component of the rhizosphere community (i.e., it is ''rhizosphere competent''). This is achieved by changing the numbers, composition, or spatial arrangement of the indigenous microbial community (e.g., the inoculant integrates into the indigenous population) such that the right mix of strains and processes are stimulated. If the inoculant survives and expresses its desired degrading properties, significant reductions in pollutant concentration can be measured. Often, however, the basic catabolic requirement is satisfied but the necessity for rhizosphere competence is ignored, resulting in the rapid decline of the numbers of the introduced strain (Young and Burns, 1993; van Veen *et al.*, 1997). This results in a low amount of degradation and can only be satisfactory if the degradative potential is carried on a transmissible plasmid which is passed rapidly to one or more of the indigenous species (see Section IV.B). Some of the characteristics of an ideal rhizoremediation inoculant are summarized in Table 4.

If plants are introduced to the polluted site at the same time as the carefully selected degraders, the development of a microbiologically

TABLE IV

SOME PROPERTIES OF AN IDEAL RHIZOREMEDIATION INOCULANT SPECIES OR MICROBIAL COMMUNITY

- Has rhizosphere ''competence'' genes
- Does not disrupt beneficial microbial processes
- Can degrade a range of target xenobiotics at both high and low concentrations
- Is resistant to other organic and heavy metal pollutants
- Displays chemotaxis—locates and moves toward pollutant ''hotspots''
- Increases bioavailability by producing surfactants or can access phytosurfactant solubilized pollutants
- Functions as a donor in horizontal transfer of catabolic plasmids
- Can be detected and tracked once released
- Serves as a reporter of contaminant concentrations and their location
- Produces plant growth-promoting compounds

Adapted from Burns and Stach (2002).

defined rhizosphere may best be ensured by treating seeds (Pfender, 1996) or the roots of pot-grown plants (Miller and Dyer, 2002) with microbial inocula prior to sowing or planting (bacterization). This guarantees high initial densities and preemptive colonization of the root and may counteract competition from the resident microflora. There are well-developed inoculant technologies and many successful examples from the exploitation of the two highly integrated rhizosphere symbioses: mycorrhiza and rhizobia.

C. DEFINED PLANT–MICROBE COMBINATIONS

It is implicit in the options and examples previously described (Sections VI.A. and VI.B.) that there are some plant and microbial combinations that have a synergistic relationship in which the product of their interaction is of greater value to the bioremediation process than the value of the individual components. The possibility of the existence of a specific plant–microbe interaction has already been discussed (Section V). The mutual benefit is realized by microbes responding to plant signals and developing in an environment with pollutant carbon and energy sources while the plant benefits from the destruction of potentially toxic chemicals and the many consequences of enhanced microbial activity. An appreciation of this synergy is demonstrated in the increasing number of remediation

experiments which involve carefully selected and screened plant–microbe pairs.

In a laboratory experiment, Pfender (1996) planted millet seeds coated with *Pseudomonas* strain SR3 in a soil contaminated with PCP. The bacterium reduced PCP concentration from 175 mg/kg to 3 mg/kg in 4 weeks and thereby protected the growing plants from phytotoxicity. In the absence of the inoculum, millet grew poorly and accumulated PCP in root tissues. However, in this instance, there was no obvious rhizosphere stimulus as nonplanted soils inoculated with *Pseudomonas* strain SR3 showed the same amount and rate of PCP degradation.

Miller and Dyer (2002) inoculated the roots of crested wheatgrass seedlings with an unidentified microbial consortium from a PCP-contaminated soil. They reported that the seedlings were tolerant to PCP and three times more PCP was mineralized in comparison with soil containing noninoculated seedlings. The importance of the plant was confirmed when root exudates of wheatgrass were shown to stimulate the degradation of PCP by the consortium 100-fold when added to growth media.

In a complicated series of experiments by Siciliano and Germida, (1997 and 1998), the capacity of a large number of plant–microbe associations to degrade mixtures of chlorinated benzoic acids (CBA) was investigated. CBAs arise from the degradation of PCBs and many chlorinated herbicides and are common soil pollutants. Using a total of 16 forage grasses and three known CBA degraders (*Ps. aeruginosa* R75, *Ps. savastonoi* CB 35, *Alacaligenes* sp. BR60), many combinations were found that significantly reduced soil concentrations of 2,3-dichlorobenzoic acid, 2,5-dichlorobenzoic acid, and 3-chlorobenzoic acid. The authors recommended seed coating as a better approach than adding the inoculant directly to soil and highlighted the complexity of the system in relation to pollutant concentration, distribution, and bioavailability. Drawing on their own work and that of others, they pointed out the likely importance of the composition of the indigenous population prior to introducing the plant and the need for more than one type of inoculant species, including those that are plant growth-promoting rhizobacteria.

PAHs are a second group of compounds that are suitable targets for rhizoremediation because not only are they persistent and common toxic pollutants (Wilcke, 2000; Samanta *et al.*, 2002), but they are also less mobile than many other organics and are retained in the soil surface layers accessible to plant roots. To date, most of the research has concentrated on the low ring number PAHs such as naphthalene,

pyrene, benzapyrene, and phenanthrene (Smith *et al.*, 1999; Stach and Burns, 2002; Table 3). The necessary extracellular depolymerization of compounds, such as coronene, benzo-perylene, and indenopyrene, to prepare them for uptake and degradation by suitable bacteria and fungi is a major challenge for bioremediationists. Kuiper *et al.* (2001) isolated naphthalene-degrading pseudomonads from the rhizosphere of native grasses growing in a highly contaminated soil. They then selected one strain, *Pseudomonas putida* PCL-1444, that had superior annual ryegrass root-colonizing qualities. This microbe reduced the toxicity of naphthalene to the grass and, over a period of 5 days, the concentration of the PAH declined by 99%. A subsequent publication (Kuiper *et al.*, 2002) identified glucose and succinic acid as the annual ryegrass root exudates principally responsible for the increased expression of the naphthalene degradation pathway in *P. putida* PCL-1444. Both the upper and lower pathways were induced by salicylic acid, an intermediate in naphthalene degradation. Another potential advantage of this particular plant–microbe pair (but, presumably, most others) was the ability of the growing plant root system to penetrate physical barriers and transport the associated inoculant to the more distant and otherwise inaccessible soil. In the absence of a strong and specific chemotactic response, this is an important way of distributing degradative microbes in what would otherwise be inaccessible soil zones possibly containing hotspots of the target pollutant. Recently, Olsen *et al.* (2003) looked at mulberry trees that had been growing in PAH-contaminated land for many years. Fifteen-year-old trees had extensive root systems penetrating up to 2 m into soil. This, and the knowledge that mulberry roots produce flavanoids capable of stimulating pollutant degradation (Donnelly *et al.*, 1994; Fletcher *et al.*, 1995; Leigh *et al.*, 2002), suggests that even subsurface soils may be susceptible to rhizoremediation.

Brazil *et al.* (1995) recognized the importance of root colonization in any successful rhizoremediation strategy and genetically engineered a rhizosphere-competent *Pseudomonas fluorescens* strain (F113pcb) to degrade polychlorinated biphenyls. This strain was introduced on sugar beet seeds prior to sowing in a nonsterile soil microcosm and shown to colonize the developing roots as effectively as the wild type. Using a *lacZ* reporter fusion, the researchers showed that *Bph* genes producing 2,3-dihydroxybiphenyl 1,2-dioxygenease were expressed by the inoculant in the rhizosphere.

Yee *et al.* (1998) inserted the *tomA* (toluene *o*-monooxygenase) genes from *Burkholderia cepacia* $PR1_{23}$ into *P. fluorescens* and coated wheat seeds with the recombinant bacterium. *P. fluorescens* established itself

in the rhizosphere, constitutively expressed the monooxygenase, and degraded 63% of the initial trichloroethylene (TCE) in 4 days. The appropriate negative controls showed only 9% degradation. Shim *et al.* (2000) also engineered indigenous rhizosphere bacteria (originally from poplar) to express constitutively toluene *o*-monooxygenase from another *B. cepacia* strain, G4. The recombinants colonized the rhizosphere at high densities (up to 3.1×10^5 CFU/cm root) and retained their abilities to express the monooxygenase for the 29-day duration of the experiment.

Poplar, a plant that is fast growing, deep rooting, and which has a high transpiration rate (Shim *et al.*, 2000), was also used by Tesar *et al.* (2002) in an outdoor pot experiment with a view to identifying the clone that best stimulated a diesel-degrading rhizosphere. One clone (Brandaris) grew well in diesel-contaminated soils when compared with 15 other clones. The authors then isolated a number of strains from the rhizosphere which utilized *n*-alkanes as carbon sources and contained genes encoding the key enzyme alkane hydroxylase. In all, three potentially useful plant–microbe combinations were identified.

Many contaminated sites contain a mixture of organic (and inorganic) contaminants and, therefore, rhizoremediation strategies must take into account the toxicities and degradabilities of all the pollutants. Increasingly, researchers will screen their promising microbe–plant combinations against a range of related organics and other compounds. Consequently, the stage prior to field evaluation is to measure the efficacy of the system in realistic laboratory microcosms containing contaminated soil collected from the site under investigation.

VII. Conclusions

It is estimated that there are over 350,000 hectares of contaminated land in the U.K. alone, representing a potential hazard to humans and wildlife and a source of water and air pollution. Not surprisingly, great efforts are being made to detect, monitor, and ameliorate pollution. Bioremediation is an appealing approach because microbial species, individually or collectively, have an immense capacity to transform and mineralize a vast array of pollutant organics. In addition, the ''natural'' and ''sustainable'' process of converting pollutants into carbon dioxide and water has significant appeal to scientists, policymakers, and environmentalists. Specifically, rhizoremedation may be chosen if: (1) *in situ* treatment is the most attractive economic option as compared with physical, chemical, and thermal treatments;

(2) the pollution is restricted to (or at least concentated in) the layer of soil that the plant roots can penetrate or access as a result of transpiration (although pumping up polluted ground water and distributing it over the bioactive rhizosphere layer may be worth considering); (3) the visual impact of the cleanup process is important; (4) the site is difficult to access with large vehicles and machinery or is part of a fragile ecosystem; and (5) the achievable level of decontamination and the time scale are compatible with the proposed use of the site.

Establishing the constraints to natural (i.e., that brought about by the native rhizosphere) pollutant attenuation is essential before embarking on any remediation strategy. In most cases, a chronically contaminated soil will contain a large number of microbial strains that are, at the very least, resistant to the toxic effects of the pollutants—after all, these brownfield soils are the first choice when searching for pollutant degraders. If the appropriate microbial community or genotypic potential already exists and has been identified (see Burns and Stach, 2002), then various *in situ* rhizostimulation scenarios should be considered (Section VI.A–C). Using plants for the general or even directed stimulation of the indigenous microorganisms is one thing; supporting the establishment of one or more new microbial species (rhizoaugmentation) is a second, more adventurous choice and one which would be difficult to make with any degree of confidence given the current state of our knowledge.

As has been mentioned (Section II), our understanding of microbial ecology in the rhizosphere has been limited to cultivation-based techniques and is therefore subject to culture bias. In the last few years, the availability of molecular tools has opened up the soil microbial ecology black box (Tiedje *et al.*, 1999) and has enabled detailed and culture-independent studies of rhizosphere microbial identity. However, until very recently, linking the identity of microorganisms with their function in the environment remained a major unfulfilled objective in microbial ecology (Dejonghe et al., 2001; Gray and Head, 2001). Exciting advances in methodology now allow the phylogenetic identification of microorganisms responsible for specific steps in carbon cycling. One of the most powerful methods is the stable isotope-probing approach (SIP) in which ^{13}C-labeled substrate is pulse-applied to the system and becomes incorporated into the nucleic acids of those microorganisms utilizing the substrate. The resulting heavy DNA (Radajewski *et al.*, 2000) or RNA (Manefield *et al.*, 2002) fractions can be isolated by density gradient centrifugation and subjected to further molecular analysis. By labeling rhizodeposits with ^{13}C (through

growing the plant in a $^{13}CO_2$ atmosphere), it may be possible to understand the dynamics of competition and carbon-sharing in the rhizosphere, giving insights into how best to establish inoculants. SIP also has the potential for use in identifying those microorganisms playing a key role in processing xenobiotic carbon in the rhizosphere (i.e., by pulsing with ^{13}C-labeled pollutant). Until now, understanding pollutant degradation (characterization of the genetics and enzymatic basis of catabolic pathways) largely comes from culture-based studies. However, the enrichment process routinely used to isolate microorganisms with the desired degradative traits selects for those microorganisms best suited for growth under somewhat artificial culture conditions and not necessarily for those which are responsible for biodegradation *in situ* (Stach and Burns, 2002). If we can identify which microorganisms (or communities of microorganisms) are actively biodegrading *in situ*, this will be invaluable in helping us both to choose microbial inoculants for use in rhizoaugmentation and to design rational rhizostimulation strategies.

Search and discovery and conventional plant breeding will provide pollutant-tolerant plants (Adam and Duncan, 2003), many of them with degradative rhizospheres. However, genetic engineering has the power to generate improved or virtually new species and those studying phytoaccumulation or phytoextraction of metals from soil have increasingly been screening transgenic plants (Berken *et al.*, 2002; Dhankher *et al.*, 2002; Dushenkov *et al.*, 2002; Valls and de Lorenzo, 2002; Bennett *et al.*, 2003; Dushenkov, 2003). Plant molecular biologists with an interest in the degradation of organics should focus on traits that, for example, show increased translocation of photosynthesate to the roots (and from there out into the soil), generate root surface proteins that encourage the attachment of catabolic bacteria, or stimulate synthesis and exudation of inducers specific for a particular degradative pathway. An ingenious approach is shown by the work of Drake *et al.* (2002), which studied an antibody-expressing transgenic *Nicotiana* and suggested that root exudates may bind to their pollutant antigens, presumably forming nontoxic or nonbioavailable complexes. The literature on the manipulation of plants to modify root exudate patterns has been reviewed (Rengel, 2002). Inevitably, there are some anxieties about the impact of GM plants and their crop residues on the soil microbial community and the stability of gene products in soil. Appropriate impact studies will be necessary prior to the use of GM plants for remediation (Bruinsma *et al.*, 2003).

The current potential for genetically modifying the other component of the partnership, the inoculant microorganisms, is even greater and there are many examples of these novel strains being tested in

contaminated soils with or without plants (Brazil *et al.*, 1995; Yee *et al.*, 1998; Shim *et al.*, 2000; Section VI.C).

Ultimately, of course, the plant could be manipulated in order to bypass the microflora entirely by expressing high levels of extracellular enzymes (esterases, hydrolases, and oxidoreductases) or by efficiently translocating the pollutants into the root cells and using intracellular (glutathione-S-transferases) enzymes that catalyze their degradation. The introduction of entire catabolic networks into plants is a real possibility.

In conclusion, the reliable use of new and emerging remediation technologies will depend on a much better understanding of plant–soil–microbe interactions. However, this knowledge will need to be applied in the context of cost–benefit analyses and risk assessments which consider human health and the broader ecological impact of pollution and its amelioration (Menzie et al., 2000).

Glossary of Latin Plant Names

Annual ryegrass (*Lolium multiflorum*)
Barley (*Hordeum vulgare*)
Birdsfoot trefoil (*Lotus corniculatus*)
Burr medic (*Medicago polymorpha*)
Bush bean (*Phaseolus vulgaris*)
Chicory (*Chicorium intybus*)
Common brome (*Bromus inermis*)
Common zinnia (*Zinnia angustifolia*)
Crested wheatgrass (*Agropyron desertorum*)
Dahurian wild rye (*Elymus dauricus*)
Daisy fleabane (*Erigeron annuus*)
Dill (*Anethum graveolens*)
Early goldenrod (*Solidago juncea*)
Fall panicum (*Panicum dichotomiflorum*)
Flat pea (*Lathyrus sylvestris*)
Green foxtail (*Setaria viridus*)
Green pea (*Pisum sativum*)
Hard fescue (*Festuca ovina*)
Hound's tongue (*Cynoglossium officinale*)
Hybrid poplar (*Populus deltoides*)
Hycrest crested wheatgrass (*Agropyron cristatum x Agropyron desertorum*)
Ryegrass (*Lolium perenne*)
Sand dropseed (*Sporobolus crytandrus*)
Slender oat (*Avena barbata*)
Soybean (*Glycine max*)
Intermediate wheatgrass (*Agropyron intermedium*)
Jack pine (*Pinus banksiana*)
Kentucky bluegrass (*Poa pratensis*)

Lucerne (*Medicago sativa*)
Lupin (*Lupinus polyphyllus*)
Maize (*Zea Mays*)
Meadow brome (*Bromus biebersteinii*)
Millet (*Panicum miliaceum*)
Mulberry (*Morus* sp.)
Oat (*Avena sativa*)
Pepper (*Capsicum annuum*)
Poplar (*Populus* sp.)
Radish (*Raphanus sativus*)
Rape (*Brassica napus*)
Red clover (*Trifolium pratense*)
Red fescue (*Festuca rubra*)
Red pine (*Pinus resinosa*)
Reed canarygrass (*Phalaris arundinaceae*)
Rice (*Oryza sativa*)
Rose clover (*Trifolium hirtum*)
Spear thistle (*Cirsium vulgare*)
Spearmint (*Mentha spicata*)
Streambank wheatgrass (*Agropyron riparum*)
Sudan grass (*Sorghum vulgare*)
Sugar beet (*Beta vulgaris*)
Sugar cane (*Saccharum officinarum*)
Summer cypress (*Kochia scoparia*)
Sunflower (*Helianthus annuus*)
Switch grass (*Panicum virgatum*)
Tall fescue (*Festuca arundinacea*)
Timothy grass (*Phleum pratense*)
Wheat (*Triticum aestivum*)
Wheatgrass (*Agropyron* sp.)
White clover (*Trifolium repens*)
White pine (*Pinus strobus*)

References

Abdelhafid, R., Houot, S., and Barriuso, E. (2000). Dependence of atrazine degradation on C and N availability in adapted and nonadapted soils. *Soil Biology & Biochemistry* **32,** 389–401.

Abraham, W.-R., Nogales, B., Golyshin, P. N., Pieper, D. H., and Timmis, K. N. (2002). Polychlorinated biphenyl-degrading microbial communities in soils and sediments. *Current Opinion in Microbiology* **5,** 246–253.

Adam, G., and Duncan, H. (2003). The effect of diesel fuel on common vetch (*Vicia sativa L.*) plants. *Environmental Geochemistry and Health* **25,** 123–130.

Aitchinson, E. W., Kelley, S. L., Alvarez, P. J. J., and Schnoor, J. L. (2000). Phytoremediation of 1,4-dioxane by hybrid poplar trees. *Water Environment Research* **72,** 313–321.

Alexander, M. (1994). ''Biodegradation and Bioremediation.'' Academic Press, Inc., San Diego, CA.

Alexander, M. (1995). How toxic are toxic chemicals in soil? *Environmental Science and Technology* **29,** 2713–2717.

Anderson, T. A., Guthrie, E. A., and Walton, B. T. (1993). Bioremediation in the rhizosphere. *Environmental Science and Technology* **27,** 2630–2636.

Anderson, T. A., Kruger, E. L., and Coats, J. R. (1994). Biological degradation of pesticide wastes in the root zone of soils collected at an agrochemical dealership. *In* ''Bioremediation through Rhizosphere Technology'' (T. A. Anderson and J. R. Coats, eds.), pp. 199–209. American Chemical Society, Washington, DC.

Anderson, T. A., White, D. C., and Walton, B. T. (1995). Degradation of hazardous organic compounds by rhizosphere microbial communities. *In* ''Biotransformations: Microbial Degradation of Health Risk Compounds'' (V. P. Singh, ed.), pp. 205–225.

Angle, J. S., Gagliardi, J. V., McIntosh, M. S., and Levin, M. A. (1996). Enumeration and expression of bacterial counts in the rhizosphere. *In* ''Soil Biochemistry, Vol. 9'' (G. Stotzky and J.-M. Bollag, eds.), pp. 233–251. Marcel Dekker, New York.

Banfield, J. F., Barker, W. W., Welch, S. A., and Taunton, A. (1999). Biological impact on mineral dissolution: Application of the lichen model to understanding mineral weathering in the rhizosphere. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 3404–3411.

Banks, M. K., Lee, E., and Schwab, A. P. (1999). Evaluation of dissipation mechanisms for benzo(a)pyrene in the rhizosphere of tall fescue. *Journal of Environmental Quality* **28,** 294–298.

Baudoin, E., Benizri, E., and Guckert, A. (2002). Impact of growth stage on the bacterial community structure along maize roots, as determined by metabolic and genetic fingerprinting. *Applied Soil Ecology* **19,** 135–145.

Bennett, L. E., Burkhead, J. L., Hale, K. L., Terry, N., Pilon, M., and Pilon-Smits, E. A. H. (2003). Analysis of transgenic Indian mustard plants for phytoremediation of metal-contaminated mine tailings. *Journal of Environmental Quality* **32,** 432–440.

Berken, A., Mulholland, M. M., LeDuc, D. L., and Terry, N. (2002). Genetic engineering of plants to enhance selenium phytoremediation. *Critical Reviews in Plant Sciences* **21,** 567–582.

Bianciotto, V., Andreotti, S., Balestrini, R., Bonfante, P., and Perotto, S. (2001). Mucoid mutants of the biocontrol strain *Pseudomonas fluorescens* CHA0 show increased ability in biofilm formation on mycorrhizal and nonmycorrhizal carrot roots. *Molecular Plant–Microbe Interactions* **14,** 255–260.

Binet, P., Portal, J. M., and Leyval, C. (2000). Dissipation of 3-6-ring polycyclic aromatic hydrocarbons in the rhizosphere of ryegrass. *Soil Biology & Biochemistry* **32,** 2011–2017.

Binet, P., Portal, J. M., and Leyval, C. (2001). Application of GC-MS to the study of anthracene disappearance in the rhizosphere of ryegrass. *Organic Geochemistry* **32,** 217–222.

Binnie, J., Cape, J. N., Mackie, N., and Leith, I. D. (2002). Exchange of organic solvents between the atmosphere and grass—The use of open-top chambers. *The Science of the Total Environment* **285,** 53–67.

Bowen, G., and Rovitra, A. D. (1991). The rhizosphere—The hidden half of the hidden half. *In* ''Plant Roots the Hidden Half'' (Y. Waisel, A. Eshel, and U. Kafkafi, eds.), pp. 641–669. Marcel Dekker, Inc., New York.

Boyle, J. J., and Shann, J. R. (1995). Biodegradation of phenol, 2,4-DCP, 2,4-D, and 2,4,5-T in field-collected rhizosphere and nonrhizosphere soils. *Journal of Environmental Quality* **24,** 782–785.

Boyle, J. J., and Shann, J. R. (1998). The influence of planting and soil characteristics on mineralization of 2,4,5-T in rhizosphere soil. *Journal of Environmental Quality* **27,** 704–709.

Brazil, G. M., Kenefick, L., Callanan, M., Haro, A., deLorenzo, V., Dowling, D. N., and O'Gara, F. (1995). Construction of a rhizosphere Pseudomonad with potential to degrade polychlorinated biphenyls and detection of *bph* gene expression in the rhizosphere. *Applied and Environmental Microbiology* **61,** 1946–1952.

Breedveld, G. D., and Sparrevik, M. (2000). Nutrient-limited biodegradation of PAH in various soil strata at a creosote contaminated site. *Biodegradation* **11,** 391–399.

Briggs, G. G., Bromilow, R. H., and Evans, A. A. (1982). Relationships between lipophilicity and root uptake and translocation of non-ionised chemicals by barley. *Pesticide Science* **13,** 495.

Brimecombe, M. J., De Leij, F. A., and Lynch, J. M. (2001). The effect of root exudates on rhizosphere microbial populations. *In* ''The Rhizosphere: Biochemistry and Organic Substances at the Soil-Plant Interface'' (R. Pinton, Z. Varanini, and P. Nannipieri, eds.), pp. 95–140. Marcel Dekker, Inc., New York.

Bringhurst, R. M., Cardon, Z. G., and Gage, D. J. (2001). Galactosides in the rhizosphere: Utilization by *Sinorhizobium meliloti* and development of a biosensor. *Proceedings of the National Academy of Science USA* **98,** 4540–4545.

Bruinsma, M., Konalchuk, G. A., and van Veen, J. A. (2003). Effects of genetically modified plants on microbial communities and processes in soil. *Biology and Fertility of Soils* **37,** 329–337.

Brunner, W., Sutherland, F. H., and Focht, D. D. (1985). Enhanced biodegradation of polychlorinated biphenyls in soil by analog enrichment and bacterial inoculation. *Journal of Environmental Quality* **14,** 324–328.

Burdman, S., Okon, Y., and Jurkevitch, E. (2000). Surface characteristics of *Azospirillum brasilense* in relation to cell aggregation and attachment to plant roots. *Critical Reviews in Microbiology* **26,** 91–110.

Burken, J. G., and Schnoor, J. L. (1998). Predictive relationships for uptake of organic contaminants by hybrid poplar trees. *Environmental Science and Technology* **32,** 3379.

Burns, R. G. (1988). Laboratory models in the study of soil microbiology. *In* ''Handbook of Laboratory Model Systems for Microbial Ecosystem Research'' (J. W. T. Wimpenny, ed.), pp. 51–98. CRC Press, FL.

Burns, R. G., Rogers, S., and McGhee, I. (1996). Remediation of inorganics and organics in industrial and urban soils. *In* ''Contaminants and the Soil Environment'' (R. Naidu, R. S. Kookuna, D. P. Oliver, S. Rogers, and M. J. McLaughlin, eds.). Kluwer, The Netherlands.

Burns, R. G., and Stach, J. E. M. (2002). Microbial ecology of soil biofilms: Substrate bioavailability, bioremediation, and complexity. *Developments in Soil Science* **28B,** 17–42.

Buyanovsky, G. A., Kremer, R. J., Gajda, A. M., and Kazemi, H. V. (1995). Effect of corn plants and rhizosphere populations on pesticide degradation. *Bulletin of Environmental Contamination and Toxicology* **55,** 689–696.

Buyer, J. S., Roberts, D. P., and Russek-Cohen, E. (2002). Soil and plant effects on microbial community structure. *Canadian Journal of Microbiology* **48,** 955–964.

Casavant, N. C., Thompson, D., Beattie, G. A., Phillips, G. J., and Halverson, L. J. (2003). Use of a site-specific recombination-based biosensor for detecting bioavailable toluene and related compounds on roots. *Environmental Microbiology* **5,** 238–249.

Chaineau, C. H., Morel, J. L., and Oudot, J. (2000). Biodegradation of fuel oil hydrocarbons in the rhizosphere of maize. *Journal of Environmental Quality* **29,** 569–578.

Chaineau, C. H., Yepremian, C., Vidalie, J. F., Ducreux, J., and Ballerini, D. (2003). Bioremediation of a crude oil-polluted soil: Biodegradation, leaching and toxicity assessments. *Water Air and Soil Pollution* **144,** 419–440.

Chelius, M. K., and Triplett, E. W. (2001). The diversity of archaea and bacteria in association with the roots of *Zea mays* L. *Microbial Ecology* **41,** 252–263.

Chernin, L., and Chet, I. (2002). Microbial enzymes and biocontrol of plant pathogens. *In* ''Enzymes in the Environment'' (R. G. Burns and R. P. Dick, eds.), pp. 171–226. Marcel Dekker, New York.

Chow, M. L., Radomski, C. C., McDermott, J. M., Davies, J., and Axelrood, P. E. (2002). Molecular characterization of bacterial diversity in Lodgepole pine (*Pinus contorta*) rhizosphere soils from British Columbia forest soils differing in disturbance and geographic source. *FEMS Microbiology Ecology* **42,** 347–357.

Crowley, D. E. (2001). Function of siderophores in the plant rhizosphere. *In* ''The Rhizosphere: Biochemistry and Organic Substances at the Soil–Plant Interface'' (R. Pinton, Z. Varanini, and P. Nannipieri, eds.), pp. 223–261. Marcel Dekker, Inc., New York.

Crowley, D. E., Alvey, S., and Gilbert, E. S. (1997). Rhizosphere ecology of xenobiotic-degrading microorganisms. *In* ''Phytoremediation of Soil and Water Contaminants'' (E. L. Kruger, T. A. Anderson, and J. R. Coats, eds.), pp. 20–36. American Chemical Society, Washington, DC.

Crowley, D. E., Brennerova, M. V., Irwin, C., Brenner, V., and Focht, D. D. (1996). Rhizosphere effects on biodegradation of 2,5-dichlorobenzoate by a bioluminescent strain of root-colonizing *Pseudomonas fluorescens*. *FEMS Microbiology Ecology* **20,** 79–89.

Cunningham, S. D., Shann, J. R., Crowley, D. E., and Anderson, T. A. (1997). Phytoremediation of contaminated water and soil. *In* ''Phytoremediation of Soil and Water Contaminants'' (E. L. Kruger, T. A. Anderson, and J. R. Coats, eds.), pp. 2–17. American Chemical Society, Washington, DC.

Curl, E. A., and Truelove, B. (eds.). (1986). ''The Rhizosphere.'' Springer, New York.

Dakora, F. D., and Phillips, D. A. (2002). Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil* **245,** 35–47.

Dale, J. W. (1998). ''Molecular Genetics of Bacteria.'' John Wiley and Sons, Chichester, UK.

Davis, L. C., Castro-Diaz, S., Zhang, Q., and Erickson, L. E. (2002). Benefits of vegetation for soils with organic contaminants. *Critical Reviews in Plant Sciences* **21,** 457–491.

de Lipthay, J. R., Barkay, T., and Sorensen, S. J. (2001). Enhanced degradation of phenoxyacetic acid in soil by horizontal transfer of the *tfd*A gene encoding a 2,4-dichlorophenoxyacetic acid dioxygenase. *FEMS Microbiology Ecology* **35,** 75–84.

De Meyer, G., Capieau, K., Audenaert, K., Buchala, A., Metraux, J.-P., and Hofte, M. (1999). Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseusomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. *Molecular Plant–Microbe Interactions* **12,** 450–458.

de Neergaard, A., and Magid, J. (2001). Influence of the rhizosphere on microbial biomass and recently formed organic matter. *European Journal of Soil Science* **52,** 377–384.

De Rore, H., Demolder, K., De Wilde, K., Top, E., Houwen, F., and Verstraete, W. (1994). Transfer of the catabolic plasmid RP4:Tn4371 to indigenous soil bacteria and its

effect on respiration and biphenyl breakdown. *FEMS Microbiology Ecology* **15,** 71–81.

Dec, J., and Bollag, J. M. (2000). Phenoloxidase-mediated interactions of phenols and anilines with humic materials. *Journal of Environmental Quality* **29,** 665–676.

Dejonghe, W., Boon, N., Seghers, D., Top, E. M., and Verstraete, W. (2001). Bioaugmentation of soils by increasing microbial richness: Missing links. *Environmental Microbiology* **3,** 649–657.

Dejonghe, W., Goris, J., El Fantroussi, S., Hofte, M., De Vos, P., Verstraete, W., and Top, E. M. (2000). Effect of dissemination of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation plasmids on 2,4-D degradation and on bacterial community structure in two different soil horizons. *Applied and Environmental Microbiology* **66,** 3297–3304.

Dhankher, O. P., Li, Y. J., Rosen, B. P., Shi, J., Salt, D., Senecoff, J. F., Sashti, N. A., and Meagher, R. B. (2002). Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and gamma-glutamylcysteine synthetase expression. *Nature Biotechnology* **20,** 1140–1145.

Dietz, A. C., and Schnoor, J. L. (2001). Advances in phytoremediation. *Environmental Health Perspectives* **109**(Suppl 1), 163–138.

Dinkla, I. J. T., Gabor, E. M., and Janssen, D. B. (2001). Effects of iron limitation on the degradation of toluene by *Pseudomonas* strains carrying the TOL (pWWO) plasmid. *Applied and Environmental Microbiology* **67,** 3406–3412.

Dinkla, I. J. T., and Janssen, D. B. (2003). Simultaneous growth on citrate reduces the effects of iron limitation during toluene degradation in Pseudomonas. *Microbial Ecology* **45,** 97–107.

Donnelly, P. K., Hedge, R. S., and Fletcher, J. S. (1994). Growth of PCB-degrading bacteria on compounds from photosynthetic plants. *Chemosphere* **28,** 981–988.

Dormaar, J. F. (1969). Phospholipids in chernozenic soils of southern Alberta. *Canadian Journal of Soil Science* **11,** 134–137.

Drake, P. M. W., Chargelegue, D., Vine, N. D., Van Dolleweerd, C. J., Obregon, P., and Ma, J. K. C. (2002). Transgenic plants expressing antibodies: A model for phytoremediation. *FASEB Journal* **16,** 1855–1860.

Duineveld, B. M., Kowalchuk, G. A., Keijzer, A., van Elsas, J. D., and van Veen, J. A. (2001). Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Applied and Environmental Microbiology* **67,** 172–178.

Dunning Hotopp, J. C., and Hausinger, R. P. (2001). Alternative substrates of 2,4-dichlorophenoxyacetate/alpa-ketoglutarate dioxygenase. *Journal of Molecular Catalysis B: Enzymatic* **15,** 155–162.

Dushenkov, S. (2003). Trends in phytoremediation of radionuclides. *Plant and Soil* **249,** 167–175.

Dushenkov, S., Skarzhinskaya, M., Glimelius, K., Gleba, D., and Raskin, I. (2002). Bioengineering of a phytoremediation plant by means of somatic hybridization. *International Journal of Phytoremediation* **4,** 117–126.

Dzantor, E. K., and Woolston, J. E. (2001). Enhancing dissipation of arochlor 1248 (PCB) using substrate amendment in rhizosphere soil. *Journal of Environmental Science and Health* **A36,** 1861–1871.

Fang, C., Radosevich, M., and Fuhrmann, J. J. (2001). Atrazine and phenanthrene degradation in grass rhizosphere soil. *Soil Biology & Biochemistry* **33,** 671–678.

Ferro, A., Kennedy, J., Doucette, W., Nelson, S., Jauregui, G., McFarland, B., and Bugbee, B. (1997). Fate of benzene in soils planted with alfalfa: Uptake, volatilization, and degradation. *In* ''Phytoremediation of Soil and Water Contaminants'' (E. L. Kruger, T. A. Anderson, and J. R. Coats, eds.), pp. 223–237. American Chemical Society, Washington DC.

Ferro, A. M., Sims, R. C., and Bugbee, B. (1994). Hycrest crested wheatgrass accelerates the degradation of pentachlorophenol in soil. *Journal of Environmental Quality* **23,** 272–279.

Filer, K., and Harker, A. R. (1997). Identification of the inducing agent of the 2,4-dichlorophenoxyacetic acid pathway encoded by plasmid pJP4. *Applied and Environmental Microbiology* **63,** 317–320.

Fletcher, J. S., Donnelly, P. K., and Hegde, R. S. (1995). Biostimulation of PCB-degrading bacteria by compounds released from plant roots. *In* ''Bioremediation of Recalcitrant Xenobiotics'' (R. E. Hinchee, D. B. Anderson, and R. E. Hoeppel, eds.), pp. 131–136. Battelle Memorial Institute, Columbus, Ohio.

Germida, J. J., Frick, C. M., and Farrell, R. E. (2002). Phytoremediation of oil-contaminated soils. *Developments in Soil Science* **28B,** 169–186.

Gilbert, E. S., and Crowley, D. E. (1997). Plant compounds that induce polychlorinated biphenyl biodegradation by *Arthrobacter* sp. strain B1B. *Applied and Environmental Microbiology* **63,** 1933–1938.

Gomes, N. C. M., Heuer, H., Schonfeld, J., Costa, R., Mendonca-Hagler, L., and Smalla, K. (2001). Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. *Plant and Soil* **232,** 167–180.

Goodwin, T. W., and Mercer, E. I. (1983). ''Introduction to Plant Biochemistry.'' Permagon Press, Oxford, UK.

Gramss, G., Voigt, K.-D., and Kirsche, B. (1999). Oxidoreductase enzymes liberated by plant roots and their effects on soil humic material. *Chemosphere* **38,** 1481–1494.

Gransee, A., and Wittenmayer, L. (2000). Qualitative and quantitative analysis of water-soluble root exudates in relation to plant species and development. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* **163,** 381–385.

Gray, N. D., and Head, I. M. (2001). Linking genetic identity and function in communities of uncultured bacteria. *Environmental Microbiology* **3,** 481–492.

Gundersen, J. L., Macintyre, W. G., and Hale, R. C. (1997). pH-dependent sorption of chlorinated guaiacols on estuarine sediments: The effects of humic acids and TOC. *Environmental Science and Technology* **31,** 188–193.

Gunther, T., Dornberger, U., and Fritsche, W. (1996). Effects of ryegrass on biodegradation of hydrocarbons in soil. *Chemosphere* **33,** 203–215.

Haby, P. A., and Crowley, D. E. (1996). Biodegradation of 3-chlorobenzoate as affected by rhizodeposition and selected carbon substrates. *Journal of Environmental Quality* **25,** 304–310.

Hatzinger, P., and Alexander, M. (1995). Effect of aging of chemicals in soil on their biodegradability and extractability. *Environmental Science and Technology* **29,** 537–545.

Hiltner, L. (1904). Über neuerr Erfahrungen und Probleme auf dem Gebeit der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründungüng und Brache. *Arbeiten der Deutschen Landwirtschaftsgesellschaft Berlin* **98,** 59–78.

Hoagland, R. E., Zablotowicz, R. M., and Locke, M. A. (1997). In integrated phytoremediation strategy for chloroacetamide herbicides in soil. *In* ''Phytoremediation of Soil and

Water Contaminants'' (E. L. Kruger, T. A. Anderson, and J. R. Coats, eds.), pp. 92–105. American Chemical Society, Washington DC.

Hsu, T.-S., and Bartha, R. (1979). Accelerated mineralisation of two organophosphate insecticides in the rhizosphere. *Applied and Environmental Microbiology* **37,** 36–41.

Janssen, P. H., Yates, P. S., Grinton, B. E., Taylor, P. M., and Sait, M. (2002). Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria, Actinobacteria, Proteobacteria* and *Verrucobacteria*. *Applied and Environmental Microbiology* **68,** 2391–2396.

Jensen, L. E., and Nybroe, O. (1999). Nitrogen availability to *Pseudomonas fluorescens* DF57 is limited during decomposition of barley straw in bulk soil and in the barley rhizosphere. *Applied and Environmental Microbiology* **65,** 4320–4328.

Joergensen, R. G. (2000). Ergosterol and microbial biomass in the rhizosphere of grassland soils. *Soil Biology & Biochemistry* **32,** 647–652.

Joner, E. J., Johansen, A., Loibner, A. P., DeLaCruz, M. A., Szolar, O. H. J., Portal, J.-M., and Leyval, C. (2001). Rhizosphere effects on microbial community structure and dissipation and toxicity of polycyclic aromatic hydrocarbons (PAHs) in spiked soil. *Environmental Science and Technology* **35,** 2773–2777.

Ka, J. O., Yu, Z., and Mohn, W. W. (2001). Monitoring the size and metabolic activity of the bacterial community during biostimulation of fuel-contaminated soil using competitive PCR and RT-PCR. *Microbial Ecology* **42,** 267–273.

Kandeler, E., Marschner, P., Tscherko, D., Gahoonia, T. S., and Nielsen, N. E. (2002). Microbial community composition and functional diversity in the rhizosphere of maize. *Plant and Soil* **238,** 301–312.

Kelley, S. L., Aitchinson, E. W., Deshpande, M., Schnoor, J. L., and Alvarez, P. J. J. (2001). Biodegradation of 1,4-dioxane in planted and unplanted soil: Effect of bioaugmentation with *Amycolata* sp. CB1190. *Water Research* **35,** 3791–3800.

Kim, J.-E., Wang, C.-J. J., and Bollag, J.-M. (1998). Interaction of reactive and inert chemicals in the presence of oxidoreductases: Reaction of the herbicide bentazon and its metabolites with humic monomers. *Biodegradation* **8,** 387–392.

Knaebel, D. B., and Vestal, J. R. (1992). Effects of intact rhizosphere microbial communities on the mineralization of surfactants in surface soils. *Canadian Journal of Microbiology* **38,** 643–653.

Kowalchuk, G. A., Buma, D. S., de Boer, W., Klinkhamer, P. G. L., and van Veen, J. A. (2002). Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. *Antonie van Leeuwenhoek* **81,** 509–520.

Kruger, E. L., Anhalt, J. C., Sorenson, D., Nelson, B., Chouhy, A. L., Anderson, T. A., and Coats, J. R. (1997). Atrazine degradation in pesticide-contaminated soils: Phytoremediation potential. *In* ''Phytoremediation of Soil and Water Contaminants'' (E. L. Kruger, T. A. Anderson, and J. R. Coats, eds.), pp. 54–64. American Chemical Society, Washington, DC.

Kuiper, I., Bloemberg, G. V., and Lugtenberg, B. J. J. (2001). Selection of a plant–bacterium pair as a novel tool for rhizostimulation of polyaromatic hydrocarbon-degrading bacteria. *Molecular Plant–Microbe Interactions* **14,** 1197–1205.

Kuiper, I., Kravchenko, L. V., Bloemberg, G. V., and Lugtenberg, B. J. J. (2002). *Pseudomonas putida* strain PCL1444, selected for efficient root colonization and naphthalene degradation, effectively utilizes root exudate components. *Molecular Plant–Microbe Interactions* **15,** 734–741.

Leigh, M. B., Fletcher, J. S., Fu, X., and Schmitz, F. J. (2002). Root turnover: An important source of microbial substrates in rhizosphere remediation of recalcitrant contaminants. *Environmental Science and Technology* **36,** 1579–1583.

Li, P. J., Sun, T. H., Stagnitti, F., Zhang, C. G., Zhang, H. R., Xiong, X. Z., Allinson, G., Ma, X. J., and Allinson, M. (2002). Field-scale bioremediation of soil contaminated with crude oil. *Environmental Engineering Science* **19,** 277–289.

Liesack, W., Janssen, P. H., Rainey, F. A., Ward-Rainey, N. L., and Stackebrandt, E. (1997). Microbial diversity in soil: The need for a combined approach using molecular and cultivation techniques. *In* ''Modern Soil Microbiology'' (J. D. van Elsas, J. T. Trevors, and E. M. H. Wellington, eds.), pp. 375–439. Marcel Dekker, New York.

Liste, H.-H., and Alexander, M. (1999). Rapid screening of plants promoting phenanthrene degradation. *Journal of Environmental Quality* **28,** 1376–1377.

Liste, H.-H., and Alexander, M. (2000a). Plant-promoted pyrene degradation in soil. *Chemosphere* **40,** 7–10.

Liste, H.-H., and Alexander, M. (2000b). Accumulation of phenanthrene and pyrene in rhizosphere soil. *Chemosphere* **40,** 11–14.

Macek, T., Mackova, M., and Kas, J. (2000). Exploitation of plants for the removal of organics in environmental remediation. *Biotechnology Advances* **18,** 23–34.

Mandelbaum, R. T., Allan, D. L., and Wackett, L. P. (1995). Isolation and characterization of a *Pseudomonas* sp. that mineralizes the s-triazine herbicide atrazine. *Applied and Environmental Microbiology* **61,** 1451–1457.

Manefield, M., Whiteley, A. S., Griffiths, R. I., and Bailey, M. J. (2002). RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Applied and Environmental Microbiology* **68,** 5367–5373.

Marchand, A.-L., Piutti, S., Lagacherie, B., and Soulas, G. (2002). Atrazine mineralization in bulk soil and maize rhizosphere. *Biology and Fertility of Soils* **35,** 288–292.

Margesin, R., and Schinner, F. (2001). Bioremediation (natural attenuation and biostimulation) of diesel oil-contaminated soil in an alpine glacier skiing area. *Applied and Environmental Microbiology* **67,** 3127–3133.

Marilley, L., and Aragno, M. (1999). Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. *Applied Soil Ecology* **13,** 127–136.

Marilley, L., Vogt, G., Blanc, M., and Aragno, M. (1998). Bacterial diversity in the bulk soil and rhizosphere fractions of *Lolium perenne* and *Trifolium repens* as revealed by PCR restriction analysis of 16S rDNA. *Plant and Soil* **198,** 219–224.

Marschner, P., Neumann, G., Kania, A., Weiskopf, L., and Lieberei, R. (2002). Spatial and temporal dynamics of the microbial community structure in the rhizosphere of cluster roots of white lupin (*Lupinus albus* L.). *Plant and Soil* **246,** 167–174.

Master, E. R., and Mohn, W. W. (2001). Induction of *bphA*, encoding biphenyl dioxygenase, in two polychlorinated biphenyl-degrading bacteria, psychotolerant *Pseudomonas* strain Cam-1 and mesophilic *Burkholderia* strain LB400. *Applied and Environmental Microbiology* **67,** 2669–2676.

McFall, S. M., Chugani, S. A., and Chakrabarty, A. M. (1998). Transcriptional activation of the catechol and chlorocatechol operons: Variations on a theme. *Gene* **223,** 257–267.

McGhee, I., and Burns, R. G. (1995). Bioremediation of soil contaminated with the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA). *Applied Soil Ecology* **2,** 143–154.

Mehmannavaz, R., Prasher, S. O., and Ahmad, D. (2002). Rhizospheric effects of alfalfa on biotransformation of polychlorinated biphenyls in a contaminated soil augmented with *Sinorhizobium meliloti*. *Process Biochemistry* **37,** 955–963.

Meijer, S. N., Ockenden, W. A., Sweetman, A., Breivik, K., Grimalt, J. O., and Jones, K. C. (2003). Global distillation and budget of PCBs and HCB in background surface soils: Implications for sources and environmental processes. *Environmental Science and Technology* **37,** 667–672.

Menzie, C., Burke, A. M., Grasso, D., Harnois, M., Mageo, B., McDonald, D., Montgomery, C., Nichols, A., Pignatello, J., Price, B., Price, R., Rose, J., Shatkin, J. A., and Smets, B. (2000). An approach for incorporating information on chemical availability in soils into risk assessment and risk-based decision making. *Human and Ecological Risk Assessment* **6,** 479–510.

Mihelcic, J. R., Mitzell, R. J., and Stapleton, J. M. (1993). Bioavailability of sorbed and separate phase chemicals. *Biodegradation* **4,** 141–153.

Miller, E. K., and Dyer, W. E. (2002). Phytoremediation of pentachlorophenol in the crested wheatgrass (*Agropyron cristatum x desertorum*) rhizosphere. *International Journal of Phytoremediation* **4,** 223–238.

Miya, R. K., and Firestone, M. K. (2000). Phenanthrene-degrader community dynamics in rhizosphere soil from a common annual grass. *Journal of Environmental Quality* **29,** 584–592.

Neilson, J. W., Pierce, S. A., and Maier, R. M. (1999). Factors influencing expression of luxCDABE and nah genes in *Pseudomonas putida* RB1353(NAH, puTK9) in dynamic systems. *Applied and Environmental Microbiology* **65,** 3473–3482.

Normander, B., and Prosser, J. I. (2000). Bacterial origin and community composition in the barley phytosphere as a function of habitat and presowing conditions. *Applied and Environmental Microbiology* **66,** 4372–4377.

Ogawa, N., McFall, S. M., Klem, T. J., Miyashita, K., and Chakrabarty, A. M. (1999). Transcriptional activation of the chlorocatechol degradative genes of *Ralstonia eutropha* NH9. *Journal of Bacteriology* **181,** 6697–6705.

Olsen, P. E., Wong, T., Leigh, M. B., and Fletcher, J. S. (2003). Allometric modeling of plant root growth and its application in rhizosphere remediation of soil contaminants. *Environmental Science and Technology* **37,** 638–643.

Pai, S. G., Riley, M. B., and Camper, N. D. (2001). Microbial degradation of mefenoxam in rhizosphere of *Zinnia angustifolia*. *Chemosphere* **44,** 577–582.

Parsek, M. R., Shinabarger, D. L., Rothmel, R. K., and Chakrabarty, A. M. (1992). Roles of CatR and cis, cis-muconate in activation of the catbC operon, which is involved in benzoate degradation in *Pseudomonasputida*. *Journal of Bacteriology* **174,** 7798–7806.

Paszczynski, A., and Crawford, R. L. (2000). Recent advances in the use of fungi in environmental remediation and biotechnology. *In* ''Soil Biochemistry,'' Vol. 10, pp. 379–422 (J. -M. Bollag and G. Stotzky, eds.), Marcel Dekker, New York.

Perkovich, B. S., Anderson, T. A., Kruger, E. L., and Coats, J. R. (1996). Enhanced mineralisation of [^{14}C]atrazine in *Kochia scoparia* rhizospheric soil from a pesticide-contaminated site. *Pesticide Science* **46,** 391–396.

Persello-Cartieaux, F., Nussaume, L., and Robaglia, C. (2003). Tales from the underground: Molecular plant–rhizobacteria interactions. *Plant Cell and Environment* **26,** 189–199.

Pfender, W. (1996). Bioremediation bacteria to protect plants in pentachorophenol-contaminated soil. *Journal of Environmental Quality* **25,** 1256–1260.

Pichtel, J., and Liskanen, P. (2001). Degradation of diesel fuel in rhizosphere soil. *Environmental Engineering Science* **18,** 145–157.

Pignatello, J. J., and Xing, B. (1996). Mechanisms of slow sorption of organic chemicals to natural particles. *Environmental Science and Technology* **30,** 1–11.

Pinton, R., Varanini, Z., and Nannipieri, P. (eds.). (2001). ''The Rhizosphere: Biochemistry and Organic Substances at the Soil–Plant Interface.'' Marcel Dekker, New York.

Piutti, S., Hallet, S., Rousseaux, S., Philippot, L., Soulas, G., and Martin-Laurent, F. (2002). Accelerated mineralisation of atrazine in maize rhizosphere soil. *Biology and Fertility of Soils* **36,** 434–441.

Radajewski, S., Ineson, P., Parekh, N. R., and Murrell, J. C. (2000). Stable-isotope probing as a tool in microbial ecology. *Nature* **403,** 646–649.

Ramos, C., Molbak, L., and Molin, S. (2000). Bacterial activity in the rhizosphere analyzed at the single-cell level by monitoring ribosome contents and synthesis rates. *Applied and Environmental Microbiology* **66,** 801–809.

Read, D. B., Bengough, A. G., Gregory, P. J., Crawford, J. W., Robinson, D., Scrimgeour, C. M., Young, I. M., Zhang, K., and Zhang, X. (2003). Plant roots release phospholipid surfactants that modify the physical and chemical properties of soil. *New Phytologist* **157,** 315–326.

Read, D. B., and Gregory, P. J. (1997). Surface tension and viscosity of axenic maize and lupin root mucilages. *New Phytologist* **137,** 623–628.

Reddy, B. R., and Sethunathan, N. (1994). Mineralization of p-nitrophenol in the rhizosphere of rice. *Agriculture, Ecosystems and Environment* **47,** 313–317.

Reilley, K. A., Banks, M. K., and Schwab, A. P. (1996). Dissipation of polycyclic aromatic hydrocarbons in the rhizosphere. *Journal of Environmental Quality* **25,** 212–219.

Rengel, Z. (2002). Genetic control of root exudation. *Plant and Soil* **245,** 59–70.

Rice, P. J., Anderson, T. A., and Coats, J. R. (1997). Evaluation of the use of vegetation for reducing the environmental impact of deicing agents. *In* ''Phytoremediation of Soil and Water Contaminants'' (E. L. Kruger, T. A. Anderson, and J. R. Coats, eds.), pp. 162–176. American Chemical Society, Washington, DC.

Robinson, G. K., and Lenn, M. J. (1994). The bioremediation of polychlorinated biphenyls (PCBs): Problems and perspectives. *Biotechnology and Genetic Engineering Reviews* **12,** 139–188.

Robinson, S. L., Novak, J. T., Widdowson, M. A., Crosswell, S. B., and Fetterolf, G. J. (2003). Field and laboratory evaluation of the impact of tall fescue on polyaromatic hydrocarbon degradation in an aged creosote-contaminated surface soil. *Journal of Environmental Engineering–ASCE* **129,** 232–240.

Roling, W. F. M., Milner, M. G., Jones, D. M., Lee, K., Daniel, F., Swannell, R. J. P., and Head, I. M. (2002). Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. *Applied and Environmental Microbiology* **68,** 5537–5548.

Samanta, S. K., Singh, O. V., and Jain, R. K. (2002). Polycyclic aromatic hydrocarbons: Environmental pollution and bioremediation. *Trends in Biotechnology* **20,** 243–248.

Sandmann, E. R. I. C., and Loos, M. A. (1984). Enumeration of 2,4-D-degrading microorganisms in soils and crop plant rhizospheres using indicator media: High populations associated with sugarcane (*Saccharum officinarum*). *Chemosphere* **13,** 1073–1084.

Schellenberg, K., Leuenberger, C., and Schwarzenbach, R. P. (1984). Sorption of chlorinated phenols by natural sediments and aquifer materials. *Environmental Science and Technology* **18,** 652–657.

Schmidt, S. K., and Scow, K. M. (1996). Use of soil bioreactors and microcosms in bioremediation research. *In* ''Manual of Environmental Microbiology'' (C. J. Hurst, ed.). American Society for Microbiology Press, Washington, DC.

Schroder, P., and Collins, C. (2002). Conjugating enzymes involved in xenobiotic metabolism of organic xenobiotics in plants. *International Journal of Phytoremediation* **4,** 247–265.

Schwab, A. P., and Banks, M. K. (1994). Biologically mediated dissipation of polyaromatic hydrocarbons in the root zone. *In* ''Bioremediation through Rhizosphere Technology'' (T. A. Anderson and J. R. Coats, eds.), pp. 132–141. American Chemical Society, Washington, DC.

Schwaner, N. E., and Kroer, N. (2001). Effect of plant species on the kinetics of conjugal transfer in the rhizosphere and relation to bacterial metabolic activity. *Microbial Ecology* **42,** 458–465.

Shaw, L. J., Beaton, Y., Glover, L. A., Killham, K., Osborn, D., and Meharg, A. A. (2000). Bioavailability of 2,4-dichlorophenol associated with water-soluble humic material. *Environmental Science and Technology* **34,** 4721–4726.

Shim, H., Chauhan, S., Ryoo, D., Bowers, K., Thomas, S. M., Canada, K. A., Burken, J. G., and Wood, T. K. (2000). Rhizosphere competitiveness of trichloroethylene-degrading, poplar-colonizing recombinant bacteria. *Applied and Environmental Microbiology* **66,** 4673–4678.

Shupack, D. P., and Anderson, T. A. (2000). Mineralization of propylene glycol in root zone soil. *Water, Air and Soil Pollution* **118,** 53–64.

Siciliano, S. D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumier, D., Ouellette, D., Roy, R., Whyte, L. G., Banks, M. K., Schwab, P., Lee, K., and Greer, C. W. (2001). Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Applied and Environmental Microbiology* **67,** 2469–2475.

Siciliano, S. D., and Germida, J. J. (1997). Bacterial inoculants of forage grasses that enhance degradation of 2-chlorobenzoic acid in soil. *Environmental Toxicology and Chemistry* **16,** 1098–1104.

Siciliano, S. D., and Germida, J. J. (1998a). Degradation of chlorinated benzoic acid mixtures by plant–bacteria associations. *Environmental Toxicology and Chemistry* **17,** 728–733.

Siciliano, S. D., and Germida, J. J. (1998b). Mechanisms of phytoremediation: Biochemical and ecological interactions between plants and bacteria. *Environmental Reviews* **6,** 65–79.

Siciliano, S. D., Germida, J. J., Banks, K., and Greer, C. W. (2003). Changes in microbial community composition and function during a polyaromatic hydrocarbon phyto-remediation field trial. *Applied and Environmental Microbiology* **69,** 483–489.

Siciliano, S. D., and Greer, C. W. (2000). Plant–bacterial combinations to phytoremediate soil contaminated with high concentrations of 2,4,6-trinitrotoluene. *Journal of Environmental Quality* **29,** 311–316.

Simon, H. M., Dodsworth, J. A., and Goodman, R. M. (2000). Crenarchaeota colonize terrestrial plant roots. *Environmental Microbiology* **2,** 495–505.

Six, J., Conant, R. T., Paul, E. A., and Paustian, K. (2002). Stabilization mechanisms of soil organic matter: Implications for C-saturation of soils. *Plant and Soil* **241,** 155–176.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., and Berg, G. (2001). Bulk and rhizosphere soil bacterial communities studied by gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. *Applied and Environmental Microbiology* **67,** 4742–4751.

Smit, E., Leeflang, P., Glandorf, B., van Elsas, J. D., and Wernars, K. (1999). Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Applied and Environmental Microbiology* **65,** 2614–2621.

Smith, M. J., Lethbridge, G., and Burns, R. G. (1999). Fate of phenanthrene, pyrene, and benzo a pyrene during biodegradation of crude oil added to two soils. *FEMS Microbiology Letters* **173,** 445–452.

Soderberg, K. H., and Baath, E. (1998). Bacterial activity along a young barley root measured by the thymidine and leucine incorporation techniques. *Soil Biology & Biochemistry* **30,** 1259–1268.

Soeder, C. J., Papaderos, A., Kleespies, M., Kneifel, H., Haegel, F.-H., and Webb, L. (1996). Influence of phytogenic surfactants (quillaya saponin and soya lecithin) on bioelimination of phenanthrene and fluoranthrene by three bacteria. *Applied Microbiology and Biotechnology* **44,** 654–659.

Sorensen, J., Jensen, L. E., and Nybroe, O. (2001). Soil and rhizosphere as habitats for *Pseudomonas* inoculants: New knowledge on distribution, activity, and physiological state derived from micro-scale and single-cell studies. *Plant and Soil* **232,** 97–108.

Sorensen, S. J., and Jensen, L. E. (1998). Transfer of plasmid RP4 in the spermosphere and rhizosphere of barley seedling. *Antonie van Leeuwenhoek* **73,** 69–77.

Stach, J. E. M., and Burns, R. G. (2002). Enrichment versus biofilm culture: A functional and phylogenetic comparison of polycyclic aromatic hydrocarbon-degrading microbial communities. *Environmental Microbiology* **4,** 169–182.

Steinberg, S. M., Pignatello, J. J., and Sawhney, B. L. (1987). Persistence of 1,2-dibromoethane in soil: Entrapment in intraparticle micropores. *Environmental Science and Technology* **21,** 1201–1208.

Stuart-Keil, K. G., Hohnstock, A. M., Drees, K. P., Herrick, J. B., and Madsen, E. L. (1998). Plasmids responsible for horizontal transfer of naphthalene catabolism genes between bacteria at a coal tar-contaminated site are homologous to pDTG1 from *Pseudomonas putida* NCIB 9816–4. *Applied and Environmental Microbiology* **64,** 3633–3640.

Tesar, M., Reichenauer, T. G., and Sessitsch, A. (2002). Bacterial rhizosphere populations of black poplar and herbal plants to be used for phytoremediation of diesel fuel. *Soil Biology & Biochemistry* **34,** 1883–1892.

Thirup, L., Johansen, A., and Winding, A. (2003). Microbial succession in the rhizosphere of live and decomposing barley roots as affected by the antagonistic strain *Pseudomonas fluorescens* DR54-BN14 or the fungicide imazalil. *FEMS Microbiology Ecology* **43,** 383–392.

Tiedje, J. M., Asuming-Brempong, S., Nusslein, K., Marsh, T. L., and Flynn, S. J. (1999). Opening the black box of soil microbial diversity. *Applied Soil Ecology* **13,** 109–122.

Toal, M. E., Yeomans, C., Killham, K., and Meharg, A. A. (2000). A review of rhizosphere carbon flow modelling. *Plant and Soil* **222,** 263–281.

Top, E. M., Springael, D., and Boon, N. (2002). Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters. *FEMS Microbiology Ecology* **42,** 199–208.

Top, E. M., van Daele, P., De Saeyer, N., and Forney, L. J. (1998). Enhancement of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation in soil by dissemination of catabolic plasmids. *Antonie van Leeuwenhoek* **73,** 87–94.

Troxler, J., Azelvandre, P., Zala, M., Defago, G., and Haas, D. (1997). Conjugative transfer of chromosomal genes between fluorescent pseudomonads in the rhizosphere of wheat. *Applied and Environmental Microbiology* **63,** 213–219.

Uren, N. C. (2001). Types, amounts and possible functions of compounds released into the rhizosphere by soil-grown plants. *In* ''The Rhizosphere: Biochemistry and Organic Substances at the Soil–Plant Interface'' (R. Pinton, Z. Varanini, and P. Nannipieri, eds.), pp. 19–40. Marcel Dekker, Inc., New York.

Valls, M., and de Lorenzo, V. (2002). Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. *FEMS Microbiology Reviews* **26,** 327–338.

van der Meer, J. R., Werlen, C., Nishino, F., and Spain, J. C. (1998). Evolution of a pathway for chlorobenzene metabolism leads to natural attenuation in contaminated groundwater. *Applied and Environmental Microbiology* **64,** 4185–4193.

van Elsas, J. D., and Bailey, M. J. (2002). The ecology of transfer of mobile genetic elements. *FEMS Microbiology Ecology* **42,** 187–197.

van Veen, J. A., van Overbeek, L. S., and van Elsas, J. D. (1997). Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews* **61,** 121–135.

Volkering, F., Breure, A. M., and Rulkens, W. H. (1998). Microbiological aspects of surfactant use for biological soil remediation. *Biodegradation* **8,** 401–417.

Walton, B. T., Hoylman, A. M., Perez, M. M., Anderson, T. A., Johnson, T. R., Guthrie, E. A., and Christman, R. F. (1994). Rhizosphere microbial communities as a plant defense against toxic substances in soils. *In* ''Bioremediation through Rhizosphere Technology'' (T. A. Anderson and J. R. Coats, eds.), pp. 82–92. American Chemical Society, Washington, DC.

Wang, C. J., Thiele, S., and Bollag, J. M. (2002). Interaction of 2,4,6-trinitrotoluene (TNT) and 4-amino-2,6-dinitrotoluene with humic monomers in the presence of oxidative enzymes. *Archives of Environmental Contamination and Toxicology* **42,** 1–8.

Wasaki, J., Yamamura, T., Shinano, T., and Osaki, M. (2003). Secreted acid phosphatase is expressed in cluster roots of lupin in response to phosphorus deficiency. *Plant and Soil* **248,** 129–136.

Weller, D. M., Raaijmakers, J. M., Gardener, B. B. M., and Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annual Review of Phytopathology* **40,** 309–348.

Wetzel, S. C., Banks, M. K., and Schwab, A. P. (1997). Rhizosphere effects on the degradation of pyrene and anthracene in soil. *In* ''Phytoremediation of Soil and Water Contaminants'' (E. L. Kruger, T. A. Anderson, and J. R. Coats, eds.), pp. 254–261. American Chemical Society, Washington, DC.

White, J. C., Kelsey, J. W., Hatzinger, P. B., and Alexander, M. (1997). Factors affecting sequestration and bioavailability of phenanthrene in soils. *Environmental Toxicology and Chemistry* **16,** 2040–2045.

Wieland, G., Neumann, G., and Backhaus, H. (2001). Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development. *Applied and Environmental Microbiology* **67,** 5849–5854.

Wilcke, W. (2000). Polycyclic aromatic hydrocarbons (PAHs) in soil—a review. *Journal of Plant Nutrition and Soil Science—Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* **163,** 229–248.

Wiltse, C. C., Rooney, W. L., Chen, Z., Schwab, A. P., and Banks, M. K. (1998). Greenhouse evaluation of agronomic and crude oil-phytoremediation potential among alfalfa genotypes. *Journal of Environmental Quality* **27,** 169–173.

Winterlin, W. L., Seiber, J. N., Craigmail, A., Baier, T., Woodrow, J., and Walker, G. (1989). Degradation of pesticide waste taken from a highly contaminated soil evaporation pit in California. *Archives of Environmental Contamination and Toxicology* **18,** 734–747.

Yang, C. H., and Crowley, D. E. (2000). Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Applied and Environmental Microbiology* **66,** 345–351.

Yee, D. C., Maynard, J. A., and Wood, T. K. (1998). Rhizoremediation of trichloroethylene by a recombinant root-colonizing *Pseudomonas fluorescens* strain expressing

toluene *ortho*-monooxygenase constitutively. *Applied and Environmental Microbiology* **64,** 112–118.

Yoshitomi, K. J., and Shann, J. R. (2001). Corn (*Zea mays* L.) root exudates and their impact on ^{14}C-pyrene mineralization. *Soil Biology & Biochemistry* **33,** 1769–1776.

Young, C. S., and Burns, R. G. (1993). Detection, survival, and activity of bacteria added to soil. *In* ''Soil Biochemistry,'' Vol. 8 (J.-M. Bollag and G. Stotzky, eds.), pp. 1–63. Marcel Dekker, New York.

Zablotowicz, R. M., Locke, M. A., and Hoagland, R. E. (1997). Aromatic nitroreduction of acifluorfen in soils, rhizospheres, and pure cultures of rhizobacteria. *In* ''Phytoremediation of Soil and Water Contaminants'' (E. L. Kruger, T. A. Anderson, and J. R. Coats, eds.), pp. 38–53. American Chemical Society, Washington, DC.

Zhou, J., Xia, B., Treves, D. S., Wu, L.-Y., Marsh, T. L., O'Neill, R. V., Palumbo, A. V., and Tiedje, J. M. (2002). Spatial and resource factors influencing high microbial diversity in soil. *Applied and Environmental Microbiology* **68,** 326–334.

Anaerobic Dehalogenation of Organohalide Contaminants in the Marine Environment

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I. Introduction—The Halogen Cycle

Halogenated organic compounds constitute one of the largest groups of environmental chemicals and the development and production of new halogenated organic compounds, including aliphatic, aromatic, and heterocyclic derivatives, has increased over the last 100 years. These compounds are integral to a variety of industrial applications, including use as solvents, degreasing agents, biocides, pharmaceuticals, plasticizers, hydraulic and heat transfer fluids, intermediates for chemical synthesis, flame retardants, and numerous other industrial functions. Halogenated compounds are also produced as by-products in various industrial processes. The majority of these compounds are chlorinated, but

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brominated, fluorinated, and iodinated compounds also have industrial applications (Häggblom and Bossert, 2003). Their use in industry and agriculture account for a large entry of these chemicals into the environment, resulting in widespread dissemination and environmental contamination, with estuarine and marine sediments as significant sinks.

Although organohalide compounds are typically considered to be anthropogenic compounds of industrial origin, they have their counterpart in several thousands of naturally occurring biogenic and geogenic organohalides, representing most classes of organic chemicals (Häggblom and Bossert, 2003; Gribble, 1998, 2000). Biogenic producers of organohalides have been found in both marine and terrestrial environments and include bacteria, fungi, plants, sponges, worms, insects, and mammals (Faulkner, 1980; Gribble, 1998, 1999, 2000; Neidleman and Giegert, 1986; Siuda and DeBerbardis, 1973; van Pee 1996, 2001). Geogenic sources of organohalides include forest fires and volcanic emissions (Isidorov *et al.*, 1990; Jordan *et al.*, 2000). Natural sources account for a significant portion of the global organohalogen budget. Over 3000 naturally produced organohalides (mainly brominated and chlorinated metabolites) have been identified to date, in almost all classes of organic chemicals (Gribble, 1994, 1998; Key *et al.*, 1997; O'Hagan and Harper, 1999).

The marine environment is a particularly rich source of biogenic organohalides, which are produced by a diversity of marine organisms, including mollusks, algae, polychaetes, jellyfish, and sponges (Ashworth and Cormier, 1967; Baker and Duke, 1973; Fielman *et al.*, 1999; Garson *et al.*, 1994; Schmitz and Gopichand, 1978). For example, marine macroalgae produce a wide range of volatile chlorinated, brominated, and iodinated hydrocarbons, the most abundant of which is bromoform (Giese *et al.*, 1999). A number of sponge species in the phylum *Porifera*, in particular *Aplysina* sp., have been shown to produce an amazing variety of brominated metabolites, including bromoindoles, bromophenols, polybrominated diphenyl ethers, and even brominated dibenzo-*p*-dioxins (Ebel *et al.*, 1997; Gribble, 1999; Norte and Fernandez, 1987; Utkina *et al.*, 2001). Bromine-containing metabolites can account for over 10% of the sponge dry weight (Turon *et al.*, 2000). These compounds may serve as a chemical defense against predators and inhibit biofouling. In addition, *Aplysina* sponges harbor large amounts of bacteria which can amount to 40% of the biomass of the animal, and it has been hypothesized that some of the organobromine compounds may in fact be synthesized by bacteria associated with the sponge (Hentschel *et al.*, 2001). Acorn worms (phylum *Hemichordata*) represent another group of marine animals with an

incredible capacity to produce organobromine compounds. 2,6-Dibromophenol, 2,4,6-tribromophenol, and brompyrroles are produced by various species, apparently as defensive agents against predation (Chen *et al.*, 1991; Fielman *et al.*, 1999; King, 1986, 1988; Yoon *et al.*, 1994). The concentration of brominated phenols may reach several hundred μmol per liter in the burrow lining of these worms.

II. Microbial Transformation of Organohalides and the Influence of Electron Donors and Alternate Electron Acceptors

Microbial degradation is one of the key factors that determine the ultimate fate of organohalides in the environment, with cleavage of the carbon–halogen bond being one of the critical steps. Microbial degradation requires the presence of enzymes that cleave this bond under physiological conditions. Evolution over the last 3.6 billion years has provided ample time for the development of metabolic pathways necessary for the biodegradation of most halogenated compounds, although many of the new, anthropogenic xenobiotic organohalides are often problematic.

Microorganisms have evolved a variety of metabolic strategies for cleaving the carbon–halogen bond (for reviews, see Häggblom, 1992; Häggblom and Bossert, 2003; Holliger *et al.*, 2003; Löffler *et al.*, 2003). Dehalogenation reactions comprise different strategies, where organohalides serve either as electron donors (and carbon sources) or electron acceptors, as follows: (1) the organohalide serves as a carbon and energy source and dehalogenation occurs in order to break down the carbon backbone, (2) the organohalide serves as an alternate electron acceptor for anaerobic respiration, termed (de)halorespiration, (3) dehalogenation occurs as a detoxification mechanism, or (4) the organohalide is dehalogenated through fortuitous reactions that do not yield any benefit to the organism. Structural features of a particular compound are central in determining which dehalogenation mechanisms are possible for the overall biodegradation of the compound. Metabolic strategies for degradation vary between types of halogenated organic compounds (e.g., aliphatic vs aromatic), as well as redox environments, (e.g., aerobic vs anaerobic) and the availability of electron donors and acceptors.

In the absence of oxygen, organic compounds can be metabolized by diverse microbial communities using alternate electron acceptors. This includes the use of organohalides as an electron acceptor for respiration (dehalorespiration). The presence and absence of other respiratory electron acceptors will have a direct impact on the

activity of the microbial community and biodegradability of organic compounds (Bossert *et al.*, 2003). In terms of microbial respiration and carbon flow in anoxic environments, the most important electron acceptors are nitrate, sulfate, Fe(III), Mn(IV), and carbonate, which result in the processes of denitrification, sulfidogenesis, iron reduction, manganese reduction, and methanogenesis, respectively (Ghiorse and Wilson, 1988). At the high sulfate concentrations found in seawater (20 to 30 mM), sulfate is one of the predominant electron acceptors affecting degradation of organic material in estuarine and marine sediments (Capone and Kiene, 1988). Iron reduction is important in some marine sediments, while denitrification can be significant in regions of high nitrate input from sewage discharge or agricultural runoff (Capone and Kiene, 1988; Nealson and Saffarini, 1994). The microorganisms that are active under the different redox conditions are physiologically and phylogenetically distinct. The prevailing electron-accepting processes that participate in dehalogenation are determined not only by the availability of electron-accepting species, but also by the concentration of electron donors, especially hydrogen, in the environment (Fennell and Gossett, 2003). Competition for hydrogen (or other simple organic electron donors) is key in determining the succession of microorganisms and redox processes (Fig. 1).

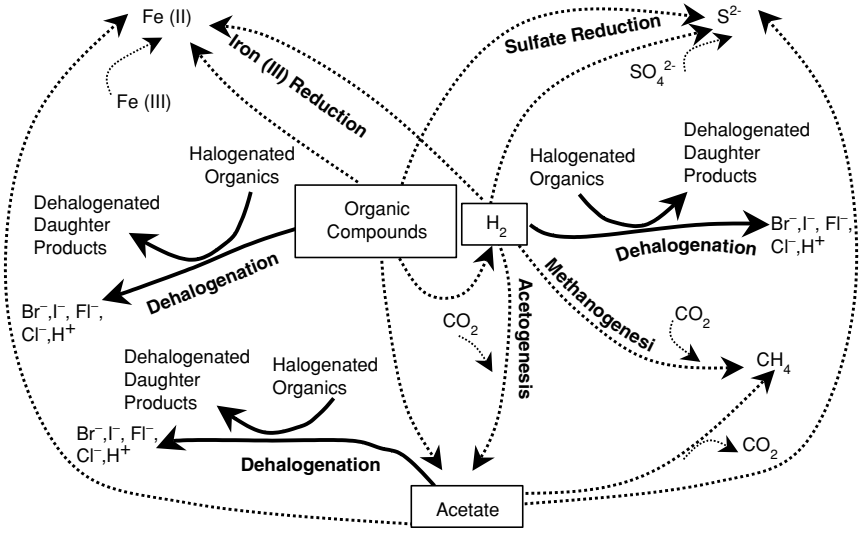


FIG. 1. Competing electron flow pathways in anaerobic sediments (after Fennell and Gossett, 2003).

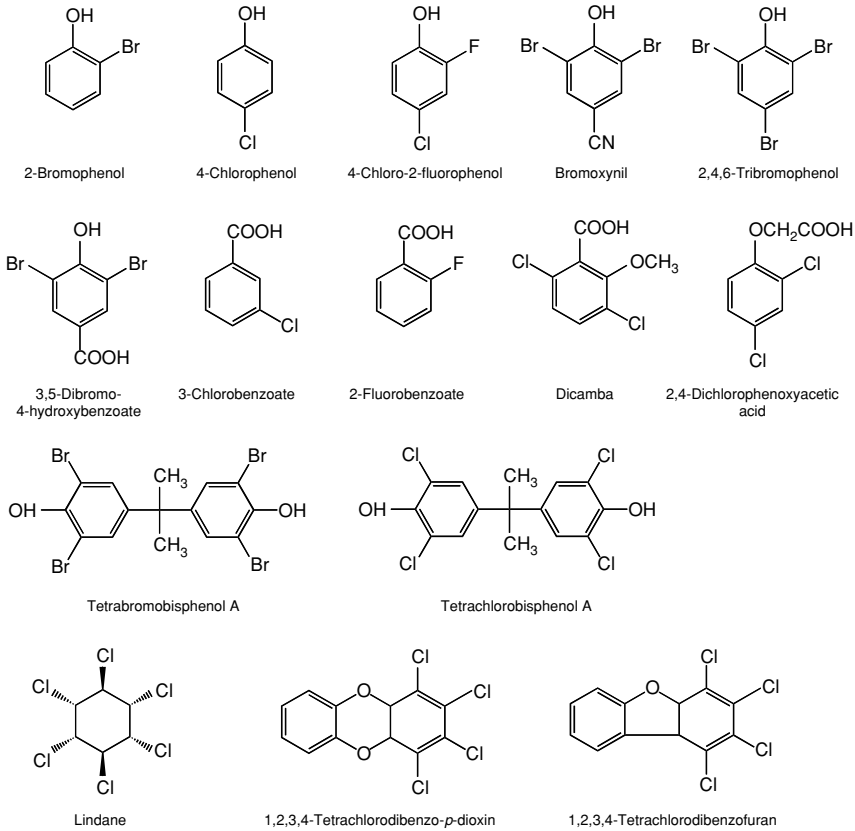


FIG. 2. Structures of halogenated compounds under study.

Our laboratories are examining the anaerobic degradation of halogenated aromatic compounds in estuarine and marine sediments and how alternate electron acceptors influence dehalogenation rates and degradation mechanisms. The objective of our work has been to determine the capacity for dehalogenation and degradation of organohalides by anaerobic microbial communities in anoxic sediments and to study how these processes can be stimulated for bioremediation of contaminated sites. Some of the halogenated compounds under study are shown in Fig. 2.

Microbial degradation of halogenated organic compounds in anoxic estuarine and marine sediments is widely observed (Table 1) and generally proceeds first via reductive dehalogenation. Several studies have shown that alternate electron acceptors, in particular sulfate, impede

TABLE I

DEHALOGENATIONS OBSERVED IN THE MARINE ENVIRONMENT

Organohalide	Environment	Redox condition	Reference
Halogenated phenols			
2,4-Dibromophenol	Marine sediment from burrow of hemichordate	Anaerobic marine broth	King, 1988
2,4-Dichlorophenol			
Halogenated phenols	Estuarine sediment enrichments	Sulfidogenic	Häggblom and Young, 1995
Monochlorinated phenols	Estuarine sediment cultures and enrichments	Denitrifying, sulfidogenic, and methanogenic	Häggblom *et al.*, 1993
4-Chloro-2-fluorophenol	Estuarine sediment enrichments	Sulfidogenic	Häggblom, 1998
4-Chloro-3-fluorophenol			
2-Chlorophenol	Isolate (*Desulfovibrio dechloracetivorans*) from marine sediment	Dehalorespiration	Sun *et al.*, 2000
Monobrominated phenols	Estuarine sediment cultures	Iron-reducing, sulfidogenic, and methanogenic	Monserrate and Häggblom, 1997
2-Bromophenol	Enrichments from estuarine sediment	Sulfidogenic	Knight *et al.*, 1999
2-Bromophenol	Enrichment culture	Sulfidogenic	Rhee *et al.*, 2003
4-Bromophenol	Marine sediment from burrow of hemichordate		Steward and Lovell, 1997
2,4,6-Tribromophenol	Pure culture isolated from hemichordate burrow	Sulfidogenic	Steward *et al.*, 1995
2,4,6-Tribromophenol	Marine sediment from burrow of hemichordate	Sulfidogenic	Watson *et al.*, 2000

2,4,6-Tribromophenol	Isolate (*Desulfovibrio* sp.) from marine sediment	Dehalorespiration	Boyle *et al.*, 1999c
Dichlorophenols (2,4-DCP, 3,4-DCP)	Marine sediment	Methanogenic	Hale *et al.*, 1997
Bromophenols	Enrichments from marine sponges	Methanogenic, sulfidogenic	Ahn *et al.*, 2002, 2003
Halogenated benzoic acids			
Monochlorinated benzoic acids	Estuarine sediment culture	Denitrifying, sulfidogenic, and methanogenic	Häggblom *et al.*, 1993
3-Chlorobenzoate 4-Chlorobenzoate	Enrichments from estuarine sediment	Denitrifying	Häggblom *et al.*, 1996
3-Chlorobenzoate	Isolate (*Desulfomonile limimaris*) from marine sediment	Dehalorespiration	Sun *et al.*, 2001
3,5-Dibromo-4-hydroxybenzoate	Estuarine sediment culture	Methanogenic, sulfidogenic, iron-reducing, denitrifying	Knight *et al.*, 2003
Fluorinated benzoic acids	Estuarine sediment cultures	Denitrifying	Vargas *et al.*, 2000
Halogenated hydrocarbons			
Chlorobenzenes	Estuarine sediment culture	Sulfidogenic	Masunaga *et al.*, 1996
1,2,4-Trichlorobenzene	Estuarine sediment culture		Yonezawa *et al.*, 1994
Tetrachloroethene	Estuarine sediment culture	Sulfidogenic	Mazur and Jones, 2001
Biocides			
Lindane(γ-hexachlorocyclohexane)	Marine sediment culture	Sulfidogenic	Boyle *et al.*, 1999a
2,4-Dichlorophenoxyacetic acid	Estuarine sediment culture	Sulfidogenic	Boyle *et al.*, 1999b
Bromoxynil	Estuarine sediment culture	Methanogenic, sulfidogenic, iron-reducing	Knight *et al.*, 2003

(*continues*)

TABLE I (*Continued*)

Organohalide	Environment	Redox condition	Reference
Polychlorinated biphenyls, dioxins, and halogenated flame retardants			
Arcolors	Estuarine sediment culture	Methanogenic, sulfidogenic	Alder *et al.*, 1993
Polychlorinated biphenyls	Estuarine sediment culture	Methanogenic	Wu and Wiegel, 1997
2,3,5,6-Tetrachlorobiphenyl	Estuarine sediment culture	Methanogenic	Holoman *et al.*, 1999
2,3,7,8-tetrachlorodibenzo-*p*-dioxin	Estuarine sediment	Methanogenic	Albrecht *et al.*, 1999
Polychlorinated dibenzo-*p*-dioxins	Estuarine sediment	Methanogenic	Barkovskii and Adriaens, 1996
1,2,3,4-tetrachlorodibenzo-*p*-dioxin	Estuarine sediment culture	Iron-reducing, sulfidogenic, and methanogenic	Vargas *et al.*, 2001; Fennell *et al.*, 2002
Tetrabromobisphenol A, Tetrachlorobisphenol A	Estuarine sediment culture	Sulfidogenic, methanogenic	Voordeckers *et al.*, 2002

the enrichment of dehalogenating populations, or that interspecific competition for electron donors inhibits anaerobic reductive dechlorination (see Bossert *et al.*, 2003; Mohn and Tiedje, 1992; Häggblom *et al.*, 2000; Suflita and Townsend, 1995). Although early studies concluded that sulfate-reducing bacteria may outcompete dehalogenating microorganisms for hydrogen, these interactions appear to be more complex (Townsend *et al.*, 1997). For example, in studies with *Desulfomonile tiedjei* DCB-1, thiosulfate and sulfite competed with 3-chlorobenzoate for reducing equivalents and their reduction was favored over dechlorination in pure cultures (DeWeerd and Suflita, 1990; DeWeerd *et al.*, 1991; Townsend and Suflita, 1997). However, in strict competition for hydrogen between sulfate-reducing and dehalogenating populations, dehalogenating bacteria appear to be able to outcompete sulfate reducers for hydrogen. For example, the hydrogen threshold achieved by tetrachloroethene-dechlorinating populations in estuarine sediments was 0.5 nmol, less than that of the hydrogen level achieved by sulfate reduction in the same system (Mazur and Jones, 2001). Recently, anaerobic dehalogenating bacteria in the genera *Desulfovibrio* (Boyle *et al.*, 1999c) and *Desulfomonile* (Sun *et al.*, 2001) that are not inhibited by sulfate have been isolated from estuarine and marine environments.

We have shown that reductive dehalogenation can proceed simultaneously with other dominant electron-accepting processes, with further degradation of the dehalogenated product coupled to methanogenesis, sulfate reduction, or iron (III) reduction (Häggblom, 1998; Häggblom *et al.*, 1993; Knight *et al.*, 1999; Monserrate and Häggblom, 1997). Differences in substrate specificity, differences in apparent rate of dehalogenation, and unique community profiles indicate that distinct microbial populations are responsible for reductive dehalogenation under different dominant terminal electron-accepting conditions (Knight *et al.*, 1999; Häggblom *et al.*, 2000). Reductive dehalogenation is a promising mechanism for the removal of organohalides from estuarine and marine sediments. While the process has much potential, a key aspect of the technology—the nature and capability of the intrinsic microbial community—is often unknown.

III. Molecular and Biochemical Characterization of Dehalogenating Consortia in Marine Environments

After initial demonstration of dehalogenating activity in anaerobic estuarine and marine sediments, we have pursued the characterization of the structure and diversity of microbial food webs active during

dehalogenation. We demonstrated that sulfidogenic and iron-reducing microbial populations can dehalogenate and mineralize chlorinated and brominated aromatic compounds and that the dehalogenating populations are distinct under different redox conditions, e.g., sulfidogenic, iron-reducing, or methanogenic (Knight *et al.*, 1999; Häggblom *et al.*, 2000). More detailed characterization of these anaerobic microbial communities, combining physiological analysis with biomolecular tools, is needed to fully understand the role that they play in the degradation of organohalides in marine sediments.

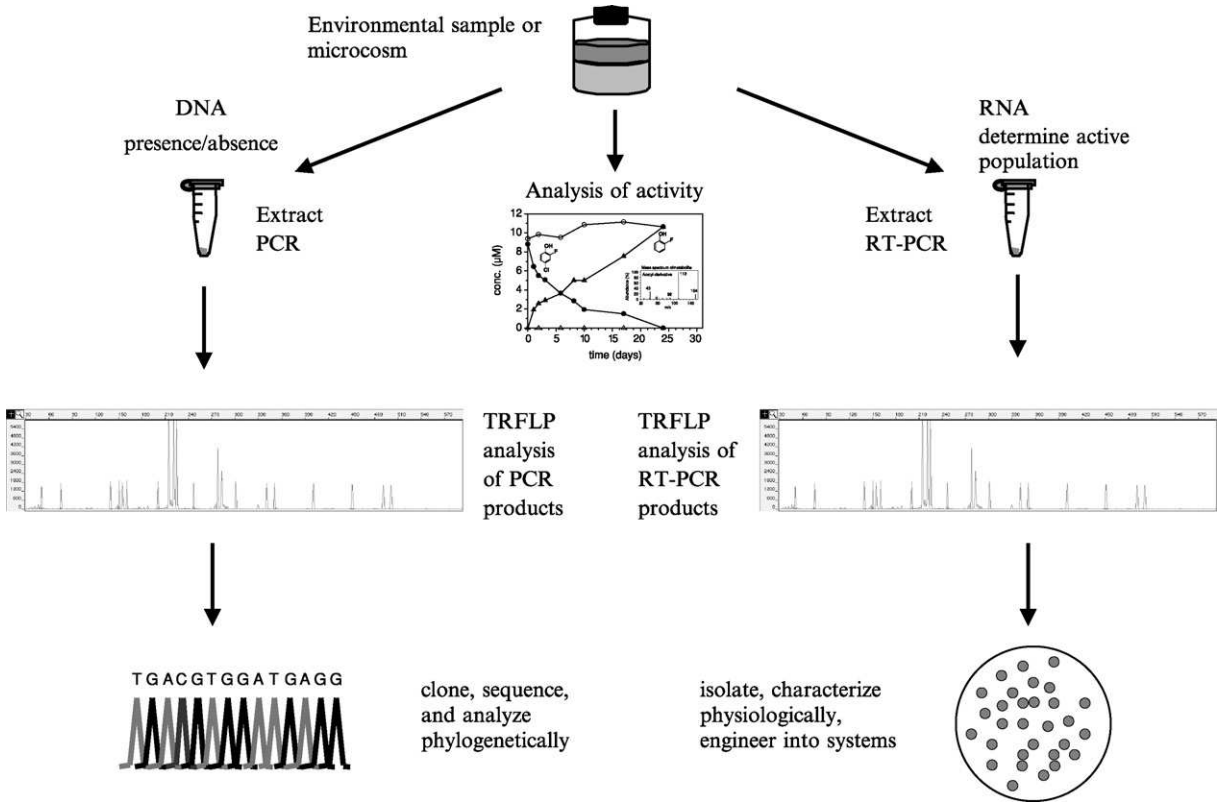
Since many bacteria generally lack defining morphological or biochemical characteristics, a molecular-based strategy of characterizing microbial communities has become necessary to identify microorganisms capable of dehalogenation. Within the last several decades, efforts have focused on identifying specific molecules present within the bacteria to differentiate the various members of the microbial community. These biomarkers can include lipids, proteins, and nucleic acids. Currently, the simplest, most popular way to identify bacteria is by PCR amplification/sequencing of 16S rRNA genes. This characterization of SSU genes has become routine in many labs (for reviews, see Head *et al.*, 1998; Hugenholtz *et al.*, 1998; Amann *et al.*, 1995; Torsvik *et al.*, 1996; Woese, 1987). This approach has been used in a variety of studies for detecting microorganisms involved in dehalogenation (Knight *et al.*, 1999; Löffler *et al.*, 2000; Fennell *et al.*, 2001; Richardson *et al.*, 2002; Becker *et al.*, 2001; Holoman *et al.*, 1998; Wu *et al.*, 2002). Most studies have been able to identify the bacteria present within microcosms, bioreactors, and in the field, but presence/absence does little to elucidate activity and cannot absolutely determine the microorganism(s) capable of or responsible for the bulk of the dehalogenation. This limitation has been partially remedied by assaying ribosomal RNA during dehalogenation to monitor the active populations. The relationship between ribosomal RNA content and growth rate has been well established for bacteria (for references, see Kerkhof and Ward, 1993). Later work extended the range of growth rates exhibiting a relationship between rRNA content and growth to environmentally realistic doubling times (Kemp *et al.*, 1993; Kerkhof and Ward, 1993; Poulsen *et al.*, 1993). The RNA-based approach was implemented in a 2-chlorophenol bioreactor by Becker *et al.* (2001) with oligonucleotide probes to determine whether the *Desulfovibribionaceae* were involved in dehalogenation. However, the specific microorganism(s) responsible for dehalogenation could not be determined using this group-specific approach. A more specific method is

outlined below and in Fig. 3 (see color insert) with what we have termed ''knock-out enrichments.''

Knock-out enrichment assays coupled with time course assays of ribosomal activity were used to determine which consortia members are responsible for dehalogenation in a previously characterized sulfate-reducing, 2-bromophenol (2BP) dehalogenating-consortium. 2BP dehalogenation occurred in the presence or absence of sulfate. In the absence of sulfate, debromination occurred concurrently with hydrogen utilization and phenol accumulated. T-RFLP analyses coupled with reverse transcription-PCR elucidated the involved phylotypes. *Desulfovibrio* strain 2BP-48 was actively producing ribosomes in enrichments exhibiting 2-BP debromination, but not in enrichments exhibiting phenol degradation alone (Häggblom *et al.*, 2001; Fennell *et al.*, unpublished data; Rhee *et al.*, unpublished data). This organism was obtained in co-culture with a second *Desulfovibrio* strain, BP212, which did not carry out dehalogenation or phenol degradation. The dehalogenating strain reductively dehalogenated *ortho*-substituted halogens of phenols (2BP, 2,6-dibromophenol, and 2-iodophenol) using lactate or formate as electron donor with production of phenol as the dehalogenation end product. The strain could couple reductive dehalogenation to growth and is capable of simultaneous sulfidogenesis and reductive dehalogenation in the presence of sulfate.

IV. Detection of Putative Reductive Dehalogenase-encoding Genes in Dehalogenating Consortia and Marine Sediments

An alternative to the indirect method using ribosomes to elucidate dehalogenating microorganisms in consortia or natural environments involves the use of functional genes. Recently, several membrane-bound reductive dehalgenases (RDH) have been purified and characterized: the tetrachloroethene reductase (*pceA*) gene from *Dehalospirillum multivorans* (Neumann *et al.*, 1998) and a *Desulfitobacterium* sp. (Suyama *et al.*, 2002), the 2-chlorophenol reductase (*cprA*) gene from *Desulfitobacterium dehalogenans* (van de Pas *et al.*, 1999), and the trichloroethene reductase gene (*tceA*) from *Dehalococcoides ethenogenes* (Magnuson *et al.*, 2000). This has led to the detection of putative RDH genes from an anaerobic bioreactor (von Wintzingerode *et al.*, 2001) and a debrominating consortium from marine sediments (Rhee *et al.*, 2003). Several sets of degenerate PCR primers to amplify RDH gene fragments have been designed and putative RDH genes have been cloned and identified. The phylogenetic tree in Fig. 4 shows the diversity of RDH genes and shows the relationship


Environmental sample or microcosm
DNA
presence/absence
Extract
PCR
Analysis of activity
conc. (μM)
time (days)
RNA
determine active population
Extract
RT-PCR
TRFLP analysis of PCR products
TRFLP analysis of RT-PCR products
TGACGTGGATGAGG
clone, sequence, and analyze phylogenetically
isolate, characterize physiologically, engineer into systems

between known RDH genes and RDH gene clones from environments. The amino acid sequences deduced from the C-terminal region of the enzymes were compared and showed high similarity. However, Rhee *et al.* (2003) determined that several of these putative RDH genes were linked to transposonlike sequences and may, in fact, be pseudogenes. Thus, it is recommended that additional assays at the mRNA level may be necessary to identify expressed RDH genes in natural samples to monitor dehalogenating populations.

V. Dehalogenation by Anaerobic Bacteria in Marine Sponges—Natural Sources of Dehalogenating Bacteria

Marine sponges are natural sources of brominated organic compounds (up to 12% of the dry weight) such as bromoindoles, bromophenols, and bromopyrroles. *Aplysina aerophoba* sponges harbor large amounts of bacteria, which can amount to 40% of the biomass of the animal (Hentschel *et al.*, 2001). We have demonstrated that the sponge harbors a large population of anaerobic dehalogenating bacteria (Ahn *et al.*, 2002, 2003). TRFLP/DGGE 16S rRNA gene analysis suggested that distinct dehalogenating microbial populations were enriched on the different halogenated compounds. With primers designed for reductive dehalogenase genes, we could also show the presence of several putative reductive dehalogenase genes in sponge-associated microorganisms. Phylogenetic analysis showed that the dehalogenase target genes formed three distinct groups separate from other known reductive dehalogenase gene sequences (Fig. 4). The diversity of gene motifs detected suggests that the sponge microbial community is capable of dehalogenating a broad range of organohalide compounds. Sponges are thus an unexplored source of anaerobic bacteria with diverse dehalogenating capabilities. Anaerobic dehalogenating bacteria have also been found in the burrows of bromophenol- and bromopyrrole-producing hemichordates (King, 1986, 1988; Steward *et al.*, 1995), suggesting that dehalorespiration might be widely distributed in natural marine environments, a reflection of the prevalence of biogenic brominated organic compounds in the environment.

FIG. 3. Three-pronged experimental approach to characterizing microbial populations responsible for dehalogenation. DNA-based assays can determine presence/absence and phylogenetic affiliation. RNA-based assays can monitor the active populations involved in dehalogenation. Chemical analysis of activity can demonstrate dehalogenating processes, delineate metabolic pathways, and verify molecular measurements.

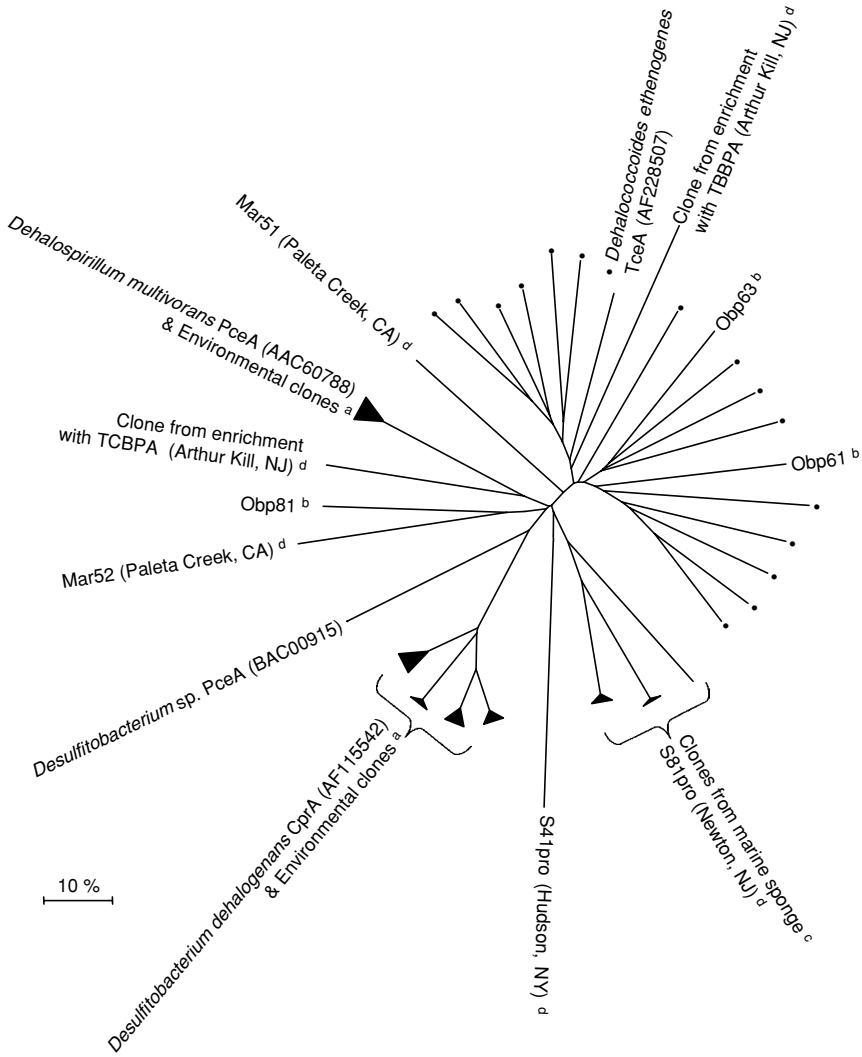


FIG. 4. Diversity of RDH genes. The phylogenetic tree shows the relationship of the amino acid sequence deduced for the C-terminal region between known RDH genes and RDH gene motifs cloned from marine environments. Functional genes were cloned from dehalorespiring bacteria. Symbols and notes: •, putative gene from *Dehalococcoides ethenogenes* genome (Smidt, 2001); a, environmental clone (von Wintzingerode *et al.*, 2001); b, clone from 2-bromophenol enrichment culture (Rhee *et al.*, 2003); c, clone from marine sponge (Ahn *et al.*, 2003); d, clone from marine sediment using degenerate primer of Rhee *et al.* (2003); TCBPA, tetrachlorobisphenol A; TBBPA, tetrabromobisphenol A. The bar represents 10% estimated sequence divergence.

VI. *In situ* Enhancement of Anaerobic Microbial Dechlorination of Polychlorinated Dibenzo-*p*-dioxins and Dibenzofurans in Marine and Estuarine Sediments

The management of marine and estuarine sediments contaminated with toxic organic compounds, including polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), is a major problem with far-reaching economic and ecological consequences. Enhancement of microbial degradation of PCDD/Fs *in situ* is an attractive remediation alternative that could potentially detoxify sediments, avoid the problematic redistribution of contaminants that is associated with dredging, and decrease the cost of sediment management. Application of bioremediation to PCDD/F-contaminated marine and estuarine sediments has been limited by lack of fundamental knowledge about the microorganisms responsible for their degradation, including anaerobic reductive dechlorination, the first step required for the ultimate complete degradation of highly chlorinated congeners.

Engineered bioremediation systems employing reductive dechlorination to remove organohalides such as the chlorinated solvents have been used successfully in freshwater aquifers and have been studied for bioremediation of haloaromatic-contaminated soils and sediments. The strategy for enhancing dechlorination stipulates addition of electron donors to stimulate the use of the chlorinated compounds as electron acceptors. Microbial dehalogenation of dioxins has been demonstrated in a variety of soils and sediments (Adriaens and Grbić-Galić, 1994; Beurskens *et al.*, 1995; Ballerstedt *et al.*, 1997; Bunge *et al.*, 2001). Recently, a *Dehalococcoides* strain has been shown to dechlorinate dioxins and can be transferred with dioxins as the sole electron acceptor (Bunge *et al.*, 2003). Low aqueous solubilities and strong sorption of the PCDD/F to sediments however reduce their availability as an electron acceptor for dehalorespiring bacteria and may intrinsically limit growth by bacteria using these compounds (Harms, 1998)—even if adequate electron donor is present. Through addition of alternate halogenated electron acceptors—''haloprimers''—the growth of dehalogenating bacteria or expression of their dehalogenases is potentially decoupled from low substrate (PCDD/F) driving force, if the added halogenated compounds are utilized by microorganisms that can then simultaneously dechlorinate PCDD/F. Adding haloprimers has previously been shown to enhance dechlorination of polychlorinated biphenyls (PCB) (Hartkamp-Commandeur, 1996; Bedard *et al.*, 1998; De Weerd and Bedard, 1999; Cho *et al.*, 2002). This effect has also been observed for dechlorination PCDD, for example. Beurskens *et al.* (1995) demonstrated

dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) in a sediment-free culture enriched from Rhine River sediment on hexachlorobenzene (HCB). Laboratory-scale studies of dioxin dechlorination carried out on sediments from the Passaic River, NJ, a tidally influenced river with sulfidogenic dioxin-contaminanted sediments, were performed by Adriaens and coworkers. They have shown stimulation of biological dechlorination of the dioxin contaminants by the estuarine populations by adding hydrogen or simple organic electron donors such as fatty acids (Barkovskii and Adriaens, 1996; Albrecht *et al.*, 1999). Furthermore, the same group observed a stimulatory effect on dioxin dechlorination by addition of 2-monobromodibenzo-*p*-dioxin (2MBDD) as a surrogate halogenated electron acceptor (Albrecht *et al.*, 1999).

In current work, we are using 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) and 1,2,3,4-tetrachlorodibenzofuran (1,2,3,4-TeCDF) as models to examine the potential for microbial reductive dechlorination of polychlorinated dioxins and dibenzofurans (PCDD/Fs). We are characterizing the PCDD/F dechlorinating capability of native dehalogenating bacteria from different estuarine and marine sites using a variety of electron donor and haloprimer amendments to enhance PCDD/F dechlorination under methanogenic and sulfate-reducing conditions. Haloprimers could potentially be useful in facilitating biotreatment of dredged sediments in confined disposal facilities, for *in situ* bioremediation applications, or for obtaining high concentrations of dehalogenating biomass to amend to sediments.

We examined bromophenols as potential haloprimers because they are produced naturally in marine environments and have been shown to be dehalogenated in the presence of ongoing sulfate reduction (important for marine systems). In addition, after dehalogenation the phenol moiety is rapidly degraded and results in no residual contamination (e.g., Häggblom and Young, 1995; Häggblom *et al.*, 2000). We demonstrated that dechlorination of 1,2,3,4-TeCDD in estuarine sediment cultures from Arthur Kill, NJ, that had been previously enriched on bromophenols was much more extensive than in fresh, unacclimated sediments (Vargas *et al.*, 2001). Although dechlorination of PCDDs was slower in sulfate-reducing sediments, some activity did occur. The bromophenols are dehalogenated to phenol which is mineralized via sulfate reduction or fermented in the absence of sulfate to produce hydrogen and acetate—thus becoming an electron donor (two-for-one action). Complete dehalogenation and mineralization of the haloprimer is highly desirable in bioremediation applications since the treatment should not result in additional residual contaminants in the system.

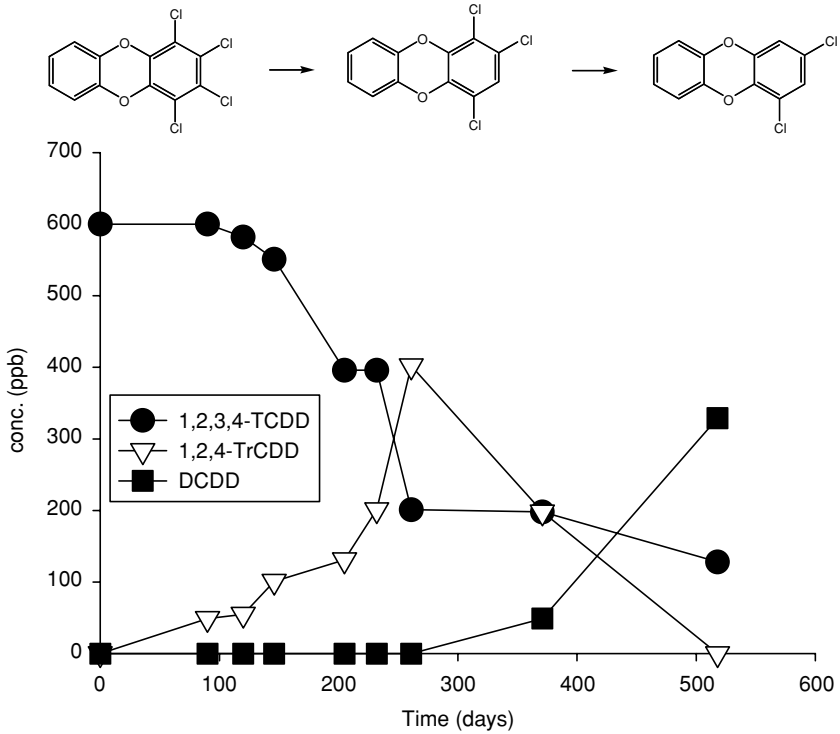


FIG. 5. Reductive dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin in a methanogenic estuarine sediment slurry. Metabolites were identified and quantified by gas chromatography—mass spectrometry (modified from Vargas *et al.*, 2000).

In methanogenic sediment slurries from the Arthur Kill NJ, intertidal strait, 1,2,3,4-TeCDD was reductively dechlorinated in the lateral position to 1,2,3,4-trichlorodibenzo-*p*-dioxin, and further to di- and monochlorinated dibenzo-*p*-dioxins (Fig. 5) (Vargas *et al.*, 2000; Fennell *et al.*, 2002). Lateral dechlorination of polychlorinated dioxins is central to their detoxification, since this would avoid the formation of the toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin product. Bioremediation would be especially useful under conditions where the microbial population removes the lateral chlorines.

Addition of haloprimers or electron donors to stimulate *in situ* bioremediation of organohalide-contaminated sediments is a challenging engineering problem since the addition mechanism must avoid redistribution of the contaminants. Increased engineering research directed at environmentally acceptable amendment of sediments, for example,

in combination with *in situ* stabilization by capping, is an area that is gaining more attention. Fundamental microbial studies are revealing the potential for remedial applications in marine systems as more cost-effective mechanisms for dealing with the enormous areas of near-shore sediment contamination.

VII. Summary

Microbially mediated dehalogenation processes contribute to the global cycling of both biogenic and anthropogenic halogenated organic compounds. Detailed information on biodegradation mechanisms for a variety of organohalides and on the microorganisms mediating these processes has greatly increased our understanding of the cycling and fate of these unique and widespread compounds in our environment. The marine environment appears to be a particularly rich source of dehalogenating microorganisms. It is well established by laboratory and field studies that anaerobic dehalogenation of sediment contaminants, such as PCBs, pesticides, and dioxins, occurs intrinsically and can be enhanced via various methods. Specific dehalogenating bacterial populations can be enriched on various organohalides. Biodehalogenation processes are likely to be significantly affected by the prevailing terminal electron-accepting condition, and thus, biotransformation of organohalide contaminants in marine and estuarine environments will vary as a function of the redox conditions within the sediment profile. Fundamental knowledge of the activities and interactions of dehalogenating microorganisms is providing a strong basis for development of new bioremediation technologies for removal of harmful halogenated compounds from our environment.

References

Adriaens, P., and Grbić-Galić, D. (1994). Reductive dechlorination of PCDD/F by anaerobic cultures and sediments. *Chemosphere* **29,** 2253–2259.

Ahn, Y.-B., Rhee, S.-K., Fennell, D. E., Kerkhof, L. J., Hentschel, U., and Häggblom, M. M. (2002). Reductive dehalogenation of haloaromatics by microorganisms associated with the marine sponge *Aplysina aerophoba*. Abstract Q-21, American Society for Microbiology 102nd General Meeting, Salt Lake City, May 19–23, 2002.

Ahn, Y.-B., Rhee, S-K., Fennell, D. E., Kerkhof, L. J., Hentschel, U., and Häggblom, M. M. (2003). Reductive dehalogenation of brominated phenolic compounds by microorganisms associated with the marine sponge *Aplysina aerophoba*. *Appl. Environ. Microbiol.* **69**, 4159–4166.

Albrecht, I. D., Barkovskii, A. L., and Adriaens, P. (1999). Production and dechlorination of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in historically contaminated estuarine sediments. *Environ. Sci. Technol.* **33,** 737–744.

Alder, A. C., Häggblom, M. M., Oppenheimer, S. R., and Young, L. Y. (1993). Reductive dechlorination of polychlorinated biphenyls in freshwater and marine sediments. *Environ. Sci. Technol.* **27,** 530–538.

Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59,** 143–169.

Ashworth, R. B., and Cormier, M. J. (1967). Isolation of 2,6-dibromophenol from the marine hemichordate, *Balanoglossus biminiensis. Science* **155,** 1558–1559.

Baker, J. T., and Duke, C. C. (1973). Isolation from the hypobranchial glands of marine molluses of 6-bromo-2,2-dimethylthioindolin-3-one and 6-bromo-2-methylthioindoleninone as alternative precursors to Tyrian purple. *Tetrahedron Lett.* **14,** 2481–2482.

Ballerstedt, H., Kraus, A., and Lechner, U. (1997). Reductive dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin and its products by anaerobic mixed cultures from Saale River sediment. *Environ. Sci. Technol.* **31,** 1749–1753.

Barkovskii, A. L., and Adriaens, P. (1996). Microbial dechlorination of historically present and freshly spiked chlorinated dioxins and diversity of dioxin-dechlorinating populations. *Appl. Environ. Microbiol.* **62,** 4556–4562.

Becker, J. G., Berardesco, G., Rittmann, B. E., and Stahl, D. A. (2001). Successional changes in an evolving anaerobic chlorophenol-degrading community used to infer relationships between population structure and system-level processes. *Appl. Environ. Microbiol.* **67,** 5705–5714.

Becker, J. G., Stahl, D. A., and Rittmann, B. E. (1999). Reductive dehalogenation and conversion of 2-chlorophenol to 3-chlorobenzoate in a methanogenic sediment community: Implications for predicting the environmental fate of chlorinated pollutants. *Appl. Environ. Microbiol.* **65,** 5169–5172.

Bedard, D. L., Van Dort, H., and DeWeerd, K. A. (1998). Brominated biphenyls prime extensive microbial reductive dehalogenation of Aroclor 1260 in Housatonic River sediment. *Appl. Environ. Microbiol.* **64,** 1786–1795.

Beurskens, J. E. M., Toussaint, M., de Wolf, J., van der Steen, J. M. D., Slot, P. C., Commandeur, L. C. M., and Parsons, J. R. (1995). Dehalogenation of chlorinated dioxins by an anaerobic microbial consortium from sediment. *Environ. Toxicol. Chem.* **14,** 939–943.

Bossert, I. D., Häggblom, M. M., and Young, L. Y. (2003). Microbial ecology of dehalogenation. *In* ''Dehalogenation: Microbial Processes and Environmental Applications'' (M. M. Häggblom and I. D. Bossert, eds.) pp. 33–52. Kluwer Academic Publishers, Boston.

Boyle, A. W., Häggblom, M. M., and Young, L. Y. (1999a). Dehalogenation of lindane (γ-hexachlorocyclohexane) by anaerobic bacteria from marine sediments and by sulfate-reducing bacteria. *FEMS Microbiol. Ecol.* **29,** 379–387.

Boyle, A. W., Knight, V. K., Häggblom, M. M., and Young, L. Y. (1999b). Transformation of 2,4-dichlorophenoxyacetic acid in four different marine and estuarine sediments: Effects of sulfate, hydrogen, and acetate on dehalogenation and side chain cleavage. *FEMS Microbiol. Ecol.* **29,** 105–113.

Boyle, A. W., Phelps, C. D., and Young, L. Y. (1999c). Isolation from estuarine sediments of a *Desulfovibrio* strain which can grow on lactate coupled to the reductive dehalogenation of 2,4,6-tribromophenol. *Appl. Environ. Microbiol.* **65,** 1133–1140.

Bunge, M., Ballerstedt, H., and Lechner, U. (2001). Regiospecific dechlorination of spiked tetra- and trichlorodibenzo-*p*-dioxins by anaerobic bacteria from PCDD/F-contaminated Spittelwasser sediments. *Chemosphere* **43,** 675–681.

Bunge, M., Adrian, L., Kraus, A., Lorenz, W. G., Andreesen, J. R., Görisch, H., and Lechner, U. (2003). Reductive dehalogenation of chlorinated dioxins by the anaerobic bacterium *Dehalococcoides ethenogenes* sp. strain CBDB1. *Nature* **421,** 357–360.

Capone, D. G., and Kiene, R. P. (1988). Comparison of microbial dynamics in marine and freshwater sediments: Contrasts in anaerobic carbon metabolism. *Limnol. Oceanogr.* **33,** 725–749.

Chen, C. P., Lincoln, D. E., Woodin, S. A., and Lovell, C. R. (1991). Purification and properties of a unique flavin-containing chloroperoxidase from the capitellid polychaete *Notomastus lobatus*. *J. Biol. Chem.* **266,** 23909–23915.

Cho, Y.-C., Ostrofsky, E. B., Sokol, R. C., Frohnhoefer, R. C., and Rhee, G.-Y. (2002). Enhancement of microbial PCB dechlorination by chlorobenzoates, chlorophenols, and chlorobenzenes. *FEMS Microbiol Ecol.* **42,** 51–58.

DeWeerd, K. A., Concannon, F., and Suflita, J. M. (1991). Relationship between hydrogen consumption, dehalogenation, and the reduction of sulfur oxyanions by *Desulfomonile tiedjei*. *Appl. Environ. Microbiol.* **57,** 1929–1934.

DeWeerd, K. A., and Suflita, J. M. (1990). Anaerobic aryl reductive dehalogenation of halobenzoates by cell extracts of *Desulfomonile tiedjei*. *Appl. Environ. Microbiol.* **56,** 2999–3005.

DeWeerd, K. A., and Bedard, D. L. (1999). Use of halogenated benzoates and other halogenated aromatic compounds to stimulate the microbial dechlorination of PCBs. *Environ. Sci. Technol.* **33,** 2057–2063.

Ebel, R., Brenzinger, M., Kunze, A., Gross, H. J., and Proksch, P. (1997). Wound activation of protoxins in marine sponge *Aplysina aerophoba*. *J. Chem. Ecol.* **23,** 1451–1461.

Faulkner, D. J. (1980). Natural organohalogen compounds. *In* ''Handbook of Environmental Chemistry, Vol. 1, Part A. Anthropogenic Compounds'' (O. Hutzinger ed.), pp. 229–254. Springer-Verlag, Berlin.

Fennell, D. E., and Gossett, J. M. (2003) Microcosms for site-specific evaluation of enhanced biological reductive dehalogenation. (Häggblom M. M. Bossert I. D. eds.), pp. 385–420. Kluwer Academic Publishers, Boston.

Fennell, D. E., Ahn, Y.-B., Rhee, S.-K., Kerkhof, L. J., and Häggblom, M. M. (2002). Stimulation of dioxin dechlorination in marine and estuarine sediments. Abstract Q-141, American Society for Microbiology 102nd General Meeting, Salt Lake City, May 19–23, 2002.

Fennell, D. E., Carroll, A. B., Zinder, S. H., and Gossett, J. M. (2001). Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. *Environ. Sci. Technol.* **35,** 1830–1839.

Fielman, K. T., Woodin, S. A., Walla, M. D., and Lincoln, D. E. (1999). Widespread occurrence of natural halogenated organics among temperate marine infauna. *Marine Ecol. Prog. Ser.* **181,** 1–12.

Garson, M. J., Zimmermann, M. P., Battershill, C. N., Holden, J. L., and Murphy, P. T. (1994). The distribution of brominated long-chain fatty acids in sponge and symbiont cell types from the tropical marine sponge *Amphimedon terpenensis*. *Lipids* **29,** 509–516.

Ghiorse, W. C., and Wilson, J. T. (1988). Microbial ecology of the terrestrial subsurface. *Adv. Appl. Microbiol.* **33,** 107–172.

Giese, B., Laturnus, F., Adams, F. C., and Wiencke, C. (1999). Release of volatile iodinated C_1–C_4 hydrocarbons by marine macroalgae from various climate zones. *Environ. Sci. Technol.* **33,** 2432–2439.

Gribble, G. W. (1998). Naturally occurring organohalogen compounds. *Acc. Chem. Res.* **31,** 141–152.

Gribble, G. W. (1994). The natural production of chlorinated compounds. *Environ. Sci. Technol.* **28,** 310A–319A.

Gribble, G. W. (1999). The diversity of naturally occurring organobromine compounds. *Chem. Soc. Rev.* **28,** 335–346.

Gribble, G. W. (2000). The natural production of organobromine compounds. *Environ. Sci. Pollut. Res.* **7,** 37–49.

Häggblom, M. M. (1992). Microbial breakdown of halogenated aromatic pesticides and related compounds. *FEMS Microbiol. Rev.* **103,** 29–72.

Häggblom, M. M. (1998). Reductive dehalogenation by a sulfate-reducing consortium. *FEMS Microbiol. Ecol.* **26,** 35–41.

Häggblom, M. M., and Bossert, I. D. (2003). Organohalides—a global perspective. *In* ''Dehalogenation: Microbial Processes and Environmental Applications'' (M. M. Häggblom and I. D. Bossert eds.), pp. 3–29. Kluwer Academic Publishers, Boston.

Häggblom, M. M., Knight, V. K., and Kerkhof, L. J. (2000). Anaerobic decomposition of halogenated aromatic compounds. *Environmental Pollution* **107,** 199–207.

Häggblom, M. M., Rivera, M. D., and Young, L. Y. (1996). Anaerobic degradation of halogenated benzoic acids coupled to denitrification observed in a variety of sediment and soil samples. *FEMS Microbiol Lett.* **144,** 213–219.

Häggblom, M. M., and Young, L. Y. (1995). Anaerobic degradation of halogenated phenols by sulfate-reducing consortia. *Appl. Environ. Microbiol.* **61,** 1546–1550.

Häggblom, M. M., Rivera, M. D., and Young, L. Y. (1993). Influence of alternative electron acceptors on the anaerobic biodegradability of chlorinated phenols and benzoic acids. *Appl. Environ. Microbiol.* **59,** 1162–1167.

Häggblom, M. M., Ahn, Y. B., Fennell, D. E., Kerkhof, L. J., and Rhee, S. K. (2001). Molecular characterization of anaerobic microbial dehalogenating communities in marine and estuarine sediments. SERDP–ESTCP Partners in Environmental Technology Symposium, November 27–29, 2001, Washington, DC.

Hale, D. D., Rogers, J. E., and Wiegel, J. (1997). Reductive dechlorination of chlorophenols in slurries of low-organic carbon marine sediments and subsurface soils. *Appl. Microbiol. Biotechnol.* **47,** 742–748.

Harms, H. (1998). Bioavailability of dioxin-like compounds for microbial degradation. *In* ''Biodegradation of Dioxins and Furans'' (Rolf-Michael Wittich, ed.), pp. 135–163, Springer.

Hartkamp-Commandeur, L. C. M., Gerritse, J., Govers, A. J., and Parsons, J. R. (1996). Reductive dehalogenation of polychlorinated biphenyls by anaerobic microorganisms enriched from Dutch sediments. *Chemosphere* **32,** 1275–1286.

Head, I. M., Saunders, J. R., and Pickup, R. W. (1998). Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbiol. Ecol.* **35,** 1–21.

Hentschel, U., Schmid, M., Wagner, M., Fieseler, L., Gernert, C., and Hacker, J. (2001). Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiol. Ecol.* **35,** 305–312.

Holliger, C., Regeard, C., Diekert, G. (2003). Dehalogenation by anaerobic bacteria. *In* ''Dehalogenation: Microbial Processes and Environmental Applications'' (M. M. Häggblom and I. D. Bossert eds.), pp. 115–157. Kluwer Academic Publishers, Boston.

Holoman, T. R. P., Elberson, M. A., Cutter, L. A., May, H. D., and Sowers, K. R. (1998). Characterization of a defined 2,3,5,6-tetrachlorobiphenyl-ortho-dechlorinating microbial community by comparative sequence analysis of genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **64,** 3359–3367.

Hugenholtz, P., Goebel, B. M., and Pace, N. R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* **180,** 4765–4774.

Isidorov, V. A., Zenkevich, I. G., and Ioffe, B. V. (1990). Volatile organic compounds in solfataric gases. *J. Atmos. Chem.* **10,** 329–340.

Jordan, A., Harnisch, J., Borchers, R., Le Guern, F., and Shinohara, H. (2000). Volcanogenic halocarbons. *Environ. Sci. Technol.* **34,** 1122–1124.

Kemp, P., Lee, S., and LaRoche, J. (1993). Estimating the growth rate of slowly growing marine bacteria from RNA content. *Appl. Environ. Microbiol.* **59,** 2594–2601.

Kerkhof, L., and Ward, B. B. (1993). Comparison of nucleic acid hybridization and fluorometry for measurement of the relationship between RNA/DNA ratio and growth rate in a marine bacterium. *Appl. Environ. Microbiol.* **59,** 1303–1309.

Key, B. D., Howell, R. D., and Criddle, C. S. (1997). Fluorinated organics in the biosphere. *Environ. Sci. Technol.* **31,** 2445–2454.

King, G. M. (1988). Dehalogenation in marine sediments containing natural sources of halophenols. *Appl. Environ. Microbiol.* **54,** 3079–3085.

King, G. M. (1986). Inhibition of microbial activity in marine sediments by a bromophenol from a hemichordate. *Nature* **323,** 257–259.

Knight, V. K., Berman, M. H., and Häggblom, M. M. (2003). Biotransformation of 3,5-dibromo-4-hydroxybenzonitrile (Bromoxynil) under denitrifying, Fe(III)-reducing, sulfidogenic, and methanogenic conditions. *Environ. Toxicol. Chem.* **22,** 540–544.

Knight, V. K., Kerkhof, L. J., and Häggblom, M. M. (1999). Community analyses of sulfidogenic 2-bromophenol dehalogenating and phenol degrading consortia. *FEMS Microbiol. Ecol.* **29,** 137–147.

Löffler, F. E., Sun, Q., Li, J., and Tiedje, J. M. (2000). 16S rRNA gene based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. *Appl. Environ. Microbiol.* **66,** 1369–1374.

Löffler, F. E., Cole, J. R., Ritalahti, K. M., and Tiedje, J. M. (2003). Diversity of dechlorinating bacteria. *In* ''Dehalogenation: Microbial Processes and Environmental Applications'' (Häggblom M. M. and Bossert I. D. eds.), pp. 53–87. Kluwer Academic Publishers, Boston.

Magnuson, J. K., Romine, M. F., Burris, D. R., and Kingsley, M. T. (2000). Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes:* Sequence of tceA and substrate range characterization. *Appl. Environ. Microbiol.* **66,** 5141–5147.

Masunaga, S., Susarla, S., and Yonezawa, Y. (1996). Dechlorination of chlorobenzenes in anaerobic estuarine sediment. *Water Sci. Technol.* **33,** 173–180.

Mazur, C. S., and Jones, W. J. (2001). Hydrogen concentrations in sulfate-reducing estuarine sediments during PCE dehalogenation. *Environ. Sci. Technol.* **35,** 4783–4788.

Mohn, W. W., and Tiedje, J. M. (1992). Microbial reductive dehalogenation. *Microbiol. Rev.* **56,** 482–507.

Monserrate, E., and Häggblom, M. M. (1997). Dehalogenation and biodegradation of brominated phenols and benzoic acids under iron-reducing, sulfidogenic, and methanogenic conditions. *Appl. Environ. Microbiol.* **63,** 3911–3915.

Nealson, K. H., and Saffarini, D. (1994). Iron and manganese in anaerobic respiration: Environmental significance, physiology, and regulation. *Annu. Rev. Microbiol.* **48,** 311–343.

Neidleman, S. L., and Giegert, J. (1986). ''Biohalogenation: Principles, Basic Roles and Applications.'' Ellis Horwood Limited, Chichester, UK.

Neumann, A., Wohlfarth, G., and Diekert, G. (1998). Tetrachloroethene dehalogenase from *Dehalospirillum multivorans:* Cloning, sequencing of the encoding genes, and expression of the pceA gene in *Escherichia coli. J. Bacteriol.* **180,** 4140–4145.

Norte, M., and Fernández, J. J. (1987). Isolation and synthesis of aplysinadiene, a new rearranged dibromotyrosine derivative from *Aplysina aerophoba. Tetrahedron Lett.* **28,** 1693–1696.

O'Hagan, D., and Harper, D. B. (1999). Fluorine-containing natural products. *J. Fluorine Chem.* **100,** 127–133.

Poulsen, L. K., Ballard, G., and Stahl, D. A. (1993). Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. *Appl. Environ. Microbiol.* **59,** 1354–1360.

Rhee, S.-K., Fennell, D. E., Häggblom, M. M., and Kerkhof, L. J. (2003). Detection of reductive dehalogenase motifs in PCR fragments from a sulfidogenic 2-bromophenol-degrading consortium enriched from estuarine sediment. *FEMS Microbiology Ecology* **43,** 317–324.

Richardson, R. E., Bhupathiraju, V. K., Song, D. L., Goulet, T. A., and Alvarez-Cohen, L. (2002). Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques. *Environ. Sci. Technol.* **36,** 2652–2662.

Schmitz, F. J., and Gopichand, Y. (1978). (7E, 13>, 15Z)-14, 16-Dibromo-7, 13,15-hexadecatrien-5-ynoic acid. A novel dibromo acetylenic acid from the marine sponge *Xestospongia muta. Tetrahedron Lett.* **19,** 3637–3640.

Siuda, J. F., and DeBerbardis, J. F. (1973). Naturally occurring halogenated organic compounds. *Lloydia* **36,** 107–143.

Smidt, H. (2001) Molecular characterization of anaerobic dehalogenation by *Desulfitobacterium dehalogenans*. Ph.D. thesis. University of Wageningen, The Netherlands.

Steward, C. C., Dixon, T. C., Chen, Y. P., and Lovell, C. R. (1995). Enrichment and isolation of a reductively debrominating bacterium from the burrow of a bromometabolite-producing marine hemichordate. *Can. J. Microbiol.* **41,** 637–642.

Steward, C. C., and Lovell, C. R. (1997). Respiration and assimilation of 4-bromophenol by estuarine sediment bacteria. *Microb. Ecol.* **33,** 198–205.

Suflita, J. M., and Townsend, G. T., (1995). The microbial ecology and physiology of aryl dehalogenation reactions and implications for bioremediation. *In* ''Microbial Transformation and Degradation of Toxic Organic Chemicals'' (L. Y. Young and C. E. Cerniglia, eds.), pp. 243–268. Wiley-Liss, New York.

Sun, B., Cole, J. R., and Tiedje, J. M. (2001). *Desulfomonile limimaris* sp. nov., an anaerobic dehalogenating bacterium from marine sediments. *Int. J. Syst. Evol. Microbiol.* **51,** 365–371.

Sun, B., Cole, J. R., Sanford, R. A., and Tiedje, J. M. (2000). Isolation and characterization of *Desulfovibrio dechloracetivorans* sp. nov., a marine dechlorinating bacterium growing by coupling the oxidation of acetate to the reductive dechlorination of 2-chlorophenol. *Appl. Environ. Microbiol.* **66,** 2408–2413.

Suyama, A., Yamashita, M., Yoshino, S., and Furukawa, K. (2002). Molecular characterization of the PceA reductive dehalogenase of *Desulfitobacterium* sp. strain Y51. *J. Bacteriol.* **184,** 3419–3425.

Torsvik, V., Sorheim, R., and Goksoyr, J. (1996). Total bacterial diversity in soil and sediment communities: A review. *J. Ind. Microbiol. Biotechnol.* **17,** 170–178.

Townsend, G. T., and Suflita, J. M. (1997). Influence of sulfur oxyanions on reductive dehalogenation activities in *Desulfomonile tiedjei*. *Appl. Environ. Microbiol.* **63,** 3594–3599.

Townsend, G. T., Ramanand, K., and Suflita, J. M. (1997). Reductive dehalogenation and mineralization of 3-chlorobenzoate in the presence of sulfate by microorganisms from a methanogenic aquifer. *Appl. Environ. Microbiol.* **63,** 2785–2791.

Turon, X., Becerro, M. A., and Uriz, M. J. (2000). Distribution of brominated compounds within the sponge *Aplysina aerophoba:* Coupling of x-ray microanalysis with vryofixation techniques. *Cell Tissue Res.* **301,** 311–322.

Utkina, N. K., Denisenko, V. A., Scholokova, O. V., Virovaya, M. V., Gerasimenko, A. V., Popov, D. Y., Krasokhin, V. B., and Popov, A. M. (2001). Spongiadioxins A and B, two new polybrominated dibenzo-*p*-dioxins from an Australian marine sponge *Dysidea dendyi*. *J. Nat. Prod.* **64,** 151–153.

van de Pas, B. A., Smidt, H., Hagen, W. R., van der Oost, J., Schraa, G., Stams, A. J., and de Vos, W. M. (1999). Purification and molecular characterization of ortho-chlorophenol reductive dehalogenase, a key enzyme of halorespiration in *Desulfitobacterium dehalogenans*. *J. Biol. Chem.* **274,** 20287–20292.

van Pée, K.-H. (1996). Biosynthesis of halogenated metabolites by bacteria. *Annu. Rev. Microbiol.* **50,** 375–399.

van Pée, K.-H. (2001). Microbial biosynthesis of halometabolites. *Arch. Microbiol.* **175,** 250–258.

Vargas, C., Song, B. K., Camps, M., and Häggblom, M. M. (2000). Anaerobic degradation of fluorinated aromatic compounds. *Appl. Microbiol. Biotechnol.* **53,** 342–347.

Vargas, C., Fennell, D. E., and Häggblom, M. M. (2001). Anaerobic reductive dechlorination of chlorinated dioxins in estuarine sediments. *Appl. Microbiol. Biotechnol.* **57,** 786–790.

von Wintzingerode, F., Schlotelburg, C., Hauck, R., Hegemann, W., and Göbel, U. B. (2001). Development of primers for amplifying genes encoding CprA-and PceA-like reductive dehalogenases in anaerobic microbial consortia, dechlorinating trichlorobenzene, and 1,2-dichloropropane. *FEMS Microbiol. Ecol.* **35,** 189–196.

Voordeckers, J., Fennell, D. E., Jones, K., and Häggblom, M. M. (2002). Anaerobic biotransformation of tetrabromobisphenol A, tetrachlorobisphenol A, and bisphenol A in estuarine sediments. *Environ. Sci. Technol.* **36,** 696–701.

Watson, J., Matsui, G. Y., Leaphart, A., Wiegel, J., Rainey, F. A., and Lovell, C. R. (2000). Reductively debrominating strains of *Propionigenium maris* from burrows of bromophenol-producing marine infauna. *Int. J. Syst. Evol. Microbiol.* **50,** 1035–1042.

Woese, C. R. (1987). Bacterial evolution. *Microbiol. Rev.* **51,** 221–271.

Wu, Q., and Wiegel, J. (1997). Two anaerobic polychlorinated biphenyl-dehalogenating enrichments that exhibit different para-dechlorination specificities. *Appl. Environ. Microbiol.* **63,** 4826–4832.

Wu, Q. Z., Watts, J. E. M., Sowers, K. R., and May, H. D. (2002). Identification of a bacterium that specifically catalyzes the reductive dechlorination of polychlorinated biphenyls with doubly flanked chlorines. *Appl. Environ. Microbiol.* **68,** 807–812.

Yonezawa, Y., Fukui, M., Masunaga, S., and Urushigawa, Y. (1994). Dechlorination of 1,2,4-trichlorobenzene in the sediment of Ise Bay. *Chemosphere* **28,** 2179–2184.

Yoon, K. S., Chen, Y. P., Lovell, C. R., Lincoln, D. E., Knapp, L. W., and Woodin, S. A. (1994). Localization of the chloroperoxidase of the capitellid polychaete *Notomastus lobatus*. *Biol. Bull.* **187,** 215–222.

Biotechnological Application of Metal-reducing Microorganisms

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I. Introduction

For more than a century, microbial metabolism has been known to alter the oxidation state of inorganic species, resulting, for example, in both the oxidation and reduction of metals (Ehrlich, 1996). However, studies over the last two decades have shown that a diversity of specialist *Bacteria* and *Archaea* can use such activities to conserve energy for growth. Much recent interest has focused on organisms that can respire using metals as electron acceptors under anaerobic conditions (for

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example, ''dissimilatory'' processes as described in Ahmann *et al.*, 1994; Lovley *et al.*, 1991; Lovley and Phillips, 1988; Lovley *et al.*, 1987; Myers and Nealson, 1988 and these studies have opened up new and fascinating areas of research with potentially exciting practical applications. Microorganisms have also evolved metal resistance processes that often incorporate changes in the oxidation state of toxic metals, e.g., resistance to Hg(II) (Barkay, 2003), As(V) (Mukhopadhyay *et al.*, 2002), and Ag(I) (Silver, 2003). Several such resistance mechanisms, which do not support anaerobic growth, have been studied in detail using the tools of molecular biology. The molecular basis of respiratory metal reduction processes have not, however, been studied in such fine detail, although rapid advances are expected in this area with the imminent availability of complete genome sequences for key metal-reducing bacteria, in combination with genomic and proteomic tools. This research is being driven forward by both the need to understand the fundamental basis of a range of biogeochemical cycles and by the possibility of harnessing such activities for a range of biotechnological applications. These include the bioremediation of metal-contaminated land and water (Lloyd and Lovley, 2001), the oxidation of xenobiotics under anaerobic conditions (Lovley and Anderson, 2000), metal recovery in combination with the formation of novel biocatalysts (Yong *et al.*, 2002a), and even the generation of electricity from sediments (Bond *et al.*, 2002). However, for microbial metabolism to make an impact in these areas, it is crucial not only that the physiological basis of metal reduction is better understood, but also that such bioprocesses are integrated with new technologies developed from other areas of science and technology (e.g., engineering, electrochemistry, and materials science). The aim of this chapter is to give an up-to-date overview of the range of metals (and metalloids) reduced by microorganisms, the mechanisms involved, and the possible applications for these processes. Although such biotechnologies are relatively ''immature,'' several important examples have already been tested at field or pilot scale. Particular emphasis will be placed on these studies (e.g., the bioremediation of waters and sediments contaminated with mercury and uranium, respectively), and, where possible, additional areas that need to be developed in order to support parallel studies for other bioprocesses will be highlighted.

II. Fe(III) and Mn(IV) Reduction

A wide range of *Archaea* and *Bacteria* are able to conserve energy though the reduction of Fe(III) (ferric iron) to Fe(II) (ferrous iron). Many of these organisms are also able to grow through the reduction of

Mn(VI) to Mn(II). The environmental relevance of Fe(III) and Mn(VI) reduction has been well documented (Lovley, 1991; Thamdrup, 2000). Indeed, geochemical and microbiological evidence suggests that the reduction of Fe(III) may have been an early form of respiration on Earth (Vargas *et al.*, 1998) and is a candidate for the basis of life on other planets (Nealson and Cox, 2002). On modern Earth, Fe(III) can be the dominant electron acceptor for microbial respiration in many subsurface environments (Lovley and Chapelle, 1995), and Fe(III)-reducing microorganisms can also play a role in the remediation of such environments. For example, recent studies have shown that a range of important xenobiotics that contaminate aquifers can also be degraded under anaerobic conditions by Fe(III)- and Mn(IV)-reducing microorganisms (Anderson *et al.*, 1998; Lovley, 1997; Lovley and Anderson, 2000; Lovley *et al.*, 1989). In addition to playing an important role in the biodegradation of organic material, Fe(III)- and Mn(IV)-reducing microorganisms can influence the mineralogy of sediments through the reductive dissolution of insoluble Fe(III) and Mn(IV) oxides. Bound toxic metals can be released, leading to the possibility of using Fe(III)- and Mn(IV)-reducing bacteria for bioleaching of metals from some soils and sediments. In addition, depending on the chemistry of the water, a range of reduced minerals can also be formed; these include magnetite (Fe_3O_4), siderite ($FeCO_3$), and rhodochrosite ($MnCO_3$). This can result in changes in the structure of sediments, giving the potential to control water flow in contaminated aquifers, and may also give a route to producing biominerals of potential commercial value, such as the magnetic mineral magnetite. Fe(III)- and Mn(IV)-reducing microorganisms can also impact the mobility of other high-valence contaminant metals through direct enzymatic reduction and also via indirect reduction catalyzed by biogenic Fe(II). The bioreduction of U(VI), Cr(VI), and Tc(VII) by Fe(III)-reducing microorganisms will be discussed in detail in later sections and can result in immobilization of these potentially toxic and mobile metals in sediments or bioreactors (Lloyd and Lovley, 2001). Finally, these organisms are also able to reduce electrodes, giving the possibility of harnessing electricity from anaerobic communities in the subsurface (Bond *et al.*, 2002).

A. Diversity of Fe(III)- and Mn(IV)-reducing Microorganisms

Early studies on the reduction of Fe(III) and Mn(IV), e.g., Roberts, 1947, focused on organisms that can grow predominantly via fermentation of sugars such as glucose, with metals utilized as minor electron acceptors; typically $< 5\%$ of the reducing equivalents used for metal

reduction (Lovley, 1991). It has not been shown conclusively that metal reduction via this form of metabolism increases cell yields. More recent studies demonstrated that a wide range of *Bacteria* and *Archaea* can couple the oxidation of hydrogen and short-chain fatty acids to the reduction of Fe(III), although it has not been shown that these reactions support growth. Such examples include the reduction of Fe(III) by a *Vibrio* sp. (Jones *et al.*, 1984), *Wolinella succinogenes* (Lovley and Phillips, 1988), and *Archaea* such as *Archaeoglobus fulgidus, Pyrococcus furiosus*, and *Pyrodictium abyssi* (Vargas *et al.*, 1998). Sulfate-reducing bacteria are also known to reduce Fe(III) (Coleman *et al.*, 1993) and other metals including Cr(VI) and U(VI) that are important environmental contaminants (see Sections III.A and V.A), although, again, growth has not been demonstrated using these electron acceptors. A paper suggesting the contrary (Tebo and Obraztsova, 1998) has been disputed on the basis of inconsistencies in the cell yields when grown on a range of metals (Lovley, 2001).

The first organisms that were shown unequivocally to conserve energy for growth through the reduction of Fe(III) or Mn(IV) were *Shewanella oneidensis* (formerly *Alteromonas putrefaciens* and then *Shewanella putrefaciens*) and *Geobacter metallireducens* (formerly strain GS-15) (Lovley *et al.*, 1989; Lovley *et al.*, 1987; Myers and Nealson, 1988). Since these pioneering studies in the late 1980s, numerous organisms have been isolated that can grow using Fe(III) and Mn(IV) as electron acceptors. A detailed overview of more than 30 of these strains is given in a recent review (Lovley, 2001). Most organisms that are known to grow through the reduction of Fe(III) or Mn(IV) are relatives of *Geobacter metallireducens* and fall within the family *Geobacteraceae*, in the delta subdivision of the class *Proteobacteria*. This group comprises the Genera *Geobacter, Desulfuromonas, Desulfuromusa*, and *Pelobacter* (Lonergan *et al.*, 1996; Lovley, 2001). These organisms, with the exception of the *Pelobacter* species, are able to completely oxidize a wide range of organic compounds, including xenobiotics, when respiring using Fe(III) or Mn(IV) (Lovley *et al.*, 1993); *Pelobacter* species are more restricted in the range of electron donors utilized, although they can couple hydrogen oxidation to metal reduction. This is in contrast to *Shewanella oneidensis* and close relatives in the gamma subdivision of the *Proteobacteria* (a range of *Shewanella, Ferrimonas*, and *Aeromonas* species) that are generally able to use only a restricted range of small organic acids and hydrogen as electron donors for Fe(III) and Mn(IV) reduction. Other Fe(III)-respiring bacteria that have been characterized include *Geothrix fermentans* (Coates *et al.*, 1999), *Geovibrio ferrireducens* (Caccavo *et al.*,

1996) and the related thermophile *Deferribacter thermophilus* (Green *et al.*, 1997), *Ferribacter limneticum*, which is unusual in that it can reduce Fe(III) but not Mn(IV), and *Sulfurospirillum barnesii* (Oremland *et al.*, 1994) (see sections on Se(VI) and As(V) reduction). Acidophilic bacteria that are able to grow through the reduction of Fe(III) include *Thiobacillus ferroxidans*, which uses sulfur as an electron donor for metal reduction (Pronk *et al.*, 1992). Several hyperthermophilic *Archaea* and *Bacteria* have also been shown to grow using Fe(III) as an electron acceptor, including *Pyrobaculum islandicum, Pyrobaculum aerophilum*, and *Thermotoga maritima* (Vargas *et al.*, 1998). The environmental distribution of Fe(III)-reducing prokaryotes remains poorly understood, but most studies show that members of the family *Geobacteraceae* are the key components of Fe(III)-reducing communities in subsurface environments, including those contaminated with toxic metals and xenobiotics (Holmes *et al.*, 2002; Röling *et al.*, 2001; Rooney-Varga *et al.*, 1999; Snoeyenbos-West *et al.*, 2000; Stein *et al.*, 2001).

The mechanisms of Fe(III)-reduction, and to a lesser degree Mn(IV) reduction, have been studied in most detail in *Shewanella oneidensis* and *Geobacter sulfurreducens*. Indeed, research on these organisms has been given added impetus through the availability of the genome sequences (available at http://www.tigr.org), and suitable genetic systems for the generation of deletion mutants for both of these organisms (Coppi *et al.*, 2001; Myers and Myers, 2000). Although the terminal reductase has yet to be identified unequivocally in either organism, the involvement of *c*-type cytochromes is implicated in electron transport to Fe(III) and Mn(IV) by several studies (Beliaev *et al.*, 2001; Gaspard *et al.*, 1998; Leang *et al.*, 2003; Lloyd *et al.*, 2003; Magnuson *et al.*, 2000; Myers and Myers, 1993; Myers and Myers, 1997). In some examples, activities have also been localized to the outer membrane or surface of the cell, consistent with a role in direct transfer of electrons to Fe(III) and Mn(IV) oxides that are highly insoluble at circumneutral pH (DiChristina *et al.*, 2002; Gaspard *et al.*, 1998; Lloyd *et al.*, 2002; Myers and Myers, 1992; Myers and Myers, 2001) (Fig. 1A, see color insert). In addition to the proposed direct transfer of electrons to Fe(III) and Mn(IV) minerals and potentially toxic metals, soluble ''electron shuttles'' are also able to transfer electrons between metal-reducing prokaryotes and the mineral surface (Fig. 1B). This alleviates the requirement for direct contact between the microorganism and the mineral, and provides a mechanism for enhancing activities of Fe(III)- and Mn(IV)-reducing bacteria during the bioremediation of sediments contaminated with organics or some metals. For example, humics and other extracellular

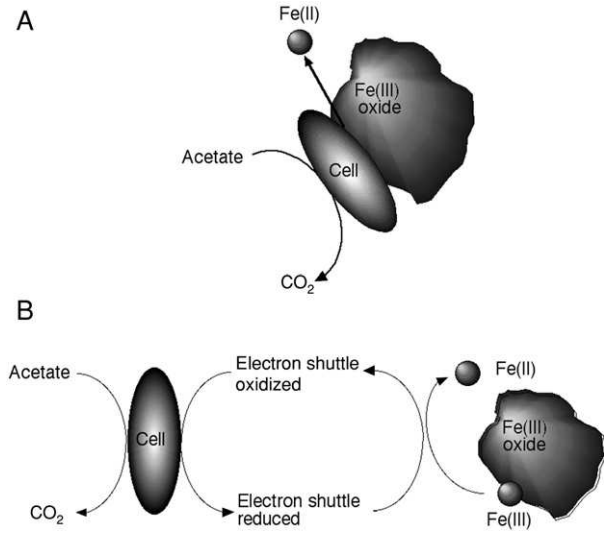

FIG. 1. Mechanisms of reduction of insoluble Fe(III) oxides, via (A) direct contact with the surface of the cell or (B) an extracellular electron shuttle.

quinones are utilized as electron acceptors by Fe(III)-reducing bacteria (Lovley *et al.*, 1996), and the reduced hydroquinone moieties are able to abiotically transfer electrons to Fe(III) minerals. The oxidized humic is then available for reduction by the microorganism, leading to further rounds of electron-shuttling to the insoluble mineral (Nevin and Lovley, 2002). Very low concentrations of an electron shuttle, e.g., 100 nM of the humic analog anthraquinone-2,6-disulfonate (AQDS), can rapidly accelerate the reduction of Fe(III) oxides (Lloyd *et al.*, 1999) and possibly other insoluble metal oxides such as Mn(IV). The secretion of soluble electron shuttles by actively respiring Fe(III) and Mn(IV) reducers has also been proposed for both *S. oneidensis* and *Geobacter sulfurreducens*, and remains hotly debated in the *Geobacter* species. Early studies suggested release of a small soluble *c*-type cytochrome by *G. sulfurreducens* (Seeliger *et al.*, 1998), but more recent studies have suggested that this protein is not an effective electron shuttle (Lloyd *et al.*, 1999). Additional studies further suggested that *Geobacter* species do not release electron-shuttling compounds (Nevin and Lovley, 2002).

Studies have also suggested that a small quinone containing extracellular electron shuttle is released by *Shewanella* species and may also promote electron transfer to Fe(III) and Mn(IV) minerals (Nevin and Lovley, 2002; Newman and Kolter, 2000). *Geothrix fermentans* also

releases an electron shuttle (Nevin and Lovley, 2002). Furthermore, *Shewanella* and *Geothrix* species release Fe(III) chelates that solublize Fe(III) from insoluble Fe(III) oxides (Nevin and Lovley, 2002). Finally, an important discovery was made when it was shown that *Geobacter metallireducens* synthesized pili and flagella when grown on insoluble Fe(III) or Mn(IV) minerals, but not on soluble forms of the metals (Childers *et al.*, 2002). These results suggest that *Geobacter* species sense when soluble electron acceptors are depleted and synthesize the appropriate appendages that allow movement to Fe(III) and Mn(IV) minerals and subsequent attachment. This may be an important mechanism for delivery of these organisms to zones of sediments contaminated with metals and organics.

B. Degradation of Organic Contaminants Coupled to Fe(III) Reduction

Some *Geobacter* species have the ability to anaerobically oxidize aromatic contaminants to carbon dioxide with Fe(III) serving as the electron acceptor (Coates *et al.*, 2001; Lovley *et al.*, 1989; Lovley and Lonergan, 1990). This metabolic capability, coupled with the abundance of Fe(III) oxides in many subsurface environments, leads to Fe(III) reduction being an important process for the degradation or organic contaminants in subsurface environments. Molecular analysis of subsurface environments in which organic contaminants were being degraded under Fe(III)-reducing conditions has demonstrated that the sediments are enriched in *Geobacter* species under these conditions (Röling *et al.*, 2001; Rooney-Varga *et al.*, 1999; Snoeyenbos-West *et al.*, 2000).

It is possible to stimulate the degradation of organic contaminants coupled to Fe(III) reduction by making the Fe(III) oxides in subsurface sediments more available for microbial reduction. One method is to add Fe(III) chelators which solublize Fe(III) (Lovley *et al.*, 1994). Another strategy is to add humic acids or other extracellular quinones which can serve as electron shuttles between *Geobacter* species and Fe(III) oxides (Lovley *et al.*, 1996). Both approaches eliminate the need for *Geobacter* species to directly contact Fe(III) oxide in order to reduce it.

More recently, it has been demonstrated that *Ferroglobus placidus*, a hyperthermophilic member of the *Archaea*, is capable of oxidizing aromatic compounds with the reduction of Fe(III) (Tor and Lovley, 2001). This is the first example of an *Archaea* of any kind that can anaerobically oxidize aromatics. In addition to having implications for metabolism in naturally hot environments, such metabolism could

be useful when heat treatments are employed to aid in the extraction of organic contaminants that are trapped in sediments (Tor and Lovley, 2001).

C. Applications of Electrode-reducing Bacteria

In addition to transferring electrons to naturally occurring insoluble electron acceptors such as Fe(III) and Mn(IV) oxides, some Fe(III) reducers can transfer electrons to electrodes (Bond *et al.*, 2002; Bond and Lovley, 2003). Members of the *Geobacteraceae* were found to conserve energy to support cell growth by coupling the oxidation of acetate or aromatic compounds to electron transfer to graphite electrodes (Bond *et al.*, 2002; Bond and Lovley, 2003). The electron transfer is essentially quantitative, with electron yields similar to that for Fe(III) oxide reduction. *Geobacter* form a biofilm on the electrode surface that is extremely stable and can continue to produce electricity for weeks (Bond and Lovley, 2003).

This novel form of respiration has potential application for harvesting electricity from a diversity of waste organic matter. For example, when a graphite electrode (the anode) is emplaced in anaerobic marine sediments and connected to a graphite electrode in the overlying, aerobic water, there is a flow of electricity (Bond *et al.*, 2002; Tender *et al.*, 2002). Molecular analysis of the anode surface demonstrated that it was highly enriched in *Geobacteraceae* (Bond *et al.*, 2002; Tender *et al.*, 2002). This suggests that the *Geobacteraceae* that colonize the anode are oxidizing acetate, the key intermediate in anaerobic metabolism of organic matter in sediments, with the electrode serving as the electron acceptor. It should be feasible to harvest electricity from the degradation of a variety of types of waste organic matter in a similar manner.

III. Reduction of Other Transition Metals

A. Bioremediation of Cr(VI)

The widespread use of chromium in the metals industries and subsequent contamination problems have led to a lot of interest in this metal. Although trace quantities are required for some metabolic activities, such as glucose and lipid metabolism, chromium is considered toxic and is designated a priority pollutant in many countries. Two oxidation states dominate: Cr(VI) is the most toxic and mobile form commonly encountered, with Cr(III) less soluble and less toxic. Indeed, Cr(III) is considered 1000 times less mutagenic than Cr(VI) (Wang, 2000). Current

treatment involves reduction of Cr(VI) to Cr(III) using chemical reductants at low pH, followed by adjustment to near-neutral pH and subsequent precipitation of Cr(III). However, studies have shown that microorganisms can also reduce Cr(VI) efficiently at circumneutral pH and could be used to treat Cr(VI)-contaminated soil and water.

A wide range of facultative anaerobes are able to reduce Cr(VI) to Cr(III) including *Escherichia coli*, Pseudomonads *Shewanella oneidensis*, and *Aeromonas* species. (See Wang, 2000, for a more exhaustive list and key references.) Anaerobic conditions are generally required to induce maximum activity against Cr(VI), but some enzyme systems operate under aerobic conditions, for example, the soluble NAD(P)H-dependent reductases of *Pseudomonas ambigua* G-1 (Suzuki *et al.*, 1992) and *P. putida* (Park *et al.*, 2000). The former study is also worthy of mention as it showed reduction of Cr(VI) via a Cr(V) intermediate, also noted recently in preparations of Cr(VI) reduced by the membrane fraction of anaerobically grown *S. putrefaciens* (now S. *oneidensis*) (Myers *et al.*, 2000). Thus, Cr(VI) bioreduction seems to be initiated by a one-electron transfer from the reductase. Obligate anaerobes are also able to enzymatically reduce Cr(VI), and anaerobic growth coupled to Cr(VI) reduction has been reported for a sulfate-reducing bacterium (Tebo and Obraztsova, 1998). The reduction of Cr(VI) by sulfate-reducing bacteria is particularly well studied (Lloyd *et al.*, 2001) and has been shown to be catalyzed by cytochrome c_3 (Lovley and Phillips, 1994). Other studies have also implicated the involvement of cytochromes in Cr(VI)-reduction by bacteria: cytochrome *c* in *E. cloacae* (Wang *et al.*, 1989) and cytochromes *b* and *d* in *Escherichia coli* (Shen and Wang, 1993). Most studies have focused on planktonic cells, but more recent studies have shown that biofilms of sulfate-reducing bacteria also reduce and precipitate Cr(VI). Cr(VI) reduction was thought to be enzymatic in this study; reduction by sulfide was discounted because sulfate reduction was inhibited dramatically in the presence of chromate (Smith and Gadd, 2000). Immobilized cells of *Bacillus* sp. (Chirwa and Wang, 1997) and *P. fluorescens* LB 300 (Chirwa and Wang, 1997) have also been used to treat Cr(VI)-contaminated water.

Environmental factors that affect Cr(VI) reduction were reviewed and include competing electron acceptors, pH, temperature, redox potential, and the presence of other metals (Wang, 2000). Another study demonstrated that the presence of complexing agents can promote Cr(VI) reduction, possibly through protection of the metal reductase by chelation of Cr(III) or intermediates formed (Mabbett *et al.*, 2002). The type of electron donor supplied can also have an effect on the rate and extent of Cr(VI) reduction. Optimal electron donors, in keeping

with other dissimilatory metal reduction processes described in this chapter, are low molecular weight carbohydrates, amino acids, and fatty acids. Degradation of a range of aromatics including phenol, p-cresol, and benzene by *P. putida* DMP-1 has also been coupled to the reduction of Cr(VI) by *E. coli* 33456 in co-culture (Shen and Wang, 1995). Similar results have also been reported for a mixed culture of phenol-degrading microorganisms and the Cr(VI)-reducing *E. coli* strain (Chirwa and Wang, 2000). Finally, indirect mechanisms that also promote Cr(VI) reduction in contaminated sediments are catalyzed by biogenic sulfide (Fude *et al.*, 1994; Smillie *et al.*, 1981), and Fe(II) (Fendorf and Li, 1996). Experiments using contaminated sediments from Norman, Oklahoma, have, however, suggested that indirect mechanisms may not always be the critical control on Cr solubility, with direct enzymatic Cr(VI) reduction by a consortium of methanogens implicated (Marsh *et al.*, 2000).

B. Reduction of Pd(II); Formation of Novel Nanobiocatalysts

The reduction of soluble Pd(II) to insoluble Pd(0) has also attracted much interest as this enzymatic process may be used to recover Pd from industrial catalysts (Lloyd *et al.*, 1998) and also to synthesize nanoscale bioinorganic catalysts of considerable commercial potential (Yong *et al.*, 2002a; Baxter-Plant *et al.*, 2003). Interest in this area is driven by the widespread use of platinum group metals (PGMs), including Pd in automotive catalytic converters required to reduce gaseous emissions, and problems associated with their recycling. With approximately 5 g of PGM per catalyst, the consumption of PGMs together was 2.5×10^6 oz in 1994, with only 0.4×10^6 oz recovered (Lloyd *et al.*, 1998). The lifetime of a catalyst is only approximately 50,000 miles although many fail sooner, and future shortages and higher prices may be predicted. Chemical and electrochemical treatments are made difficult by complex solution chemistry.

An early study (Lloyd *et al.*, 1998) showed that cells of *Desulfovibrio desulfuricans* could reduce soluble Pd(II) to elemental Pd^0, supported by pyruvate, formate, or H_2 as the electron donor without requirement for biochemical cofactors. Despite the requirement of anaerobiosis for cell growth, Pd(II) reduction was insensitive to O_2 using batch suspensions (Lloyd *et al.*, 1998). Use of hydrogen as the electron donor implied the harnessing of hydrogenase with, possibly, cytochrome c_3 activity to Pd reduction. Participation of hydrogenase was implied by the inhibition of Pd(II) reduction by Cu^{2+} ions, which are known (although not specific) inhibitors of hydrogenase activity. Further confirmation was provided recently by molecular genetic techniques using

Desulfovibrio fructosovorans, where deletion of periplasmic hydrogenases had a corresponding effect on the pattern of Pd^0 deposition, localizing the Pd^0 mainly to sites corresponding to the remaining hydrogenase(s) (I. P. Mikheenko, M. Rousset, S. Dementin and L. E. Macaskie unpublished). Studies on the mechanism of deposition of Pd^0 have shown that this is a two-step process: an initial biosorption of Pd(II) is required for the best deposition of Pd^0 followed by reduction on addition of electron donor (Yong *et al.*, unpublished; see deVargas *et al.*, 2003, for discussion). An early study found that better Pd(II) uptake was obtained from $Pd(Cl_4)^{2-}$ than from $Pd(NH_3)_4{}^{2+}$ (Yong *et al.*, 2002b), at pH 2–4 (deVargas *et al.*, 2003; Yong *et al.*, 2002b). The need for acidic pH, usual in the case of platinum group metal uptake by biomass (deVargas *et al.*, 2003; Guibal *et al.*, 1999), is attributable to the requirement for protonation of anionic sites on the biomass to facilitate the uptake of the anionic precious metal species. The site of initial coordination of Pd(II) was identified by x-ray photoelectron spectroscopy as amino groups (de Vargas and Macaskie, unpublished) consistent with the formation of nucleation foci on protein amino groups, possibly on the hydrogenase enzyme itself. The biosorption step (optimally 1 h) was found to be essential for good Pd^0 deposition, which raises concern regarding the stability of the hydrogenase under acidic conditions. However, the enzyme normally yields protons from H_2 and hydrogenase activity was unaffected by exposure to pH 2 for 1 h (Mikheenko, unpublished).

Following the biosorption step, further deposition of Pd^0 onto the nucleation sites is initiated by the provision of electron donor, normally hydrogen or formate; use of the latter was O_2-sensitive (Yong *et al.*, 2002b). When formate is used, the product would be hydrogen, generated endogenously and broken down by hydrogenase to yield electrons for metal reduction. This reaction is catalyzed in *E. coli* via the formate hydrogenlysase complex (FHL) (Lloyd *et al.*, 1997), well documented in facultatively anaerobic bacteria. A rudimentary FHL has also been reported in *Desulfovibrio* sp. but this enzyme complex is much less well understood, consisting apparently of a formate dehydrogenase coupled to a hydrogenase via a cytochrome (Peck, 1993). Parameters influencing Pd^0 deposition on the biomass were investigated by Yong *et al.* (2002a,b). Using cell suspensions, both cell density and formate concentration affected the reduction rate but this was nonlinear. This was interpreted in terms of an autocatalytic reaction via the already-deposited Pd^0 (Bio-Pd^0), conferred by the special properties of Pd^0 as a hydrogen carrier, which can also split formate and, indeed, can reduce more Pd(II) chemically (see Yong *et al.*,

2002a, for discussion). Therefore it seems likely that the enzymatic contribution is confined to the initial Pd^0 deposition steps and crystal initiation, and that once the Pd^0-crystals are established their further growth may occur by chemical reactions. This provides a rationale for why the cells are able to function in Pd removal from highly aggressive solutions following the initial Pd uptake (see following).

The deposition of Pd^0 (confirmed by x-ray powder diffraction analysis; Lloyd *et al.*, 1998; Yong *et al.*, 2002a) is illustrated in Figs. 2A and B. In the initial stages of Pd^0 deposition, small deposits within the periplasmic space (e.g., 2F) break through the outer membrane to form heavy extracellular deposits (2G) which are absent from Pd-unchallenged cells (2A). The deposits are visible also by SEM (2C, D).

The biomass is trifunctional (Yong *et al.*, 2002a): (1) in providing an enzyme catalyst for the initial stages of Pd^0 deposition (using formate dehydrogenase/hydrogenase/cytochrome c_3 to provide electrons initially); (2) in providing nucleation sites (foci of Pd^0 metal deposition for subsequent crystal growth), and (3) as a scaffold for crystals of Pd^0 able to autocatalyze further reactions by acting as a sink for H_2/formate-trapping and also production of highly reactive $H^{\bullet}$ chemically from formate (see Yong *et al.*, 2002a, for discussion). Nucleation is important in providing the ''correct'' crystal microarray and bioreduction is important in accelerating the initial part of the deposition reaction but thereafter the accumulated Bio-Pd^0 attracts and retains hydrogen; effectively, the Bio-Pd^0 provides a catalytic surface for the chemical reduction of more Pd(II) (or other reactions; see following) and thus is acting as a bionanocatalyst. This was demonstrated by the catalysis of breakdown of sodium hypophosphite to yield hydrogen, a reaction catalyzed by Pd^0 that usually required heating. For comparison Bio-Pd^0 was prepared under H_2, while its chemical counterpart (Chemical-Pd^0) was reduced more slowly chemically under H_2 without cells. Both preparations were washed and dried. Upon addition of sodium hypophosphite the Bio-Pd^0 promoted release of hydrogen immediately at room temperature, while the chemical reaction required 13 mins for initiation (Yong *et al.*, 2002a). The rate of H-evolution was 0.2 ml/min/mg Pd (Mikheenko and Macaskie, unpublished). In other tests, the Bio-Pd^0 was harnessed to addition of hydrogen across C=C double bonds, e.g., the formation of methyl succinate from methylene succinate using Bio-Pd^0 proceeded at a rate comparable to that observed using a commercial supported Pd-catalyst (Winterbottom *et al.*, unpublished).

The ease of production of Bio-Pd^0 and its high catalytic activity suggested possible applications in remediation, where the recalcitrance of

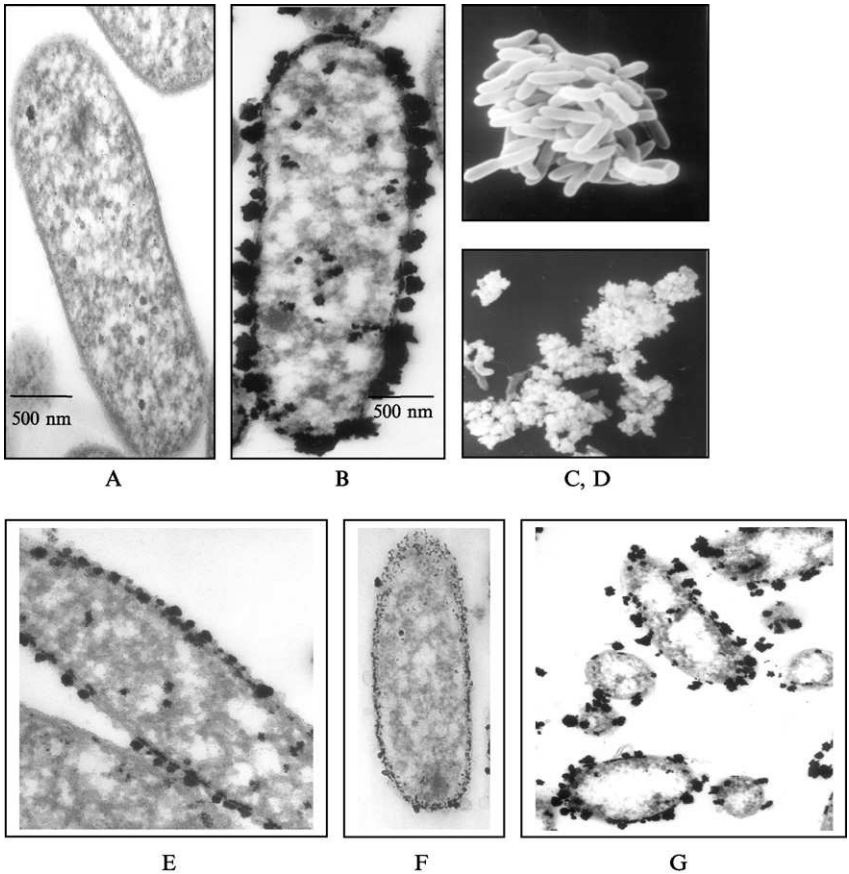

FIG. 2. Cells of *Desulfovibrio desulfuricans* loaded with Bio-Pd^0, and formation of bionanocatalyst. Cells before challenge show no electron opaque bodies (A), whereas after exposure to Pd^0, dense black deposits are visible on the cells (B). The deposits are clearly visible ''standing proud'' of the cells by scanning electron microscopy (no Pd^0: C and with Pd^0: D). The patterns of Pd-deposition and ferromagnetic properties of the cells relate to their catalytic activity (see text). Thus, cells loaded at a Pd:biomass ratio of 1:1 (E) have lower catalytic activity and ferromagnetic activity, and those loaded at a ratio of 1:3 (by one method) have high ferromagnetic activity and catalytic activity (F), while those loaded at a ratio of 1:3 (by another method) have lower ferromagnetic activity and catalytic activity (G). Note that it is possible to make a few large deposits (G) or many smaller deposits (F) for the same amount of Pd.

target molecules and/or the aggressive nature of solution matrices may present difficulties for bioremediation techniques alone. However, a major problem in the use of precious metal catalysts is their high cost.

A major resource lies in the scrap precious metals found in used automotive catalysts and electronic scrap. Recycling of precious metals is not keeping pace with demand, and a microbial method for their recovery would be commercially attractive and would help to forestall projected future shortages and increasing costs.

A novel electrobioreactor was designed for the continuous recovery of precious metals (Yong *et al.*, 2002c); Fig. 3). This comprises a central hollow ''finger'' of Pd-23% Ag alloy, which transports hydrogen as H°. The hydrogen arises from the central electrochemical chamber using $1MHNO_3$ as the electrolyte, with the Pd–Ag membrane electrode held cathodic. H_2 generated electrochemically passes as H° to a biofilm of *D. desulfuricans* on the outer face of the Pd–Ag alloy electrode. In this configuration, the biofilm-coated membrane catalyzed the reductive deposition of Pd^0 (and other precious metals) from both industrial-processing wastewater (Yong *et al.*, 2002c) and leachates from spent automotive catalysts (Yong *et al.*, 2003). The latter is remarkable since the noble metals require aqua regia for their dissolution. For use, the highly acidic leachates were partially neutralized with NaOH to pH 2–2.5, giving a solution of several molar NaCl. Batch-cell suspensions did not remove Pd from the leachate, probably attributable to the high concentration of chloride, which complexed the precious metals extensively, making them unavailable to the cells. However, use of the electrobioreactor overcame this problem, possibly because the palladized biolayer focused reducing power more intensively than did free cells. At a flow residence time of 15 min, the biofilm layer removed more than 95% of the Pd from an input solution of 2 mM Pd(II). In contrast, the Pd(II) removal, using a reactor with heat-killed biomass (or no biomass), removed ~70% of the Pd by chemical reduction alone (Yong *et al.*, 2003). The Bio-Pd^0 removed from the electrobioreactor was catalytically active (Yong *et al.*, 2002a), introducing the possibility of single-step conversion of industrial waste into new catalytic materials without the need for hydro- or pyrometallurgical reprocessing.

The catalytic property of Bio-Pd^0 was applied to environmentally problematic pollutants. The literature abounds with references to the use of microorganisms for the reduction of highly toxic Cr(VI) to less toxic and insoluble Cr(III) (see Section III.A). However Mabbett *et al.* (2002) found that in the absence of bicarbonate (and other metal-complexing ligands), Cr(VI) reduction by *D. vulgaris* was poor, probably because nascent Cr^{3+} inhibited the Cr(VI) reductase activity. However, *Desulfovibrio desulfuricans* which had been palladized (1:1 ratio of Pd^0:biomass) reduced a 700 μM solution of Cr(VI) at the expense of H_2 or formate within 24 h. Under similar conditions nonpalladized cells or

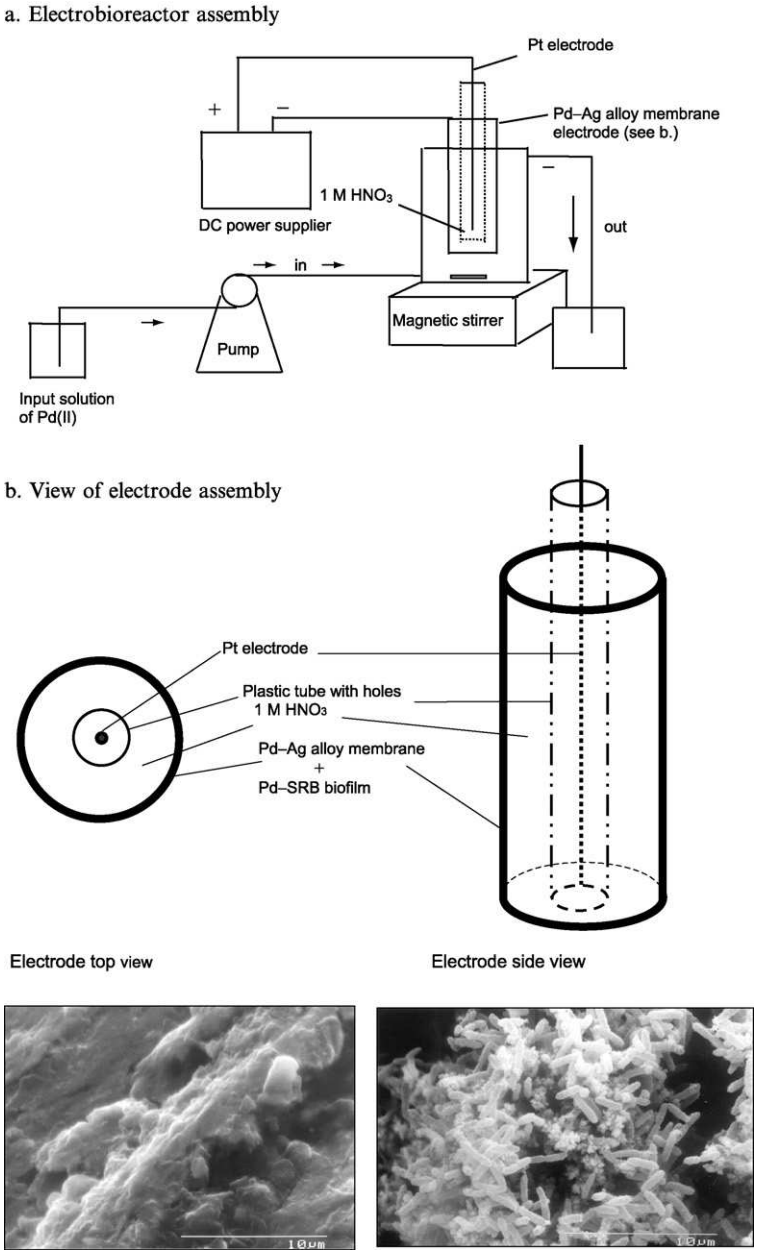

FIG. 3. Electrobioreactor for delivery of hydrogen to biofilm. (A) Electrobioreactor assembly. (B) View of electrode assembly. (C) SEM of the Pd-23% Ag solid membrane electrode surface (left) and coated with biofilm carrying Pd^0 crystals (right). Figures are adapted from Yong *et al.*, 2002c, with permission.

Pd^0 prepared under H_2 in the absence of biomass (Chem-Pd^0) reduced negligible Cr(VI) (Mabbett *et al.*, 2001; 2003). Continuous removal of Cr(VI) was observed over several weeks in a flow-through column using a 1 mM Cr(VI) input solution with formate as the electron donor at pH 3 (Mabbett, 2002). Here, the Bio-Pd^0 was held in a glass wool mat (flow residence time approx 5 h) for continuous Cr(VI) removal. Mass balance analysis showed that at pH 3 all of the Cr(VI) entering the column was accountable as Cr(III) in the exit solution (Mabbett, 2002).

As an example of recalcitrant xenobiotics, polychlorinated biphenyls (PCBs) are highly toxic, highly recalcitrant to degradation, and highly persistent industrial pollutants of major concern. Reductive dehalogenation of PCBs (i.e., removal of the chlorine substituents to leave a molecule more amenable to further chemical or biological attack) has been reported by microbial activity via reactions which are poorly understood and very slow: tens or even hundreds of days is typical (see Baxter-Plant *et al.*, 2003, for review). The ability of Bio-Pd^0 to promote reductive dehalogenation was compared using two SRB strains and Chem-Pd^0 using H_2 provided from a bottled supply, or, where kinetic data were required, using formate, where the concentration added can be accurately known. For the PCBs low activity was seen with the Chem-Pd^0, whereas Bio-Pd^0 promoted release of chloride from PCBs (Baxter-Plant *et al.*, 2003; Mabbett *et al.*, 2001; Mikheenko *et al.*, 2003). Specimen rates are shown in Table 1. Note that the catalytic ''quality'' of the Bio-Pd^0 appears to be microbial strain-associated, reinforcing the concept that the biomass exerts an influence over the pattern of Pd-deposition—a level of organization not found with Chem-Pd^0—which could be responsible for the superiority of the biomass-bound material. Analysis of the materials by x-ray powder diffraction showed the average crystal size of the biological material to be half that of the chemical Pd^0 ($\sim$11 and $\sim$23 nm, respectively; Yong *et al.*, 2002a). For routine catalytic testing, the cells were loaded with Pd^0 to a ratio of Biomass:Pd^0 of 1:1, as shown in Fig. 2E. By varying the preparation and Pd(II) exposure methods, cells could be prepared with different ratios of Pd:biomass or, for the same biomass:Pd^0 ratio, different patterns of Pd deposition. A selection of Pd-loaded biomasses was compared under the electron microscope (Figs. 2E–G) and also magnetically, using a vibrating sample magnetometer (VSM) and a superconducting quantum interference device (SQUID). The magnetic properties varied according to the deposition pattern of the Bio-Pd^0 (I. P. Mikheenko, L. E. Macaskie, M. Rousset, and S. Dementin, unpublished; Mikheenko *et al.*, 2001). Paramagnetism was expected but instead a subset of samples displayed a

TABLE I

RATES OF CHLORIDE RELEASE FROM POLYCHLORINATED BIPHENYLS

Compound	Chloride release (nmol/min/mg Pd) *D. desulfuricans* Bio-Pd^0	*D. vulgaris* Bio-Pd^0	Chem-Pd^0	Ref-Pd^0
4-chlorobiphenyl[a]	13.5 ± 1.2 (F)	5.8 ± 0.4 (F)	1.6 ± 0.6 (F)	0.8 ± 0.2 (F)
2,4,6-trichlorobiphenyl	8.3 ± 0.3 (F)	NT	2.5 ± 0.2 (F)	2.5 ± 0.2 (F)
2,3,4,5-tetrachlorobiphenyl	9.3 ± 0.2 (F)	3.8 ± 0.5 (F)	0.3 ± 0.1 (F)	0.1 ± 0.1 (F)
	8.1 ± 0.5 (H_2)	NT	NT	NT
2,2′,4,4′,6,6′ hexachlorobiphenyl	8.1 ± 0.8 (F)	NT	1.6 ± 0.2 (F)	0.8 ± 0.7 (F)

Loading of the Pd^0 onto the cells was at a mass ratio of 1:1 Pd:biomass and samples were periodically removed for analysis of chloride released (electron donor was 10 mM formate (F) or hydrogen [H_2]). Data are means ± SEM from 3 experiments. NT: not tested. The concentrations of PCBs used were 80 μg/ml as chloride, in hexane suspensions mixed with the bio-Pd^0 suspended in water. Chem-Pd^0 is Pd^0 made by chemical reduction under H_2 without biomass. Ref-Pd^0 is commercial finely divided Pd^0.

strong ferromagnetic component which was absent from Pd^0 prepared chemically under H^2 in the absence of biomass (Chem-Pd^0) and also in many of the Bio-Pd^0 samples. When comparing the dependence of magnetization on the magnetic field, the most highly catalytically active preparations (Fig. 2F) displayed a hysteresis characteristic of a strong ferromagnetic moment. Hence, the magnetic analysis showed a substantial subpopulation of smaller crystals, with the average size of the Pd particles on the cells in the ''best'' preparations (Fig. 2F) estimated from the magnetic data at ~5 nm. Thus, the crystals are essentially Pd-nanoclusters, which were made previously only by specialist physico-chemical techniques, not suitable for bulk synthesis. Preparations typified by that shown in Fig. 2G had a low catalytic activity and a low ferromagnetic component, both tending toward that of the Chem-Pd^0, which suggests that the origin of the extracellular Pd^0-deposit was largely chemical in nature, via the autocatalytic deposition superimposed on the periplasmic nanocrystals (see preceding).

In summary, although the use of microbial reductions for recovery of metals from solution is well established with respect to waste treatment, evidence is mounting that biomass can essentially be used as a templating scaffold for catalytic metal nanocrystals, synthesized enzymatically. In this respect, the biological preparations are analogous to the well-established supported metal catalysts used widely in industry.

Future studies will aim to establish the extent to which microbial preparation methods can bypass chemical methods of catalyst production.

C. Reduction of Hg(II); Bioremediation and Biosensor Development

Microbes have evolved to deal with toxic metals via several mechanisms. (See Bruins *et al.*, 2000, for an overview.) Perhaps the best studied metal resistance system is encoded by genes of the *mer* or mercury resistance operon. Here, Hg(II) is bound in the periplasm of gram-negative bacteria by the MerP protein, transported into the cell via the MerT transporter, and detoxified by reduction to relatively nontoxic volatile elemental mercury by an intracellular mercuric reductase (MerA). Mer proteins are expressed under the regulation of the activator protein, MerR, which binds Hg(II) and activates gene expression. This system is described in detail in several reviews (e.g., Hobman *et al.*, 2000) with more recent studies extending the model to include two additional transporter proteins, MerF and MerC (Wilson *et al.*, 2000). In addition to the MerA-mediated mechanism of Hg-reduction, other enzymes are also able to reduce Hg(II). A novel Fe(II)-dependent mechanism for mercury reduction has been characterized in the membrane fraction of *Thiobacillus ferrooxidans*, which may involve cytochrome *c* oxidase (Iwahori *et al.*, 2000), and *c*-type cytochromes of *Geobacter metallireducens* also reduce Hg(II) (Lovley *et al.*, 1993). In no cases, however, has the reduction of Hg(II) been shown to support microbial growth.

Mercury-resistant bacteria and the proteins that they encode have been used for the bioremediation of Hg-contaminated water and in the development of biosensors for bioavailable concentrations of Hg(II) (Bontidean *et al.*, 2002; Lloyd and Lovley, 2001). Focusing on Hg biosensors, several different components of the *mer* system have been used in prototype sensors, including the NADPH-dependent mercuric reductase (MerA) in an enzyme-linked biosensor (Eccles *et al.*, 1996), the *mer* regulatory region in a whole cell biosensor (Geiselhart *et al.*, 1991), and the MerR protein in a capacitance biosensor (Bontidean *et al.*, 2000).

Mercury-resistant bacteria have also been used to detoxify Hg(II)-contaminated water at lab and pilot scale (see Fig. 4, see color insert). Wagner-Döbler and coworkers captured reduced elemental Hg in a 20-ml immobilized cell bioreactor, inoculated with a mercury-resistant *Pseudomonas putida* and subsequently colonized with other mercury-resistant strains (Wagner-Dobler *et al.*, 2000). A companion

FIG. 4. View of the pilot plant for microbial cleanup of mercury-contaminated wastewater. The plant is located inside a blue standard container (2.5 × 2.5 × 9 m). On the left side, carbon filter, bioreactor, and pH adjustment tank can be seen. On the right side are the mercury monitors and the computer for data collection, online monitoring, and control. The volume of the bioreactor is 1 m^3. Photograph from Dr. Irene Wagner-Döbler.

study demonstrated successful removal of Hg^{2+} from chloralkali electrolysis water at laboratory scale (von Canstein *et al.*, 2002), prior to development of a pilot-plant for Hg(II) removal using this technology (Wagner-Dobler *et al.*, 2000). In the latter study, a 700-L reactor was packed with pumice granules of particle size 4 to 6 mm and inoculated with seven mercury-resistant *Pseudomonas* species. Acidic wastewater from a chloralkali factory was neutralized and amended with sucrose and yeast extract prior to introduction into the bioreactor. Concentrations of up to 10 mg/1 Hg were successfully treated with a retention efficiency of 95%, although influent spikes above this concentration had a deleterious (if reversible) effect on reactor performance. When operated in combination with an activated carbon filter, which also became colonized by bacteria, further removal of Hg to below 10 μg/L was reported. Very high loadings of Hg were retained in the reactor, estimated conservatively at 31.5 kg for the 700-L vessel.

Long-term performance of the reactors has been studied, with no loss of the entrapped Hg(0) (see Fig. 5) from the system over 16 months (von Canstein *et al.*, 2001). Although the reactors were sensitive to

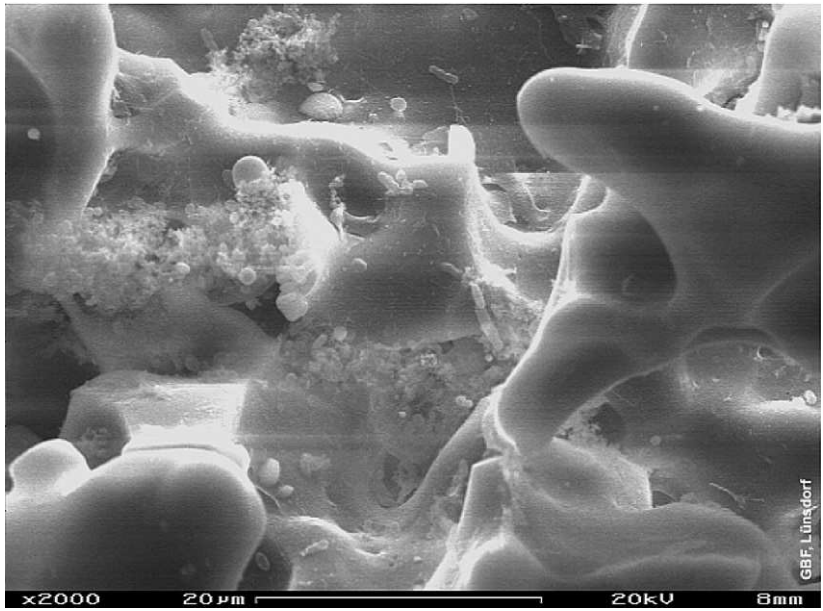

FIG. 5. Ceramic carrier granules (Siran) with mercury droplets and biofilm of *P. putida* Spi3 in a packed bed bioreactor after 2 weeks of treatment of electrolysis wastewater. Scanning electron micrograph courtesy of Heinrich Lünsdorf, GBF.

mechanical and physical stresses (shear from gas bubbles and increased temperature over 41°C, respectively), the system seems robust and able to adjust to elevated Hg(II) concentrations (up to 7.6 mg/L) within several days (von Canstein *et al.*, 2001). With a continuous selection pressure for mercury resistance, a stable and highly active mercury-reducing microbial community is established within the bioreactors; confirmed using PCR-based techniques targeting the intergenic spacer region of 16S–23S rDNA, and a functional gene target for Hg(II) reduction, *merA* (von Canstein *et al.*, 2001). The performance of the reactor system has also been studied in response to the oscillation of the mercury concentration in the bioreactor inflow (von Canstein *et al.*, 2002). At low mercury concentrations, maximum Hg(II) reduction occurred near the inflow at the bottom of the bioreactor. At higher concentrations, the zone of maximum activity migrated to the upper horizons. Molecular analysis of the microbial communities showed an increasing microbial diversity along a gradient of decreasing mercury concentrations (von Canstein *et al.*, 2002).

D. Reduction of V(V), Mo(VI), Au(III), and Ag(1)

Although most work has focused on the reduction of Fe(III) and Mn(IV), Cr(VI), Pd(II), and Hg(II), several other high-valence transition metals can also be reduced through microbial activities. For example, it has been suggested that the microbial reduction of V(V) could be responsible for the precipitation of the element in anaerobic environments and could also be used to remediate vanadium-contaminated ore-processing waste streams (Lovley, 1993). Despite an early study that showed V(V) reduction by *Micrococcus lactilyticus, Desulfovibrio desulfuricans*, and *Clostridium pasteurianum* (Woolfolk and Whiteley, 1962), followed by the observation that the ability to reduce V(V) was widespread among soil bacteria and fungi (Bautista and Alexander, 1972), few recent studies have addressed the bioreduction of this metal. To date, most work has focused on two pseudomonads: *P. vanadiumreductans* and *P. isachenkovii* isolated from a waste stream from a ferrovanadium factory and seawater, respectively (Yurkova and Lyalikova, 1991). Anaerobic cells were able to utilize a wide range of electron donors including hydrogen, sugars, and amino acids. V(V) was reduced to blue-colored V(IV) and possibly further reduced to V(III), the latter indicated by the formation of a black precipitate and by its reaction with the reagent tairon (Yurkova and Lyalikova, 1991).

Microbial reduction of Mo(VI) could also play a role in the Mo cycle, leading to the concentration of insoluble Mo in anaerobic marine sediments and reduction spots in rocks (Lovley, 1993), and by analogy could be used to remediate Mo(VI)-contaminated waters. However, comparatively few studies have addressed this process. Early work suggested that *Pseudomonas guillermondii* and a *Micrococcus* species could reduce Mo(VI) to molybdenum blue (Bautista and Alexander, 1972). More recently, similar activities have been identified in cultures of a molybdenum-resistant *Enterobacter* species (Ghani *et al.*, 1993). The organism was grown under anaerobic conditions in glucose-containing medium supplemented with 200 mM Mo(VI). Reduction of Mo(VI) was accompanied by a change in color, as Mo(V) formed and complexed with phosphate in the medium to form methylene blue. The use of metabolic inhibitors suggested that the electrons for Mo(VI) reduction were derived from the glycolytic pathway, and the terminal reductase was downstream from cytochrome *b* (Ghani *et al.*, 1993).

The ability to reduce Mo(VI) has also been identified in pre-grown cells of the sulfate-reducing bacterium *Desulfovibrio desulfuricans* (Tucker *et al.*, 1997). Here, lactate or hydrogen was used as an electron donor for enzymatic metal reduction. In parallel experiments conducted using cultures supplied with sulfate, Mo(VI) was also reduced by biogenic sulfide via an indirect mechanism (Tucker *et al.*, 1997). Cells of *D. desulfuricans* immobilized in a bioreactor have also been used to remove Mo(VI) from solution at high efficiency (Tucker *et al.*, 1998). The organism was unable to grow using Mo(VI) as an electron acceptor (Tucker *et al.*, 1997), and it is unlikely that actively growing cultures of sulfate-reducing bacteria would play a direct role in reducing high concentrations of Mo(VI) in the environment given the toxicity of molybdate to these organisms (Oremland and Capone, 1988). Finally, the thermophilic acidophiles *Sulfolobus brierleyi* and *S. acidocaldarius* are able to couple S^0 oxidation to Mo(VI) reduction (Brierley and Brierley, 1982). Similar activities have also been noted in *Thiobacillus ferroxidans* (Sugio *et al.*, 1988).

The microbial reduction of Co(III) has received recent attention because radioactive cobalt-60 can be a problematic contaminant at some U.S. Department of Energy sites where radioactive waste has been stored. Co(III) is especially mobile when complexed with EDTA, and several studies have focused on the ability of Fe(III)-reducing bacteria to retard the mobility of the metal through reduction to Co(II) (Caccavo *et al.*, 1994; Gorby *et al.* 1998). The Co(II) formed does not associate strongly with EDTA (it is over 25 orders of magnitude less

thermodynamically stable than Co(III)EDTA), and absorbs to soils, offering potential for *in situ* immobilization of the metal in contaminated soils. However, Gorby *et al.* (1998) have demonstrated that reduced Co(II)EDTA can also transfer electrons abiotically to solid Mn(IV) oxides, effectively acting as an electron shuttle between the bacterial cell and the metal oxide. Mn(IV) minerals could, therefore, play a role in maintaining concentrations of mobile Co(III)EDTA in the subsurface. The precise mechanisms of dissimilatory Co(III) reduction remain to be investigated.

In addition to the bioremediation of toxic transition metals, microbial metal reduction may also prove useful in the recovery of precious metals including gold and silver. Early studies suggested that *c*-type cytochromes of the Fe(III)-reducing bacterium *G. metallireducens* were able to transfer electrons to soluble Au(III) (Lovley *et al.*, 1993). In subsequent studies a wide range of dissimilatory reducing Fe(III)-reducing *Bacteria* and *Archaea*, including the hyperthermophilic *Archaea Pyrobaculum islandicum* and *Pyrococcus furiosus*, the hyperthermophilic bacterium *Thermotoga maritima,* and the mesophilic bacteria *Shewanella alga* and *Geovibrio ferrireducens*, were shown to reduce Au(III) (as gold chloride) to insoluble Au(0) (Kashefi *et al.*, 2001). Organic electron donors were not utilized, and the site of precipitation of Au(0) varied among organisms. Most strains tested precipitated Au on the surface of the cell, while *G. ferrireducens* precipitated Au(0) in the periplasm. The ability to reduce Au(III) seems to be species specific, and closely related organisms with similar activities against a range of metals have differing activities against Au(III) (Kashefi *et al.*, 2001). For example, unlike *P. islandicum*, a close relative of *P. aerophilum*, is unable to reduce Au(III). The obligate requirement for hydrogen as an electron donor would suggest that a hydrogenase is involved in Au(III) reduction, and given the direct reduction of other metals by hydrogenase (Lloyd *et al.*, 1997), it is possible that hydrogenases may play a direct role in Au(III) reduction. This is yet to be tested. The precise involvement of Fe(III)-reducing bacteria in the deposition of Au ores also warrants attention, as it has been argued that these organisms are present in high- and moderate-temperature sedimentary environments where Au deposits have been recovered (Kashefi *et al.*, 2001). Despite potential applications for the recovery of silver from wastes, including those from photographic processes, microbial reduction of Ag(I) has been studied, but in little detail. Early reports noted that the reduction of Ag(I) may account for resistance to silver in some microorganisms (Belly and Kydd, 1982), but more recent studies have uncovered alternative strategies for resistance to Ag(I) in organisms

isolated from hospital burn wards where silver may be used as a biocide (Gupta *et al.*, 1999). Several studies, including that of Fu *et al.* (2000), have shown biosorption of Ag(I) to the surface of cells (in this case, a *Lactobacillus* sp.), followed by reduction to Ag(0). Here, the mechanism for Ag(I) reduction remains unknown.

IV. Reduction of Metalloids

A. Reduction of As(V)

Arsenic exists as the arsenate anion [As(V)] in oxic environments and binds very strongly to sediments. Under anaerobic conditions, As(V) is reduced to the more mobile and toxic As(III). The mobilization of arsenic in sediments poses a potential threat to the lives of millions worldwide, for example, those in the Ganges–Meghna–Brainmaputra delta plain who rely on contaminated wells for their drinking water (MacArthur *et al.*, 2000). It is also possible that microbial reduction of As(V) to As(III) could be used to clean soils or sediments that are contaminated with the toxic metalloid. As with several other metal reduction processes discussed, As(V) reduction can be catalyzed by biotic or abiotic mechanisms. Early evidence for a microbial role in As(V) reduction in sediments has been reviewed extensively by Oremland and Stolz (2000). These include inhibition of As(V) reduction by treatment of sediments with heat and a wide range of antimicrobials. In addition, As(V) reduction was inhibited by air but enhanced by an atmosphere of hydrogen, suggesting the involvement of anaerobic metal-reducing bacteria.

Indeed, several organisms capable of growing through dissimilatory reduction of As(V) have now been isolated. *Chrysiogenes arsenatis* is a strict anaerobe that was isolated from wastewater from a gold mine (Macy *et al.*, 1996). This organism contains a periplasmic arsenate reductase consisting of two subunits (masses 87 and 29 kDa) which contain Mo, Fe, S, and Zn cofactors (Krafft and Macy, 1998). *Sulfurospirillum arsenophilum* (previously strain MIT-13) is a gram-negative vibroid microaerobic sulfur-reducing bacterium isolated from arsenic-contaminated watershed sediments in eastern Massachusetts. It is a very close relative of *Sulfurosprillum barnessi*, which can also reduce As(V), and has a broad activity against metals including Fe(III) and Se(VI) (see sections on these metals/metalloids). An Oremland and Stolz review (2000) has mentioned the preliminary purification and characterization of an arsenate reductase from this organism, which constituted a trimeric complex of mass 120 kDa, consisting of subunits

of 65 kDa, 31 kDa, and 22 kDa. A gram-positive sulfate-reducing bacterium *Desulftomaculum auripigmentum* has also been described that reduces As(V) followed by sulfate, resulting in the formation of orpiment (As_2S_3) (Newman *et al.*, 1998). Reduction of As(V) to As(III) by the ArcC reductase also forms the basis of a well-studied microbial arsenic resistance mechanism, preceding efflux of As(III) from the cell. This mechanism has been reviewed in detail (Mukhopadhyay *et al.*, 2002) and will not be discussed further. It should be noted, however, that ArcC-mediated As(V) reduction does not support microbial growth, and there is currently little evidence linking this mechanism of As(V) reduction to the biogeochemical cycling of arsenic. Finally, on this note, it is worth mentioning that a new organism has recently been isolated that is able to use As(III) as an electron donor for aerobic growth (Santini *et al.*, 2000), closing the biological As cycle.

B. Reduction of Se(VI) and (IV) and Other Group VIB Elements

Reduction of toxic Se(VI) and Se(IV) to relatively unreactive Se(0) results in its removal from contaminated water. Several studies have demonstrated that these transformations can be catalyzed by microbes (for an overview, see Oremland and Stolz, 2000). For example, the ability to reduce Se(VI) is widespread in sediments, with biological reduction unequivocally demonstrated in 10 out of 11 sediment types (Steinberg and Oremland, 1990). Also, Se(VI) is not reduced chemically under physiological conditions of pH and temp, and Se(VI) reduction is inhibited by autoclaving of sediments. Se(VI) reduction is also inhibited by chromate and tungstate, but not by molybdate, an inhibitor of sulfate-reducers (Oremland *et al.*, 1989; Steinberg and Oremland, 1990). These results suggest the involvement of a molybdenum-containing enzyme, recently backed up by the study of Schroder (Schroder *et al.*, 1997), who identified a Mo-containing membrane-bound Se(VI) reductase in *Thauera selanatis* as will be described.

Organisms that are known to reduce Se(VI) enzymatically include *Wolinella succinogenes* (Tomei *et al.*, 1992), *Desulfovibrio desulfuricans* (Tomei *et al.*, 1995), *Pseudomonas stutzeri* (Lortie *et al.*, 1992), *Enterobacter cloaceae* (Losi and Frankenberger, 1997), and *E. coli* (Avazeri *et al.*, 1997). In these examples, Se(VI) reduction does not support growth, and seems to be incidental to the physiology of the organism. In at least one organism (*E. coli*), the involvement of broad specificity nitrate reductases is implicated by biochemical studies (Avazeri *et al.*, 1997). In addition to these rather nonspecific reactions,

specialist organisms are known to conserve energy through Se(VI) reduction, including *Thauera selanatis* (Macy and Lawson, 1993) and *Sulfurospirillum barnesii* (originally *Geospirillum barnesii* strain SES-3 (Stolz *et al.*, 1997)) and two other bacilli (*Bacillus arsenicoselanatis* and *B. selenitireducens*), both isolated from Mono Lake, California (Switzer Blum *et al.*, 1998). Of these four model organisms, the mechanism of Se(VI) reduction is best understood in *T. selanatis* (Schroder *et al.*, 1997). A periplasmic complex of approximately 180 kDa (with subunits of masses 96, 40, and 23 kDa) has been characterized and shown to contain Mo, Fe, and acid labile sulfur. Specificity for Se(VI) is high, with a K_m of 16 μM. The enzyme was unable to reduce nitrate, nitrite, chlorate, chlorite, or sulfate. Earlier studies had suggested that the enzyme that catalyzed further reduction of Se(IV) (selenite) to elemental selenium in the organism was a component of the nitrate-respiratory system, possibly a periplasmic nitrate reductase (DeMoll-Decker and Macy, 1993). It was also noted in this study that *T. selanatis* was unable to grow through the reduction of selenite. Biochemical studies are not so advanced in *S. barnessii*, although the enzyme activity contrasts with that characterized in *T. selanatis* as it is localized in the membrane fraction and may have a wider substrate specificity (Stolz *et al.*, 1997). Whole cells of *S. barnessii* can use a very wide range of electron acceptors (including Se(VI) in addition to As(V), nitrate, fumarate, and thiosulfate), and despite the proposed wide specificity of the Se(VI) reductase, the authors of this chapter suggest that a suite of specific terminal reductases are required for growth on the different electron acceptors. This hypothesis is based on the observed differential cytochrome content and cytochrome activity when grown on alternative electron acceptors; at least three different *b*-type and 2 different *c*-type cytochromes have been detected (Stolz *et al.*, 1997).

Several studies have demonstrated that microbial reduction of Se(VI) can be harnessed in bioreactors to remove the toxic metalloid from contaminated water. A pilot-scale bioreactor containing *T. selenatis* has been used successfully to treat drainage water contaminated with both Se(VI) and nitrate in the Panoche Water District, San Joaquin Valley, California (Cantafio *et al.*, 1996). Selenium oxyanion concentrations (selenate plus selenite) in the drainage water were reduced by 98%, to an average of 12 ± 9 μg/L, while nitrate and nitrite concentrations were reduced to 0.1 and 0.01 mM, respectively (98% reduction). Between 91% and 96% of the selenium recovered was elemental selenium. Acetate was the electron donor of choice and in a subsequent study was generated for Se(VI) reduction through the fermentation of

whey (Bledsoe *et al.*, 1999). A more recent study has used a 0.5 L chemostat containing *Bacillus* sp. SF-1 to treat model wastewater containing selenate (at 41.8 mg/L) (Fujita *et al.*, 2002). Excess lactate was supplied as the electron donor. Accumulation of selenite was noted at high dilution rates, but the selenite was further reduced to elemental selenium by increasing the residence time in the reactor (Fujita *et al.*, 2002).

Tellurium shares several chemical properties with selenium and sulfur, and is used as an additive in steel making (to improve ''machinability''), as an industrial catalyst, and also as a photoreceptor in copiers. Tellurite (TeO_3^{2-}) reduction has been studied in several organisms, mainly in the context of resistance to this anionic form of tellurium. As the metalloid is precipitated as elemental Te(0), this form of metabolism could also be used to treat Te(IV)-contaminated water. Basal levels of resistance to toxic Te(IV) have been attributed to the activity of a membrane-bound nitrate reductase in *Escherichia coli* (Avazeri *et al.*, 1997). An additional Te(IV) reductase was detected in the soluble fraction of anaerobically grown cells. Growth using Te(IV) as an electron acceptor was also reported in an engineered strain overexpressing nitrate-reductase but was not thought to be physiologically relevant in wild-type cells of *E. coli* (Avazeri *et al.*, 1997). *Rhodobacter sphaeroides* has also been reported to reduce Te(IV) (as well as oxyanions of Se), with a requirement for a functional photosynthetic electron transfer chain under photosynthetic conditions, or cytochromes bc_1 and c_2 when grown aerobically (Moore and Kaplan, 1992). Again, metal reduction was discussed in the context of resistance to the metalloids. Finally, plasmids are known to encode several distinct resistance determinants for Te(IV), and again Te(IV) reduction is implicated as the resistance mechanism as elemental Te is laid down in Te-resistant bacteria (Taylor, 1999). Other mechanisms of resistance may be important, however, involving cysteine-metabolizing enzymes and methyl transferases (Taylor, 1999). Finally, Te(IV) (and Se(VI)/Se(IV)) reduction and precipitation by sulfate-reducing bacteria have also been reported, in the order Te(IV)>S(VI)>Se(IV), which is in contrast to that predicted by the redox potentials alone (Lloyd *et al.*, 2001). To date, there have been no reports of microbial growth coupled to the reduction of Te(IV) by non-genetically engineered microorganisms.

One last member of the Group VIB that warrants brief discussion is polonium. ^{210}Po is the terminal member of the ^{238}U decay series, produced by the decay of ^{210}Pb via ^{210}Bi. ^{210}Po has attracted attention, accounting for all α-emitting activity in some samples of groundwater

from the Central Florida Phosphate District (Burnett *et al.*, 1987) and has also been identified as a troublesome isotope contaminating and fouling drilling equipment during oil exploration in areas rich in uranium ores (Lloyd and Macaskie, 2000). Little is known about the geochemistry of Po, but there is evidence that factors affecting the sulfur cycle may affect Po availability. Indeed, Cherrier *et al.* (1995) demonstrated the microbial uptake of Po into the cellular pool, indicating the potential for assimilative Po reduction and a biochemical role analogous to that of S. Given that a range of anaerobic bacteria are also able to reduce Se and Te oxyanions, it is likely that dissimilatory reduction of Po is possible and could be used to treat Po-contaminated water.

V. Reduction and Bioremediation of Actinides and Fission Products

Although there are natural sources of radioactivity, the release of anthropogenic radionuclides into the environment is significant and a subject of intense public concern. Large quantities of radionuclides were released as a consequence of nuclear weapons testing in the 1950s and 1960s, while the controlled discharge of process effluents produced by industrial activities allied to the generation of nuclear power continues to introduce radioactivity into the environment today. Indeed, research programs aimed at remediating large areas of land contaminated by radionuclides in the United States, at so-called ''superfund sites,'' have resulted in significant advances in the understanding of the mechanisms of metal and radionuclide reduction in the subsurface (for examples, see www.lbl.gov/NABIR). Indeed, because many radionuclides of concern are both redox active and less soluble when reduced, bioreduction offers much promise for controlling the solubility and mobility of target radionuclides in contaminated sediments, e.g., the reduction of U(VI) (the uranyl ion; UO_2^{2+}) to U(IV) (uraninite; UO_2) (Lovley and Phillips, 1992; Lovley *et al.*, 1991) or the reduction of the fission product Tc(VII) (the pertechnetate ion; TcO_4^-) to Tc(IV) (TcO_2) (Lloyd *et al.*, 2000).

A. Reduction of U(VI); *in situ* Bioremediation

The first demonstration of dissimilatory U(VI) reduction was by Lovley and coworkers (1991) who reported that the Fe(III)-reducing bacteria *Geobacter metallireducens* (previously designated strain GS-15) and *Shewanella oneidensis* (formerly *Alteromonas putrefaciens* and then *Shewanella putrefaciens*) can conserve energy for anaerobic growth via the reduction of U(VI). It should be noted, however, that

the ability to reduce U(VI) enzymatically is not restricted to Fe(III)-reducing bacteria. Other organisms, including a *Clostridium* sp. (Francis, 1994) and the sulfate-reducing bacteria *Desulfovibrio desulfuricans* (Lovley and Phillips, 1992) and *D. vulgaris* (Lovley and Phillips, 1994), also reduce uranium but are unable to conserve energy for growth via this transformation. To date, *D. vulgaris* remains the only organism in which the enzyme system responsible for U(VI) reduction has been characterized in detail. Purified tetraheme cytochrome c_3 was shown to function as a U(VI) reductase *in vitro*, in combination with hydrogenase, its physiological electron donor (Lovley and Phillips, 1994). *In vivo* studies using a cytochrome c_3 mutant of the close relative *D. desulfuricans* strain G20 confirmed a role for cytochrome c_3 in hydrogen-dependent U(VI) reduction but suggested additional pathways from organic electron donors to U(VI), which bypassed the cytochrome (Payne *et al.*, 2002).

More recent studies have identified a homologous cytochrome (PpcA), a triheme periplasmic cytochrome c_7 of the Fe(III)-reducing bacterium *Geobacter sulfurreducens* that may also play a role in U(VI) reduction (Lloyd *et al.*, 2003). The protein was able to reduce U(VI) *in vitro*, while a *ppcA* deletion mutant supplied with acetate as an electron donor had lower activity against U(VI) (Lloyd *et al.*, 2003). Additional (if indirect) evidence linking the activity of this periplasmic protein with U(VI) reduction *in vitro* included the precipitation of the reduced product U(IV) in the periplasm (see Fig. 6) and the lack of impact of protease treatment of whole cells on the ability to reduce U(VI) (Lloyd *et al.*, 2002). This final result is particularly important, as it implies that U(VI) and Fe(III) are reduced by different mechanisms in *G. sulfurreducens*. U(VI) would seem to be reduced in the periplasm, while the reduction of insoluble Fe(III) oxides was inhibited dramatically by protease treatment, presumably due to removal of surface-bound cytochromes required for reduction of the extracellular electron acceptor. The mechanism of U(VI) reduction by an *S. putrefaciens* strain has also been investigated (Wade and DiChristina, 2000). A novel screening method was used to identify mutants that were unable to reduce U(VI). Evidence was presented to suggest that the mechanism of U(VI) reduction was distinct from those of Fe(III) and Mn(IV) reduction but may share components of the nitrite-reducing pathway (Wade and DiChristina, 2000).

The potential for dissimilatory metal-reducing microorganisms to effectively bioremediate uranium has been demonstrated in a series of laboratory and field experiments. The addition of acetate to uranium-contaminated subsurface sediments stimulated U(VI) reduction

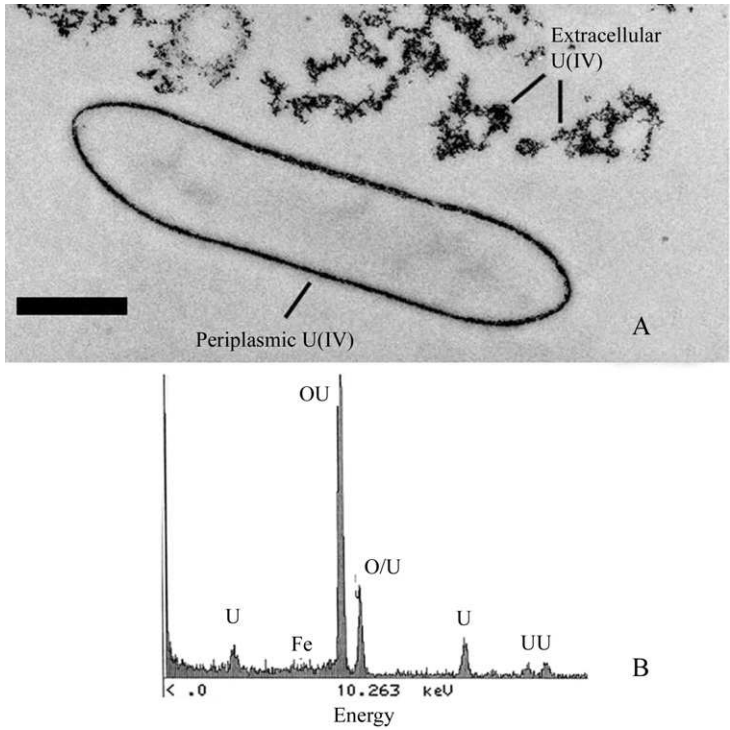

FIG. 6. Transmission electron micrograph of thin sections of *Geobacter sulfurreducens* containing electron-dense precipitates of U(IV) formed through the bioreduction of U(VI). Uranium was identified by EDS (B). Bar = 0.5 μm. Adapted from Lloyd *et al.*, 2002.

in laboratory sediment incubations (Finneran *et al.*, 2002). Nitrate had to be reduced before U(VI) reduction in part because some Fe(III)-reducing microorganisms preferentially reduce nitrate, but also because some of these organisms can oxidize U(IV) with the reduction of nitrate. Once nitrate was depleted, U(VI) was reduced concurrently with Fe(III) and prior to sulfate reduction (Finneran *et al.*, 2002). Molecular analysis of a variety of sediments demonstrated that microorganisms in the *Geobacteraceae* family were highly enriched during U(VI) reduction, accounting for nearly half of the microbial community (Holmes *et al.*, 2002). In a field study, the addition of acetate stimulated concurrent U(VI) and Fe(III) reduction with highly effective removal of U(VI) from the groundwater during the Fe(III) reduction phase (Anderson *et al.*, submitted for publication). During the period of rapid U(VI) reduction, *Geobacter* species accounted for as much as 85% of the microorganisms

in the groundwater. These studies have suggested that stimulating the activity of *Geobacter* species in subsurface environments is an effective strategy for immobilizing contaminant uranium in the subsurface.

B. Reduction of Np(V) and Pu(IV)

Although ^{238}U remains the priority pollutant in most medium- and low-level radioactive wastes, other actinides, including, ^{230}Th, ^{237}Np, ^{241}Pu, and ^{241}Am, can also be present (Lloyd and Macaskie, 2000; Macaskie, 1991). Th(IV) and Am(III) are stable across most Eh values encountered in radionuclide-contaminated waters (Fig. 7) but the potentials for Pu(V)/Pu(IV) and Np(V)/Np(IV), in common with that of U(VI)/U(IV), are more electropositive than the standard redox potential of ferrihydrite/Fe^{2+} (approximately 0 V (Thamdrup, 2000)). Thus, Fe(III)-reducing bacteria have the metabolic potential to reduce these radionuclides enzymatically or via Fe(II) produced from the reduction

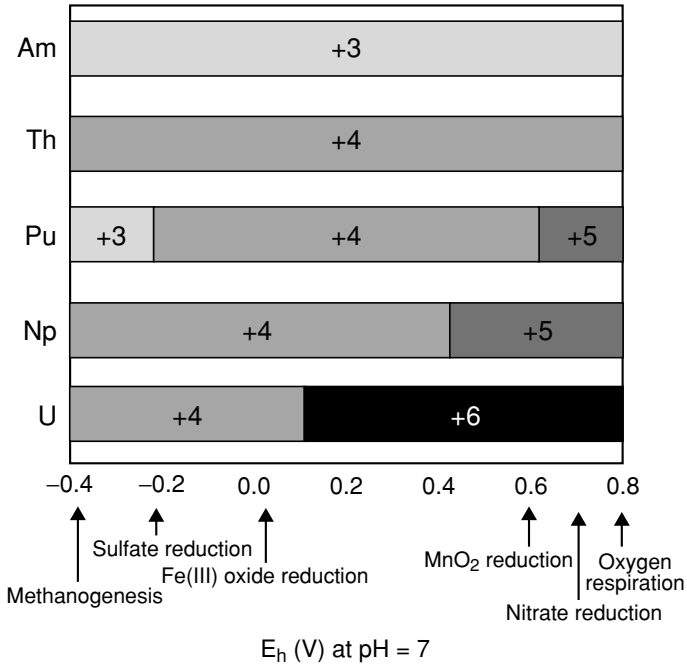

Fig. 7. Oxidation state of actinides as a function of standard reduction potential at pH 7, with redox potentials of common microbial respiratory processes (adapted from Banaszak *et al.*, 1999; Lloyd *et al.*, 2002).

of Fe(III) oxides. This is significant because the tetravalent actinides are amenable to bioremediation due to their high ligand-complexing abilities (Lloyd and Macaskie, 2000) and are also immobilized in sediments containing active biomass (Peretrukhin *et al.*, 1996). Thus, although it is possible for Fe(III)-reducing bacteria to reduce and precipitate actinides in one step (e.g., the reduction of soluble U(VI) to insoluble U(IV) [see preceding]), some transformations do not result in direct formation of an insoluble mineral phase but in the formation of a cation more amenable to bioprecipitation. This is illustrated when considering highly soluble Np(V) (NpO_2^+), which was reduced to soluble Np(IV) by *S. putrefaciens*, with the Np(IV) removed as an insoluble phosphate biomineral by a phosphate-liberating *Citrobacter* sp. (Lloyd *et al.*, 2000). Also, some studies have suggested that the reduction of Pu(IV) to (III) can be achieved by Fe(III)-reducing bacteria, although the Pu(III) was reported to reoxidize spontaneously (Rusin *et al.*, 1994). Although this may lead to solublization of sediment-bound Pu(IV), it will yield a trivalent actinide that is also amenable to bioremediation using a range of microbially produced ligands (Lloyd and Macaskie, 2000). The biochemical basis of these transformations remains uncharacterized.

C. Reduction of Tc(VII)

The fission product technetium is another long-lived radionuclide that is present in nuclear waste and has attracted considerable interest. This is due to a combination of its mobility as the soluble pertechnetate ion (Tc(VII); TcO_4-), its bioavailability as an analog of sulfate, and its long half-life (2.13×10^5 years) (Wildung *et al.*, 1979). Like Np(V), Tc(VII) has weak ligand-complexing capabilities and is difficult to remove from solution using conventional ''chemical'' approaches. Several reduced forms of the radionuclide are insoluble, however, and metal-reducing microorganisms can reduce Tc(VII) and precipitate the radionuclide as a low-valence oxide. Thus, microbial reduction of Tc(VII) to insoluble Tc(IV) offers considerable potential for the remediation of sediments and waters contaminated with the radionuclide.

Although microbial metabolism was known to decrease the solubility of Tc from earlier studies (Henrot, 1989; Pignolet *et al.*, 1989), Lloyd and Macaskie (1996) were the first to unequivocally demonstrate direct enzymatic reduction of Tc(VII) by microorganisms. In this study, a novel phosphorimager technique was used to confirm reduction of the radionuclide by *S. putrefaciens* and *Geobacter metallireducens*,

with similar activities subsequently detected in laboratory cultures of *Rhodobacter sphaeroides*, *Paracoccus denitrificans*, some Pseudomonads (Lloyd *et al.*, 2002), *E. coli* (Lloyd *et al.*, 1997), and a range of sulfate-reducing bacteria (Lloyd *et al.*, 2001, 1998; 1999). Other workers have used this technique to show that *Thiobacillus ferrooxidans* and *T. thiooxidans* (Lyalikova and Khizhnyak, 1996) and the hyperthermophile *Pyrobaculum islandicum* (Kashefi and Lovley, 2000) are also able to reduce Tc(VII). It should be stressed that Tc(VII) reduction has not been shown to support growth in any of these studies and seems to be a fortuitous biochemical side reaction in the organisms studied to date. Finally, x-ray absorption spectroscopy studies have recently identified insoluble Tc(IV) as the final oxidation state produced when Tc(VII) is reduced enzymatically by *Geobacter sulfurreducens* (Lloyd *et al.*, 2000), *E. coli* (Lloyd and Sole, unpublished), and *S. putrefaciens* (Wildung *et al.*, 2000). Other studies have also shown that Tc(VII) can be reduced via indirect microbial processes, for example, biogenic sulfide (Lloyd *et al.*, 1998), Fe(II) (Lloyd *et al.*, 2000), or U(IV) (Lloyd *et al.*, 2002). Tc(VII) reduction and precipitation by biogenic Fe(II) is particularly efficient and may offer a potentially useful mechanism for the remediation of Tc-contaminated sediments containing active concentrations of Fe(III)-reducing bacteria (Lloyd *et al.*, 2000).

The biochemical basis of Tc(VII) reduction has been best studied in *E. coli*. Initial studies demonstrated that anaerobic, but not aerobic, cultures of *E. coli* reduced Tc(VII) with the reduced radionuclide precipitated within the cell (Lloyd *et al.*, 1997). Results obtained from studies conducted with wild-type cells and 34 defined mutants defective in the synthesis of regulatory or electron transfer proteins were used to construct a model for Tc(VII) reduction by *E. coli*. The central tenet of this model is that the hydrogenase 3 component of FHL catalyzes the transfer of electrons from dihydrogen to Tc(VII). According to this model, the formate dehydrogenase component (FdhH) is required only if formate, or a precursor, is supplied as an electron donor for Tc(VII) reduction in place of hydrogen. This model has been validated by the observations that a mutant unable to synthesize hydrogenase 3 was unable to reduce Tc(VII) when either hydrogen or formate was supplied as an electron donor (Lloyd *et al.*, 1997).

The identification of hydrogenase 3 of FHL as the Tc(VII) reductase of *E. coli* opened up the way for a program to screen for organisms with naturally enhanced activities against Tc(VII). Several organisms documented to have naturally high activities of FHL or uptake hydrogenase were tested, resulting in the identification of several strains of sulfate-reducing bacteria that were able to couple the oxidation of

formate or hydrogen to Tc(VII) reduction (Lloyd *et al.*, 2001). Rates of reduction in some strains were approximately 64-fold greater than those recorded in anaerobic cultures of *E. coli* (Lloyd *et al.*, 1999). *Desulfovibrio desulfuricans* (Lloyd *et al.*, 1999) and related strains (Lloyd *et al.*, 2001) were also able to utilize formate as an efficient electron donor for Tc(VII) reduction. This is consistent with the existence of a rudimentary FHL complex (consisting of a formate dehydrogenase coupled to a hydrogenase via a cytochrome) located in the periplasm of these strains (Peck, 1993). Accordingly, the site of reduced Tc precipitation was identified as the periplasm in *D. desulfuricans* (Lloyd *et al.*, 1999), and more recent studies have confirmed a role for a periplasmic Ni–Fe hydrogenase in Tc(VII) reduction by a relative in the δ subclass of the *Proteobacteria*, the sulfate-reducing bacterium *Desulfovibrio fructosovorans* (De Luca *et al.*, 2001). Subsequent studies on the development of a bioprocess to treat Tc(VII)-contaminated water have focused on the use of immobilized cells of sulfate-reducing bacteria, such as *D. desulfuricans*, which are robust and capable of treating low concentrations of Tc(VII) against a high background of contaminating nitrate ions, is often noted in nuclear waste (Lloyd *et al.*, 1999).

VI. Future Directions

Although the environmental relevance of metal reduction processes has only recently become apparent, rapid advances in the understanding of these important biotransformations have been made. This has led the way to the development of several practical applications, most notably for the *ex situ* remediation of Hg(II)-contaminated water at pilot scale. Metal reduction processes also offer considerable potential for the treatment of contaminated water *in situ*, although advances are required in the understanding of the mechanisms of metal reduction in complex sedimentary environments, and these must be interfaced with advances in hydrology and civil engineering. Similar multidisciplinary approaches are also required if the nanoscale biominerals (e.g., Pd(0) and magnetite) produced by metal-reducing bacteria are to reach their full potential for a range of applications.

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References

Ahmann, D., Roberts, A. L., Krumholz, L. R., and Morel, F. M. M. (1994). Microbe grows by reducing arsenic. *Nature* **371,** 750.

Anderson, R. T., Rooney-Varga, J., Gaw, C. V., and Lovley, D. R. (1998). Anaerobic benzene oxidation in the Fe(III)-reduction zone of petroleum-contaminated aquifers. *Environ. Sci. Technol.* **32,** 1222–1229.

Avazeri, C., Turner, R. J., Pommier, J., Weiner, J. H., Giordano, G., and Vermeglio, A. (1997). Tellurite reductase activity of nitrate reductase is responsible for the basal resistance of *Escherichia coli* to tellurite. *Microbiol.* **143,** 1181–1189.

Banaszak, J. E., Rittman, B. E., and Reed, D. T. (1999). Subsurface interactions of actinide species and microorganisms: Implications for the bioremediation of actinide–organic mixtures. *J. Radioanal. Nucl. Chem.* **241,** 385–435.

Barkay, T., Miller, S. M., and Summers, A. (2003). Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiol. Rev.* **27,** 355–384.

Bautista, E. M., and Alexander, M. (1972). Reduction of inorganic compounds by soil microorganisms. *Soil Sci. Soc. Amer. Proc.* **36,** 918–920.

Baxter-Plant, V. S., Mikheenko, I. P., and Macaskie, L. E. (2003). Sulphate-reducing bacteria, palladium, and the reductive dehalogenation of chlorinated aromatic compounds. *Biodegradation,* **14,** 83–90.

Beliaev, A. S., Saffarini, D. A., McLaughlin, J. L., and Hunnicutt, D. (2001). MtrC, an outer membrane decahaem *c* cytochrome required for metal reduction in *Shewanella putrefaciens* MR-1. *Mol. Microbiol.* **39,** 722–730.

Belly, R. T., and Kydd, G. C. (1982). Silver resistance in microorganisms. *Dev. Ind. Microbiol.* **23,** 567–577.

Bledsoe, T. L., Cantafio, A. W., and Macy, J. M. (1999). Fermented whey—An inexpensive feed source for a laboratory scale selenium-bioremediation reactor system inoculated with *Thauera selenatis*. *App. Microbiol. Biotechnol.* **51,** 682–685.

Bond, D. R., Holmes, D. E., Tender, L. M., and Lovley, D. R. (2002). Electrode-reducing microorganisms that harvest energy from marine sediments. *Science* **295,** 483–485.

Bond, D. R., and Lovley, D. R. (2003). Electricity production by *Geobacter sulfurreducens* attached to electrodes. *Appl. Environ. Microbiol.* **69,** 1548–1555.

Bontidean, I., Csoregi, E., Corbisier, P., Lloyd, J. R., and Brown, N. L. (2002). Bacterial metal-responsive elements and their use in biosensors for monitoring of heavy metals. *In* ''The Handbook of Heavy Metals in the Environment,'' (B. Sankar, ed.), pp. 647–680. Marcel Dekker Inc., New York.

Bontidean, I., Lloyd, J. R., Hobman, J. L., Wilson, J. R., Csoregi, E., Mattiasson, B., and Brown, N. L. (2000). Bacterial metal-resistance proteins and their use in biosensors for the detection of bioavailable heavy metals. *J. Inorg. Biochem.* **79,** 225–229.

Brierley, C. L., and Brierley, J. A. (1982). Anaerobic reduction of molybdenum by *Sulfolobus* species. *Zbl. Bakt. Hyg., I. Abt. Orig.* C3, 289–294.

Bruins, M. R., Kapil, S., and Oehme, F. W. (2000). Microbial resistance to metals in the environment. *Ecotoxicol. Environ. Saf.* **45,** 198–207.

Burnett, W. C., Cowart, J. B., and Chin, P. A. (1987). Polonium in the superficial aquifer of West Central Florida. *In* ''Radon Radium and Other Radioactivity in Groundwater:

Hydrogeologic Impact and Application to Indoor Airborne Contamination,'' (B. Graves, ed.), pp. 251–269. Lewis Publ.

Caccavo, F. Jr., Coates, J. D., Rossello-Mora, R. A., Ludwig, W., Schleifer, K. H., Lovley, D. R., and McInerney, M. J. (1996). *Geovibrio ferrireducens*, a phylogenetically distinct dissimilatory Fe(III)-reducing bacterium. *Arch. Microbiol.* **165,** 370–376.

Caccavo, Jr., F., Lonergan, D. J., Lovley, D. R., Davis, M., Stolz, J. F., and McInerney, M. J. (1994). *Geobacter sulfurreducens* sp. nov., a hydrogen and acetate-oxidizing dissimilatory metal-reducing microorganism. *Appl. Environ. Microbiol.* **60,** 3752–3759.

Cantafio, A. W., Hagen, K. D., Lewis, G. E., Bledsoe, T. L., Nunan, K. M., and Macy, J. M. (1996). Pilot-scale selenium bioremediation of San Joaquin drainage water with *Thauera selenatis*. *App. Environ. Microbiol.* **62,** 3298–3303.

Cherrier, J., Burnett, W. C., and LaRock, P. A. (1995). Uptake of polonium and sulfur by bacteria. *Geomicrobiol. J.* **13,** 103–115.

Childers, S. E., Ciufo, S., and Lovley, D. R. (2002). *Geobacter metallireducens* access Fe(III) oxide by chemotaxis. *Nature* **416,** 767–769.

Chirwa, E. M. N., and Wang, Y. T. (1997). Biological reduction of hexavalent chromium by *Bacillus* sp. in a packed bed bioreactor. *Env. Sci. Technol.* **31,** 1446–1451.

Chirwa, E. M. N., and Wang, Y. T. (1997). Biological reduction of hexavalent chromium by *Pseudomonas fluorescens* LB 300 in a fixed-film reactor. *J. Environ. Eng.* **123,** 760–766.

Chirwa, E. S., and Wang, Y.-T. (2000). Simultaneous chromium(VI) reduction and phenol degradation in an anaerobic consortium of bacteria. *Wat. Res.* **34,** 2376–2384.

Coates, J. D., Bhupathiraju, V. K., Achenbach, L. A., McInerney, M. J., and Lovley, D. R. (2001). *Geobacter hydrogenophilus*, *Geobacter chapellei*, and *Geobacter grbiciae*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers. *Int. J. Syst. Evol. Microbiol.* **51,** 581–588.

Coates, J. D., Ellis, D. J., Gaw, C. V., and Lovley, D. R. (1999). *Geothrix fermentans* gen. nov., sp nov., a novel Fe(III)-reducing bacterium from a hydrocarbon-contaminated aquifer. *Int. J. Syst. Bacteriol.* **49,** 1615–1622.

Coleman, M. L., Hedrick, D. B., Lovley, D. R., White, D. C., and Pye, K. (1993). Reduction of Fe(III) in sediments by sulphate-reducing bacteria. *Nature* **361,** 436–438.

Coppi, M. V., Leang, C., Sandler, S. J., and Lovley, D. R. (2001). Development of genetic system for *Geobacter sulfurreducens*. *Appl. Environ. Microbiol.* **67,** 3180–3187.

De Luca, G., Philip, P., Dermoun, Z., Rousset, M., and Vermeglio, A. (2001). Reduction of technetium(VII) by *Desulfovibrio fructosovorans* is mediated by the nickel–iron hydrogenase. *App. Environ. Microbiol.* **67,** 4583–4587.

DeMoll-Decker, H., and Macy, J. M. (1993). The periplasmic nitrite reductase of *Thauera selanatis* may catalyze the reduction of selenite to elemental selenium. *Arch. Microbiol.* **160,** 241–247.

deVargas, I., Macaskie, L. E., and Guibal, E. (2003). Biosorption of palladium and platinum by sulphate-reducing bacteria. *J. Chem. Technol. Biotechnol.,* In Press.

DiChristina, T. J., Moore, C. M., and Haller, C. A. (2002). Dissimilatory Fe(III) and Mn(IV) reduction by *Shewanella putrefaciens* requires *ferE*, a homolog of the *pulE* (*gspE*) type II protein secretion gene. *J. Bacteriol.* **184,** 142–151.

Eccles, H., Garnham, G., Lowe, C., and Bruce, N. (1996). Biosensors for detecting metal ions capable of being reduced by reductase enzymes. USA: British Nuclear Fuels PLC.

Ehrlich, H. L. (1996). ''Geomicrobiology,'' 3rd edition. New York: Marcel Dekker Inc.

Fendorf, S., and Li, G. (1996). Kinetics of chromate reduction by ferrous iron. *Environ. Sci. Technol.* **30,** 1614–1617.

Finneran, K. T., Anderson, R. T., Nevin, K. P., and Lovley, D. R. (2002). Potential for bioremediation of uranium-contaminated aquifers with microbial U(VI) reduction. *Soil Sed. Contam.* **11,** 339–357.

Finneran, K. T., Housewright, M. E., and Lovley, D. R. (2002). Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments. *Environ. Microbiol.* **4,** 510–516.

Francis, A. J. (1994). Microbial transformations of radioactive wastes and environmental restoration through bioremediation. *J. Alloys and Compounds* **213/214,** 226–231.

Fu, J. K., Liu, Y. Y., Gu, P. Y., Tang, D. L., Lin, Z. Y., Yao, B. X., and Weng, S. Z. (2000). Spectroscopic characterization on the biosorption and bioreduction of Ag(I) by *Lactobacillus* sp. A09. *Acta Physico-Chimica Sinica* **16,** 779–782.

Fude, L., Harris, B., Urrutia, M. M., and Beveridge, T. J. (1994). Reduction of Cr(VI) by a consortium of sulfate-reducing bacteria (SRB III). *Appl. Environ. Microbiol.* **60,** 1525–1531.

Fujita, M., Ike, M., Kashiwa, M., Hashimoto, R., and Soda, S. (2002). Laboratory-scale continuous reactor for soluble selenium removal using selenate-reducing bacterium, *Bacillus* sp. SF-1. *Biotech. Bioeng.* **80,** 755–761.

Gaspard, S., Vazquez, F., and Holliger, C. (1998). Localization and solubilization of the iron(III) reductase of *Geobacter sulfurreducens*. *Appl. Environ. Microbiol.* **64,** 3188–3194.

Geiselhart, L., Osgood, M., and Holmes, D. S. (1991). *Ann. N. Y. Acad. Sci.* **646,** 53–60.

Ghani, B., Takai, M., Hisham, N. Z., Kishimoto, N., Ismail, A. K., Tano, T., and Sugio, T. (1993). Isolation and characterization of a Mo^{6+}-reducing bacterium. *App. Environ. Microbiol.* **59,** 1176–1180.

Gorby, Y. A., F., C., and Bolton, H. (1998). Microbial reduction of cobalt III $EDTA^{-}$ in the presence and absence of manganese(IV) oxide. *Environ. Sci. Technol.* **32,** 244–250.

Green, A. C., Patel, B. K. C., and Sheehy, A. J. (1997). *Deferribacter thermophilus* gen. sp. nov., a novel thermophilic manganese- and iron-reducing bacterium isolated from a petroleum reservoir. *Intern. J. System. Bacteriol.* **47,** 505–509.

Guibal, E., Larkin, A., Vincent, T., and Tobin, J. M. (1999) Platinum recovery on chitosan-based sorbents. *In* ''Biohydrometallurgy and the Environment. Towards Mining in the 21st Century'' (R. Amils and A. Ballester, eds.), pp. 265–275. Elsevier Amsterdam.

Gupta, A., Matsui, K., Lo, J. F., and Silver, S. (1999). Molecular basis for resistance to silver cations in Salmonella. *Nature Medicine* **5,** 183–188.

Henrot, J. (1989). Bioaccumulation and chemical modification of Tc by soil bacteria. *Health Phys.* **57,** 239–245.

Hobman, J. L., Wilson, J. W., and Brown, N. L. (2000). Microbial mercury reduction. *In* ''Environmental Microbe–Metal Interactions,'' (D. R. Lovley, ed.), pp. 177–197. Washington, DC.

Holmes, D. E., Finneran, K. T., and Lovley, D. R. (2002). Enrichment of *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. *Appl. Environ. Microbiol.* **68,** 2300–2306.

Iwahori, K., Takeuchi, F., Kamimura, K., and Sugio, T. (2000). Ferrous iron-dependent volatilization of mercury by the plasma membrane of thiobacillus ferrooxidans. *Appl. Environ. Microbiol.* **66,** 3823–3827.

Jones, J. G., Gardener, S., and Simon, B. M. (1984). Reduction of ferric iron by heterotrophic bacteria in lake sediments. *J. Gen. Microbiol.* **130,** 45–51.

Kashefi, K, and Lovley, K. (2000). Reduction of Fe(III), Mn(IV), and toxic metals at 100°C by *Pyrobaculum islandicum*. *Appl. Environ. Microbiol.* **66,** 1050–1056.

Kashefi, K., Tor, J. M., Nevin, K. P., and Lovley, D. R. (2001). Reductive precipitation of gold by dissimilatory Fe(III)-reducing *Bacteria* and *Archaea*. *App. Environ. Microbiol.* **67,** 3275–3279.

Krafft, T., and Macy, J. M. (1998). Purification and characterization of the respiratory arsenate reductase of *Chrysiogenes arsenatis*. *Eur. J. Biochem.* **255,** 647–653.

Leang, C., Coppi, M. V., and Lovley, D. R. (2003). OmcB, a *c*-type polyheme cytochrome, involved in Fe(III) reduction in *Geobacter sulfurreducens*. *J. Bacteriol.* **185,** 2096–2103.

Lloyd, J. R., Blunt-Harris, E. L., and Lovley, D. R. (1999). The periplasmic 9.6 kDa c-type cytochrome of *Geobacter sulfurreducens* is not an electron shuttle to Fe(III). *J. Bacteriol.* **181,** 7647–7649.

Lloyd, J. R., Chesnes, J., Glasauer, S., Bunker, D. J., Livens, F. R., and Lovley, D. R. (2002). Reduction of actinides and fission products by Fe(III)-reducing bacteria. *Geomicrobiol. J.* **19,** 103–120.

Lloyd, J. R., Cole, J. A., and Macaskie, L. E. (1997). Reduction and removal of heptavalent technetium from solution by *Escherichia coli*. *J. Bacteriol.* **179,** 2014–2021.

Lloyd, J. R., Leang, C., Hodges Myerson, A. L., Ciufo, S., Sandler, S. J., Methe, B., and Lovley, D. R. (2003). Biochemical and genetic characterization of PpcA, a periplasmic *c*-type cytochrome in *Geobacter sulfurreducens*. *Biochem. J.* **369,** 153–161.

Lloyd, J. R., and Lovley, D. R. (2001). Microbial detoxification of metals and radionuclides. *Current Opinion in Biotechnology* **12,** 248–253.

Lloyd, J. R., Mabbett, A., Williams, D. R., and Macaskie, L. E. (2001). Metal reduction by sulfate-reducing bacteria: Physiological diversity and metal specificity. *Hydrometallurgy* **59,** 327–337.

Lloyd, J. R., and Macaskie, L. E. (1996). A novel phosphorlmager-based technique for monitoring the microbial reduction of technetium. *Appl. Environ. Microbiol.* **62,** 578–582.

Lloyd, J. R., and Macaskie, L. E. (2000). Bioremediation of radioactive metals. *In* ''Environmental Microbe-Metal Interactions,'' (D. R. Lovley, ed.), pp. 277–327. ASM Press, Washington, DC.

Lloyd, J. R., Nolting, H.-F., Solé, V. A., Bosecker, K., and Macaskie, L. E. (1998). Technetium reduction and precipitation by sulphate-reducing bacteria. *Geomicrobiol. J.* **15,** 43–56.

Lloyd, J. R., Ridley, J., Khizniak, T., Lyalikova, N. N., and Macaskie, L. E. (1999). Reduction of technetium by *Desulfovibrio desulfuricans*: Biocatalyst characterization and use in a flow-through bioreactor. *Appl. Environ. Microbiol.* **65,** 2691–2696.

Lloyd, J. R., Sole, V. A., Van Praagh, C. V., and Lovley, D. R. (2000). Direct and Fe(II)-mediated reduction of technetium by Fe(III)-reducing bacteria. *Appl. Environ. Microbiol.* **66,** 3743–3749.

Lloyd, J. R., Thomas, G. H., Finlay, J. A., Cole, J. A., and Macaskie, L. E. (1999). Microbial reduction of technetium by *Escherichia coli* and *Desulfovibrio desulfuricans*: Enhancement via the use of high activity strains and effect of process parameters. *Biotechnol. Bioeng.* **66,** 123–130.

Lloyd, J. R., Yong, P., and Macaskie, L. E. (1998). Enzymatic recovery of elemental palladium by using sulfate-reducing bacteria. *Appl. Environ. Microbiol.* **64,** 4607–4609.

Lloyd, J. R., Yong, P., and Macaskie, L. E. (2000). Biological reduction and removal of pentavalent Np by the concerted action of two microorganisms. *Environ. Sci. Technol.* **34,** 1297–1301.

Lonergan, D. J., Jenter, H., Coates, J. D., Phillips, E. J. P., Schmidt, T., and Lovley, D. R. (1996). Phylogenetic analysis of dissimilatory Fe(III)-reducing bacteria. *J. Bacteriol.* **178,** 2402–2408.

Lortie, L., Gould, W. D., Rajan, W., McCready, R. G. L., and Cheng, K.-J. (1992). Reduction of selenate and selenite to elemental selenium by a *Pseudomonas stutzeri* isolate. *Appl. Environ. Microbiol.* **58,** 4042–4044.

Losi, M. E., and Frankenberger, W. T. J. (1997). Reduction of selenium oxyanions by *Enterobacter cloacae* SLD1a-1: Isolation and growth of the bacterium and its expulsion of selenium particles. *App. Environ. Microbiol.* **63,** 3079–3084.

Lovley, D., and Phillips, E. J. (1992). Reduction of uranium by *Desulfovibrio desulfuricans*. *Appl. Environ. Microbiol.* **58,** 850–856.

Lovley, D. R. (1991). Dissimilatory Fe(III) and Mn(IV) reduction. *Microbiol. Rev.* **55,** 259–287.

Lovley, D. R. (1993). Dissimilatory metal reduction. *Ann. Rev. Microbiol.* **47,** 263–290.

Lovley, D. R. (1997). Potential for anaerobic bioremediation of BTEX in petroleum-contaminated aquifers. *J. Industr. Microbiol.* **18,** 75–81.

Lovley, D. R. (2001) Dissimilatory Fe(III)- and Mn(IV)-reducing prokaryotes, *In* ''The Prokaryotes'' (M. Dworkin, S. Falcow, E. Rosenberg, E., K.-H. Schleifer, and E. Stackebrandt, eds.), Springer-Verlag, Inc., New York.

Lovley, D. R., and Anderson, R. T. (2000). Influence of dissimilatory metal reduction on fate of organic and metal contaminants in the subsurface. *Hydrogeol. J.* **8,** 77–88.

Lovley, D. R., Baedecker, M. J., Lonergan, D. J., Cozzarelli, I. M., Phillips, E. J. P., and Siegel, D. I. (1989). Oxidation of aromatic contaminants coupled to microbial iron reduction. *Nature* **339,** 297–299.

Lovley, D. R., and Chapelle, F. H. (1995). Deep subsurface microbial processes. *Rev. Geophsy.* **33,** 365–381.

Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P., and Woodward, J. C. (1996). Humic substances as electron acceptors for microbial respiration. *Nature* **382,** 445–448.

Lovley, D. R., Giovannoni, S. J., White, D. C., Champine, J. E., Phillips, E. J. P., Gorby, Y. A., and Goodwin, S. (1993). *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Arch. Microbiol.* **159,** 336–344.

Lovley, D. R., and Lonergan, D. J. (1990). Anaerobic oxidation of toluene, phenol, and p-cresol by the dissimilatory iron-reducing organism, GS-15. *Appl. Environ. Microbiol.* **56,** 1858–1864.

Lovley, D. R., and Phillips, E. J. P. (1992). Bioremediation of uranium contamination with enzymatic uranium reduction. *Environ. Sci. Technol.* **26,** 2228–2234.

Lovley, D. R., and Phillips, E. J. P. (1994). Reduction of chromate by *Desulfovibrio vulgaris* and its C_3 cytochrome. *Appl. Environ. Microbiol.* **60,** 726–728.

Lovley, D. R., Phillips, E. J. P., Gorby, Y. A., and Landa, E. (1991). Microbial reduction of uranium. *Nature* **350,** 413–416.

Lovley, D. R., Phillips, E. J. P., and Lonergan, D. J. (1989). Hydrogen and formate oxidation coupled to dissimilatory reduction of iron or manganese by *Alteromonas putrefaciens*. *Appl. Environ. Microbiol.* **55,** 700–706.

Lovley, D. R., and Phillips, E. R. (1988). Novel mode of microbial energy metabolism: Organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl. Environ. Microbiol.* **54,** 1472–1480.

Lovley, D. R., Stolz, J. F., Nord, G. L., and Phillips, E. J. P. (1987). Anaerobic production of magnetite by a dissimilatory iron-reducing microorganism. *Nature* **330,** 252–254.

Lovley, D. R., Woodward, J. C., and Chapelle, F. H. (1994). Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe(III) ligands. *Nature* **370,** 128–131.

Lyalikova, N. N., and Khizhnyak, T. V. (1996). Reduction of heptavalent technetium by acidophilic bacteria of the genus *Thiobacillus*. *Microbiol.* **65,** 468–473.

Mabbett, A. N. (2002). The reduction and recovery of chromium(VI) from industrially polluted waste water using sulphate-reducing bacteria. PhD Thesis,University of Birmingham, United Kingdom.

Mabbett, A. N., Lloyd, J. R., and Macaskie, L. E. (2002). The effect of complexing agents on the reduction of Cr(VI) by *Desulfovibrio vulgaris* ATCC 29579. *Biotechnol. Bioeng.,* **79,** 389–397.

Mabbett, A. N., Yong, P., Baxter-Plant, V. S., Mikheenko, I. P., Farr, J. P. G., and Macaskie, L. E. (2001). Effective reduction and removal of Cr(VI) and reductive dehalogenation of chlorophenol by hybrid bioinorganic catalytic processes. *In* ''Biohydrometallurgy: Fundamentals, Technology and Sustainable Development'' (V. S. T. Ciminelli and O. Garcia, eds.), pp. 335–342.

Mabbett, A. N., Yong, P., Farr, J. P. G., and Macaskie, L. E. (2003). Reduction of Cr(VI) by palladized biomass of *Desulfovibrio desulfuricans* ATCC 29577. *Biotechnol. Bioeng.,* in press.

MacArthur, J. M., Ravenscroft, P., Suafiullah, S., and Thirlwell, M. F. (2000). Arsenic in groundwater: Testing pollution mechanisms for sedimentary aquifers in Bangladesh. *Water Resources Research* **37,** 109–117.

Macaskie, L. E. (1991). The application of biotechnology to the treatment of wastes produced from nuclear fuel cycle: Biodegradation and bioaccumulation as a means of treating radionuclide-containing streams. *Crit. Rev. Biotechnol.* **11,** 41–112.

Macy, J. M., and Lawson, S. (1993). Cell yield (Ym) of *Thauera selenatis* grown anaerobically with acetate plus selenate or nitrate. *Arch. Microbiol.* **160,** 295–298.

Macy, J. M., Nunan, K., Hagen, K. D., Dixon, D. R., Harbour, P. J., Cahill, M., and Sly, L. I. (1996). *Chrysiogenes arsenatis*, gen. sp. nov., a new arsenate-respiring bacterium isolated from gold mine wastewater. *Int. J. Syst. Bacteriol.* **46,** 1153–1157.

Magnuson, T. S., Hodges-Myerson, A. L., and Lovley, D. R. (2000). Characterization of a membrane-bound NADH-dependent Fe(3+) reductase from the dissimilatory Fe(3+)-reducing bacterium *Geobacter sulfurreducens*. *FEMS Microbiol. Lett.* **185,** 205–211.

Marsh, T. L., Leon, N. M., and McInerney, M. J. (2000). Physiochemical factors affecting chromate reduction by aquifer materials. *Geomicro biol. J.* **17,** 291–303.

Mikheenko, I. P., Mikheenko, P. M., Darlington, C. N. W., Muirhead, C. M., and Macaskie, L. E. (2001). Magnetic testing of Pd-loaded bacteria. *In* ''Biohydrometallurgy: Fundamentals, Technology and Sustainable Development'' (V. S. T. Ciminelli and O. Garcia, eds.), pp. 525–532. Elsevier.

Moore, M. D., and Kaplan, S. (1992). Identification of intrinsic high-level resistance to rare-earth oxides and oxyanions in members of the class *Proteobacteria:* Characterization of tellurite, selenite, and rhodium sesquioxide reduction in *Rhodobacter sphaeroides*. *J. Bacteriol.* **174,** 1505–1514.

Mukhopadhyay, R., Rosen, B., Phung, L., and Silver, S. (2002). Microbial arsenic: From geocycles to genes and enzymes. *FEMS Microbiol. Rev.* **26,** 311.

Myers, C. R., Carstens, B. P., Antholine, W. E., and Myers, J. M. (2000). Chromium (VI) reductase activity is associated with the cytoplasmic membrane of anaerobically grown *Shewanella putrefaciens* MR-1. *J. Appl. Microbiol.* **88,** 98–106.

Myers, C. R., and Myers, J. M. (1992). Localization of cytochromes to the outer membrane of anaerobically grown *Shewanella putrefaciens* MR-1. *J. Bacteriol.* **174,** 3429–3438.

Myers, C. R., and Myers, J. M. (1993). Ferric reductase is associated with the membranes of ananerobically grown *Shewanella putrefaciens* MR-1. *FEMS Microbiol. Letts.* **108,** 15–21.

Myers, C. R., and Myers, J. M. (1997). Cloning and sequence of *cymA*, a gene encoding a tetraheme cytochrome *c* required for reduction of iron(III), fumarate, and nitrate by *Shewanella putrefaciens* MR-1. *J. Bacteriol.* **179,** 1143–1152.

Myers, C. R., and Nealson, K. H. (1988). Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science* **240,** 1319–1321.

Myers, J. M., and Myers, C. R. (2000). Role of tetraheme cytochrome *cymA* in anaerobic electron transport in cells of *Shewanella putrefaciens* MR-1 with normal levels of menaquinone. *J. Bacteriol.* **182,** 67–75.

Myers, J. M., and Myers, C. R. (2001). Role of outer membrane cytochromes OmcA and OmcB of *Shewanella putrefaciens* MR-1 in reduction of manganese dioxide. *Appl. Environ. Microbiol.* **67,** 260–269.

Nealson, K. H., and Cox, B. L. (2002). Microbial metal-ion reduction and Mars: Extraterrestrial expectations? *Curr. Opin. Microbiol.* **5,** 296–300.

Nevin, K. P., and Lovley, D. R. (2002). Mechanisms for accessing insoluble Fe(III) oxide during dissimilatory Fe(III) reduction by *Geothrix fermentans*. *Appl. Environ. Microbiol.* **68,** 2294–2299.

Nevin, K. P., and Lovley, D. R. (2002). Mechanisms for Fe(III) oxide reduction in sedimentary environments. *Geomicrobiol. J.* **19,** 141–159.

Newman, D. K., Kennedy, E. K., Coates, J. D., Ahmann, D., Ellis, D. J., Lovley, D. R., and Morel, F. M. M. (1998). Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. *Arch. Microbiol.* **165,** 380–388.

Newman, D. K., and Kolter, R. (2000). A role for excreted quinones in extracellular electron transfer. *Nature* **405,** 94–97.

Oremland, R. S., and Capone, D. G. (1988). Use of ''specific'' inhibitors in biogeochemistry and microbial ecology. *Adv. Microb. Ecol.* **10,** 285–383.

Oremland, R. S., Hollibaugh, J. T., Maest, A. S., Presser, T. S., Miller, L. G., and Culbertson, C. W. (1989). Selenate reduction to elemental selenium by anaerobic bacteria in sediments and culture: Biogeochemical significance of a novel, sulfate-independent respiration. *Appl. Envrion. Microbiol.* **55,** 2333–2343.

Oremland, R. S., and Stolz, J. (2000). Dissimilatory reduction of selenate and arsenate in nature. *In* ''Environmental Microbe-Metal Interactions'' (D. R. Lovley, ed.), pp. 199–224. ASM Press, Washington, DC.

Oremland, R. S., Switzer Blum, J., Culbertson, C. W., Visscher, P. T., Miller, L. G., Dowdle, P., and Strohmaier, R. E. (1994). Isolation, growth, and metabolism of an obligately anaerobic, selenate-respiring bacterium, strain SES-3. *Appl. Environ. Microbiol.* **60,** 3011–3019.

Park, C. H., Keyhan, M., Wielinga, B., Fendorf, S., and Matin, A. (2000). Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Appl. Environ. Microbiol.* **66,** 1788–1795.

Payne, R. B., Gentry, D. A., Rapp-Giles, B. J., Casalot, L., and Wall, J. D. (2002). Uranium reduction by *Desulfovibrio desulfuricans* strain G20 and a cytochrome *c*3 mutant. *Appl. Environ. Microbiol.* **68,** 3129–3132.

Peck, H. D. (1993). Bioenergetic strategies of the sulfate-reducing bacteria. *In* ''Sulfate-Reducing Bacteria: Contemporary Perspectives'' (J. M. Odom and R. Singleton, eds.), Springer-Verlag, New York.

Peretrukhin, V. F., Khizhniak, N. N., Lyalikova, N. N., and German, K. E. (1996). Biosorption of technetium-99 and some actinides by bottom sediments of Lake Belsso Kosino of the Moscow region. *Radiochememistry.* **38,** 440–443.

Pignolet, L., Fonsny, K., Capot, F., and Moureau, Z. (1989). Role of various microorganisms on Tc behaviour in sediments. *Health Phys.* **57,** 791–800.

Pronk, J. T., De Bruyn, J. C., Bos, P., and Kuenen, J. G. (1992). Anaerobic growth of *Thiobacillus ferroxidans*. *App. Environ. Microbiol.* **58,** 2227–2230.

Roberts, J. L. (1947). Reduction of ferric hydroxide by strains of Bacillus polymyxa. *Soil Sci.* **63,** 135–140.

Röling, W. F. M., van Breukelen, B. M., Braster, M., Lin, B., and van Verseveld, H. W. (2001). Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer. *Appl. Environ. Microbiol.* **67,** 4619–4629.

Rooney-Varga, J. N., Anderson, R. T., Fraga, J. L., Ringelberg, D., and Lovley, D. R. (1999). Microbial communities associated with anaerobic benzene degradation in a petroleum-contaminated aquifer. *Appl. Environ. Microbiol.* **65,** 3056–3063.

Rusin, P. A., Quintana, L., Brainard, J. R., Strietelmeier, B. A., Tait, C. D., Ekberg, S. A., Palmer, P. D., Newton, T. W., and Clark, D. L. (1994). Solubilization of plutonium hydrous oxide by iron reducing bacteria. *Environ. Sci. Technol.* **28,** 1686–1690.

Santini, J. M., Skly, L. I., Schnagll, R. D., and Macy, J. M. (2000). A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: Phylogenetic, physiological, and preliminary biochemical studies. *Appl. Environ. Microbiol.* **66,** 92–97.

Schroder, I., Rech, S., Krafft, T., and Macy, J. M. (1997). Purification and characterization of the selenate reductase from *Thauera selenatis*. *J. Biol. Chem.* **272,** 23765–23768.

Seeliger, S., Cord-Ruwisch, R., and Schink, B. (1998). A periplasmic and extracellular *c*-type cytochrome of *Geobacter sulfurreducens* acts as a ferric iron reductase and as an electron carrier to other acceptors or to partner bacteria. *J. Bacteriol.* **180,** 3686–3691.

Shen, H., and Wang, Y.-T. (1993). Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 3346. *Appl. Environ. Microbiol.* **59,** 3771–3777.

Shen, H., and Wang, Y.-T. (1995). Simultaneous chromium reduction and phenol degradation in a coculture of *Escherichia coli* ATCC 3346 and *Pseudomonas putida* DMP-1. *Appl. Environ. Microbiol.* **61,** 2754–2758.

Silver, S. (2003). Silver resistance. *FEMS Microbiol. Rev.* **27,** 341–354.

Smillie, R. H., Hunter, K., and Loutit, M. (1991). Reduction of chromium(VI) by bacterially produced hydrogen sulphide in a marine environment. *Water Research* **15,** 1351–1354.

Smith, W. L., and Gadd, G. M. (2000). Reduction and precipitation of chromate by mixed culture sulphate-reducing bacterial biofilms. *J. Appl. Microbiol.* **88,** 983–991.

Snoeyenbos-West, O., Nevin, K. P., Anderson, R. T., and Lovley, D. R. (2000). Enrichment of *Geobacter* species in response to stimulation of Fe(III) reduction in sandy aquifer sediments. *Microbiol Ecol.* **39,** 153–167.

Stein, L. Y., La Duc, M. T., Grundl, T. J., and Nealson, K. H. (2001). Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. *Environ. Microbiol.* **3,** 10–18.

Steinberg, N. A., and Oremland, R. S. (1990). Dissimilatory selenate reduction potentials in a diversity of sediment types. *Appl. Environ. Microbiol.* **56,** 3550–3557.

Stolz, J. F., Gugliuzza, T., Switzer Blum, J., Oremland, R. S., and Murillo, F. M. (1997). Differential cytochrome content and reductase activity in *Geospirillum barnesii* strain SES-3. *Arch. Microbiol.* **167,** 1–5.

Sugio, T., Tsujita, Y., Katagiri, T., Inagaki, K., and Tano, T. (1988). Reduction of Mo^{6+} with elemental sulfur by *Thiobacillus ferrooxidans*. *J. Bacteriol.* **170,** 5956–5959.

Suzuki, T., Miyata, H., Horitsu, K., Kawai, K., Takamizawa, Y., Tai, Y., and Okazaki, M. (1992). NAD(P)H-dependent chromium (VI) reductase of Pseudomonas ambigua G-1: A Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III). *J. Bacteriol.* **174,** 5340–5345.

Switzer Blum, J., Burns Bindi, A., Buzzelli, J., Stolz, J. F., and Oremland, R. S. (1998). *Bacillus arsenicoselenatis*, sp. nov., and *Bacillus selenitireducens* sp. nov: Two haloalkaliphiles from Mono Lake, California, that respire oxyanions of selenium and arsenic. *Arch. Microbiol.* **171,** 19–30.

Taylor, D. E. (1999). Bacterial tellurite resistance. *TIBTECH* **7,** 111–115.

Tebo, B. M., and Obraztsova, A. Y. (1998). Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors. *FEMS Micro. Lett.* **162,** 193–198.

Tender, L. M., Reimers, C. E., Stecher, H. A., Holmes, D. E., Bond, D. R., Lowy, D. A., Pilobello, K., Fertig, S. J., and Lovley, D. R. (2002). Harnessing microbially generated power on the seafloor. *Nat. Biotechnol.* **20,** 821–825.

Thamdrup, B. (2000). Bacterial manganese and iron reduction in aquatic sediments. *Adv. Microbiol. Ecol.* **16,** 41–84.

Tomei, F. A., Barton, L. L., Lemanski, C. L., and Zocco, T. G. (1992). Reduction of selenate and selenite to elemental selenium by *Wolinella succinogenes*. *Can. J. Microbiol.* **38,** 1328–1333.

Tomei, F. A., Barton, L. L., Lemanski, C. L., Zocco, T. G., Fink, N. H., and Sillerud, L. O. (1995). Transformation of selenate and selenite to elemental selenium by *Desulfovibrio desulfuricans*. *J. Industr. Microbiol.* **14,** 329–336.

Tor, J. M., and Lovley, D. R. (2001). Anaerobic degradation of aromatic compounds coupled to Fe(III) reduction by *Ferroglobus placidus*. *Environ. Microbiol.* **3,** 281–287.

Tucker, M. D., Barton, L. L., and Thomson, B. M. (1997). Reduction and immobilization of molybdenum by *Desulfovibrio desulfuricans*. *J. Environ. Qual.* **26,** 1146–1152.

Tucker, M. D., Barton, L. L., and Thomson, B. M. (1998). Reduction of Cr, Mo, Se, and U by *Desulfovibrio desulfuricans* immobilized in polyacrylamide gels. **20,** 13–19.

Vargas, M., Kashefi, K., Blunt-Harris, E. L., and Lovley, D. R. (1998). Microbiological evidence for Fe(III) reduction on early Earth. *Nature* **395,** 65–67.

von Canstein, H., Li, Y., Leonhäuser, J., Haase, E., Felske, A., Deckwer, W.-D., and Wagner-Döbler, I. (2002). Spatially oscillating activity and microbial succession of mercury-reducing biofilms in a technical scale bioremediation system. *Appl. Environ. Microbiol.* **68,** 1938–1946.

von Canstein, H., Li, Y., Timmis, K. N., Deckwer, W.-D., and Wagner-Dobler, I. (1999). Removal of mercury from chloralkali electrolysis wastewater by a mercury-resistant *Pseudomonas putida* strain. *Appl. Environ. Microbiol.* **65,** 5279–5284.

von Canstein, H. F., Li, Y., and Wagner-Döbler, I. (2001). Long-term stability of mercury reducing microbial biofilm communities analysed by 16S-23S rDNA interspacer region polymorphism. *Microbiol. Ecol.* **42,** 624–634.

von Canstein, H. F., Li, Y., and Wagner-Döbler, I. (2001). Long-term performance of bioreactors cleaning mercury contaminated wastewater and their response to

temperature and mercury stress and mechanical perturbation. *Biotechnol. Bioeng.* **74,** 212–219.

Wade, Jr., R., and DiChristina, T. J. (2000). Isolation of U(VI) reduction-deficient mutants of *Shewanella putrefaciens*. *FEMS Microbiol. Letts.* **184,** 143–148.

Wagner-Dobler, I., Lunsdorf, H., Lubbehusen, T., von Canstein, H. F., and Li, Y. (2000). Structure and species composition of mercury-reducing biofilms. *Appl. Environ. Microbiol.* **66,** 4559–4563.

Wagner-Dobler, I., von Canstein, H., Li, Y., Timmis, K. N., and Deckwer, W. D. (2000). Removal of mercury from chemical wastewater by microorganisms in technical scale. *Environ. Sci. Technol.* **34,** 4628–4634.

Wang, P. C., Mori, T., Komori, K., Sasatsu, M., Toda, K., and Ohtake, H. (1989). Isolation and characterization of an *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions. *Appl. Environ. Microbiol.* **55,** 1665–1669.

Wang, Y.-T. (2000). Microbial reduction of Cr(VI). *In* ''Environmental Microbe–Metal Interactions'' (D. R. Lovley, ed.), pp. 225–235. ASM Press, Washington, DC.

Wildung, R. E., Gorby, Y. A., Krupka, K. M., Hess, N. J., Li, S. W., Plymale, A. E., McKinley, J. P., and Fredrickson, J. K. (2000). Effect of electron donor and solution chemistry on products of dissimilatory reduction of technetium by *Shewanella putrefaciens*. *Appl. Environ. Microbiol.* **66,** 2451–2460.

Wildung, R. E., McFadden, K. M., and Garland, T. R. (1979). Technetium sources and behaviour in the environment. *J. Environ. Qual.* **8,** 156–161.

Wilson, J. R., Leang, C., Morby, A. P., Hobman, J. L., and Brown, N. L. (2000) MerF is a mercury transport protein: Different structures but a common mechanism for mercuric ion transporters? *FEBS Lett.* **472,** 78–82.

Woolfolk, C. A., and Whiteley, H. R. (1962). Reduction of inorganic compounds with molecular hydrogen by *Micrococcus lactilyticus*. *J. Bacteriol.* **84,** 647–658.

Yong, P., Rowson, N. A., Farr, J. P. G., Harris, I. R., and Macaskie, L. E. (2002a). Bioreduction and biocrystallization of palladium by *Desulfovibrio desulfuricans* NCIMB 8307. *Biotechnol. Bioeng.* **80,** 369–379.

Yong, P., Rowson, N. A., Farr, J. P. G., Harris, I. R., and Macaskie, L. E. (2002b). Bioaccumulation of palladium by *Desulfovibrio desulfuricans*. *J. Chem. Technol. Biotechnol.* **77,** 593–601.

Yong, P., Farr, J. P. G., Harris, I. R., and Macaskie, L. E. (2002c). Palladium recovery by immobilized cells of *Desulfovibrio desulfuricans* using hydrogen as the electron donor in a novel electrobioreactor. *Biotechnol. Lett.* **24,** 205–212.

Yong, P., Rowson, N. A., Farr, J. P. G., Harris, I. R., and Macaskie, L. E. (2003). A novel electrobiotechnology for the recovery of precious metals from spent automotive catalysts. *Environ. Technol.* **24,** 289–297.

Yurkova, N. A., and Lyalikova, N. N. (1991). New vanadate-reducing facultative chemolithotrophic bacteria. *Microbiology* **59,** 672–677.

Determinants of Freeze Tolerance in Microorganisms, Physiological Importance, and Biotechnological Applications

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I. Introduction

Both in their natural habitat and during industrial exploitation, microorganisms encounter a plethora of stresses, both simultaneously and sequentially. *Saccharomyces cerevisiae* has been amply studied regarding its ability to respond to environmental changes and in particular to different stress conditions (Hohmann and Mager, 1997;

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Estruch, 2000). However, as opposed to other types of stress, microbial response to freeze–thaw stress and freeze tolerance mechanisms have not been extensively studied.

During freezing and thawing, cells are exposed to changes in temperature, water content, water state, and solute concentration. It has been established that the thermal, mechanical, physico-, and biochemical events during freeze–thaw stress, as well as the resulting injury, are dependent on the cooling and warming rates, the final temperature, the freezing duration, and the suspending medium (Mazur, 1970). In the first part of this chapter, we concentrate on the events occurring during freezing and thawing as well as the cellular injury caused by freeze–thaw stress.

Microorganisms are widespread in climatic regions with frequent freeze–thaw cycles, and several cellular factors have been correlated with the extent of microbial survival following freezing and thawing. In spite of this, little is known with certainty about the genetic and molecular basis of microbial freeze tolerance. In the second part of the chapter, we discuss what is currently known about molecular and genetic mechanisms possibly underlying freeze tolerance in microorganisms.

Much research on these topics has been carried out with baker's yeast, and therefore a large part of this chapter will focus on this organism. However, where available, data on other (micro)organisms will also be discussed in order to draw more general insight. Attention will also be paid to the overlap between the molecular events under freeze–thaw stress, the injury they cause, and the tolerance mechanisms with those involved in other types of stress.

A better understanding of microbial freeze tolerance is of interest both from a fundamental point of view and for industrial purposes. Microorganisms are ubiquitous in nature. Yeast cells, for instance, are frequently found in association with plants, insects, soil, and water (Spencer and Spencer, 1996, 1997). In most natural habitats, microbes are likely to encounter temperatures below 0°C during night-frost and in winter periods. In other ecological niches, microorganisms permanently live in cold or freezing conditions. Biological cryoprotectants, such as antifreeze proteins, are found in a wide range of organisms, including microorganisms, plants, insects, and fishes, as well as other invertebrates and vertebrates. Their potential biotechnological applications have been reviewed elsewhere (Holmberg and Bülow, 1998; Breton *et al.*, 2000; Lillford and Holt, 2002). The possible uses of these proteins ranges from the frozen preservation of cell lines, tissues, organs, and foodstuffs to transgenic plants with improved

freeze tolerance. We will focus on examples of the physiological importance and putative applications of microbial freeze-tolerance mechanisms and freeze-tolerant microorganisms.

Frozen dough technology is a typical example of an industrial application where the freeze tolerance of the microorganism used is of paramount importance. The rapid, striking transition from high freeze tolerance to high freeze sensitivity during the startup of yeast fermentation constitutes a major obstacle for optimal use of the frozen dough technology. Attempts to obtain strains better suited for use in frozen doughs have been based on the few microbial freeze-tolerance mechanisms identified so far and have stimulated further research to unravel new underlying mechanisms. Therefore, the problems inherent to the frozen dough concept as well as the search for novel yeast strains better suited for use in frozen dough applications will be discussed extensively as a case study. The rapid drop in freeze tolerance during the startup of yeast fermentation also provides an unique model system for identifying novel mechanisms involved in freeze tolerance because of the rapid and dramatic change in freeze tolerance over a short time period within the same organism.

In this chapter, we will use the term ''freeze tolerance'' throughout. The classical distinction between ''stress tolerant'' and ''stress resistant'' is not meaningful in the case of freeze stress since, under freezing conditions, growth and multiplication is not possible.

II. Freeze–Thaw Events

When a cell suspension is cooled to temperatures below 0°C, both the suspending medium and the cells initially supercool (Mazur, 1977). Extracellular ice crystal formation precedes intracellular freezing (Toner *et al.*, 1993) and is determined by the freezing point of the suspending medium and the presence of ice-nucleating agents (Steponkus, 1984). Taking into consideration only its osmolar concentration, the freezing point of the cytoplasm is predicted to be above −2°C. Nevertheless, the cell interior typically remains unfrozen until it reaches −5°C to −10°C (Mazur, 1965). This intracellular supercooling can be explained by the fact that the cell membrane prevents growth of the extracellular ice into the cell interior, and the cell itself apparently does not contain nucleators of supercooled water (Mazur, 1965).

Following external freezing and internal supercooling, a chemical gradient for free water is established between the extracellular ice-containing medium and the intracellular supercooled water. This thermodynamically unstable situation can be resolved by either water

outflow or internal freezing (Mazur, 1965, 1970) (Fig. 1). At low cooling rates, ice crystal formation remains mainly extracellular, and water outflow from the cell is sufficient to restore the chemical equilibrium and minimize the extent of intracellular supercooling. If cells are cooled rapidly, extensive intracellular supercooling occurs and ultimately the chemical gradient is eliminated by freezing of the intracellular water (Mazur, 1965, 1970). The critical cooling rate at which intracellular ice crystals are formed is determined by the cell type, in particular, the volume-to-surface ratio of the cell, and the water permeability: larger, spherical cells and cells less permeable for water have a lower critical cooling rate.

It should be noted that supercooling only predisposes the cell to intracellular freezing. The origin of ice nucleation, whether spontaneous aggregation of water molecules (homogeneous nucleation), external ice-nucleating agents (heterogeneous nucleation), or even the plasma membrane itself, is a matter of dispute (Mazur, 1977; Franks *et al.*, 1983; Zachariassen and Kristiansen, 2000). It is generally believed, however, that the plasma membrane is not able to block the passage of ice crystals any more at temperatures below -5°C to -10°C, rendering extracellular ice a putative nucleator of intracellular freezing in these conditions (Mazur, 1965; Toner *et al.*, 1993; Morris *et al.*, 1998).

Once ice crystals have been formed, they tend to grow or fuse and become more spherical in order to lower their surface free energy. The rate of this process is higher for small, nonspherical ice crystals and at higher temperatures. Therefore, recrystallization occurs in the frozen condition in the presence of temperature fluctuations, but in particular during slow thawing of rapidly frozen cells (Mazur, 1970) (Figure 1). Ice crystal formation and, in particular, ice crystal growth are likely to cause mechanical stress to cellular components, especially internal membrane systems and organelles.

Both intracellular freezing and water outflow are associated with so-called solution effects: lowered amounts of cellular water, osmolarity increases, solute concentration, and eventually precipitation, pH drop, and gas bubble formation (Mazur, 1970; Steponkus, 1984). In contrast, during warming and thawing of the suspending medium, the cells experience a drop in external osmolarity, resulting in water inflow and cell expansion.

In addition, the formation of reactive oxygen species (ROS) has been associated with freeze–thaw stress. It concerns mainly superoxide radicals generated from oxygen and electrons that have leaked from the mitochondrial electron transport chain. ROS cause cellular damage, such as protein inactivation, membrane damage due to lipid

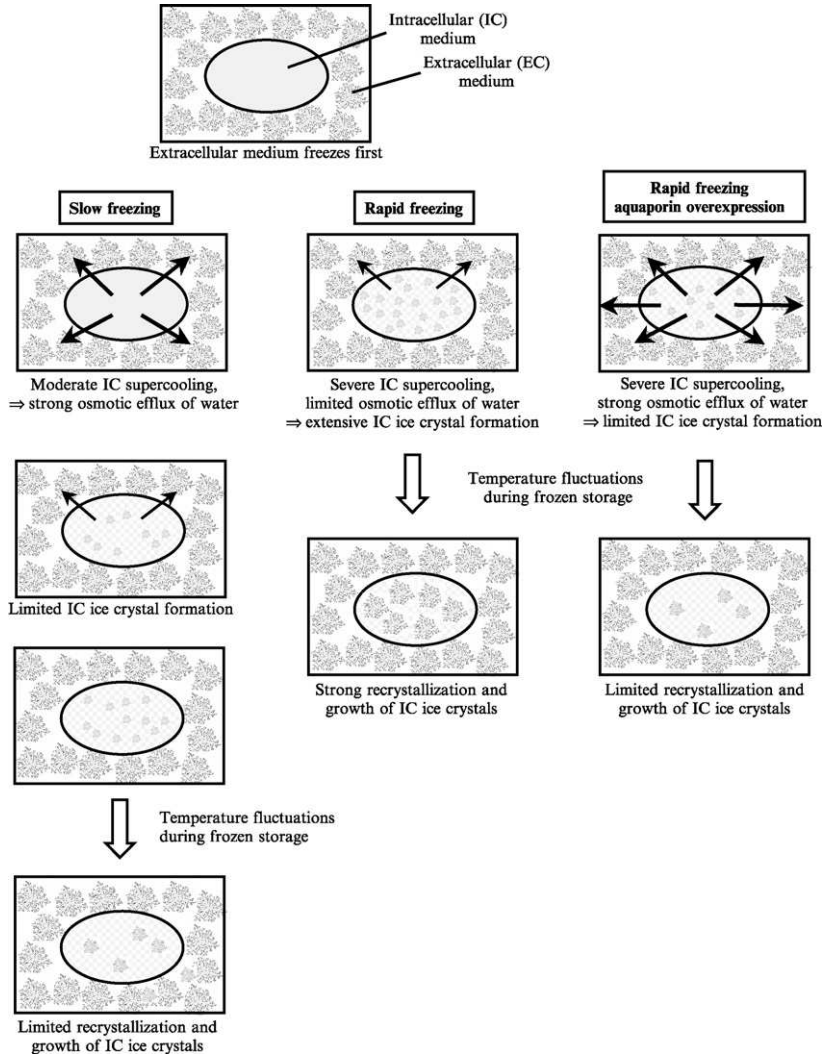

FIG. 1. Schematic outline of the status and movement of extracellular (EC) and intracellular (IC) water in a cell suspension during slow and rapid freezing as well as frozen preservation. The putative effect of aquaporin overexpression during rapid freezing is also shown. See text for more details.

peroxidation, and damage to DNA (Santoro and Thiele, 1997; Park *et al.*, 1998).

Finally, the plasma membrane plays a central role in freezing and thawing processes: Its semipermeable properties allow water outflow

and inflow via passive diffusion, and its barrier properties initially prevent extracellular ice from initiating intracellular freezing (Steponkus, 1984; Steponkus and Lynch, 1989).

Taken together, when a cell suspension is cooled down to temperatures below 0°C, at both low and high cooling rates cells are likely to be subjected to a variety of stress conditions, mainly thermal, mechanical, oxidative, osmotic, ion, and pH stress, all of which to different extents can cause cellular injury.

III. Freeze–Thaw Injury

As long as supercooling of the cell suspending medium lasts and extracellular ice crystal formation has not initiated yet, cells are only subject to a drop in temperature. Although damage to cellular structures and inhibition of cellular functions has been reported in these conditions (Grout *et al.*, 1990), cell viability seems hardly affected (Lorenz, 1974; Douzou, 1982; Park *et al.*, 1997), indicating that the temperature drop by itself is not particularly detrimental to the cells.

Upon extracellular freezing, cells are entrapped between ice crystals and are subjected to mechanical and adhesion stresses (Grout *et al.*, 1990). The concept of intracellular damage as a result of ice crystals spearing through cellular structures remains debated. In addition, the generation of electrical fields and gas bubbles in association with the ice fronts have been reported, which is possibly injurious to the cell due to destabilization and mechanical damaging of membranes (Kruuv *et al.*, 1985; Grout *et al.*, 1990; Morris *et al.*, 1998).

Both water outflow and intracellular ice crystal formation are associated with cellular injury to the plasma membrane and the cell wall. Cells are not limitlessly elastic and are able to shrink only to a certain volume without injury (Wolfe *et al.*, 1985). Upon cell shrinkage, plasma membrane material is released via endocytotic vesiculation or exocytotic extrusion, and the cell wall density increases (Steponkus, 1984; Morris *et al.*, 1998;). Membrane damage is believed to be more detrimental to cells than wall damage (Calcott and MacLeod, 1975). Intracellular ice crystals are believed to rupture the plasma membrane, resulting in a release of cellular components into the environment (Mazur, 1965, 1977). Although damage to internal membranes has also been described, (e.g., lysosome disruption (McGann *et al.*, 1988), vacuole disruption (Morris *et al.*, 1998), and release of cytochrome *c* from mitochondria (Mori *et al.*, 1986)), the surface membrane is often considered the primary site of freezing injury (Souzu, 1989a, b).

In general, cellular dehydration is found to be less detrimental than intracellular freezing. Therefore, rapidly frozen cells usually display lower survival rates than slowly frozen cells, although the length of exposure time to the solution effects already described is inversely proportional to the cooling rate (Mazur, 1970). Superimposed on the difference between slow and rapid cooling, the loss of survival is proportional to both the ultimate freezing temperature and the freezing duration (Lorenz, 1974; Park *et al.*, 1997).

For most microorganisms, an optimal cooling rate can be determined at which both major detrimental events (i.e., cellular dehydration and intracellular freezing) are minimal, and therefore survival is maximal. This optimum rate is species specific and has been determined as 7 to 10°C per minute for *S. cerevisiae* cells (Mazur, 1970). When frozen more slowly (less than 7°C per minute), yeast cells suffer mainly from dehydration and the prolonged exposure to solution effects. When frozen more rapidly (more than 10°C per minute), intracellular ice crystal formation seems to be the main damaging factor (Schwartz and Diller, 1983a, b). The existence of an optimum cooling rate confirms that freeze damage is not due to a single factor. Survival is affected by at least two factors that depend in an opposite way on the cooling velocity: cellular dehydration and intracellular ice crystal formation. This hypothesis is called the two-factor hypothesis of freezing injury (Mazur, 1970; Grout *et al.*, 1990).

Although freezing seems to be the most detrimental step when cells are subjected to freeze–thaw stress (Park *et al.*, 1997; Fonseca *et al.*, 2002), it is likely that the loss of cell viability is to a certain extent also attributable to the thawing step. Due to the loss of semipermeable and barrier properties of the cell membrane, cells show an altered osmotic behavior or even become osmotically inactive during thawing. As a consequence, cells display a smaller cell volume compared to the initial one or may even remain contracted during warming. Moreover, plasma membrane material released during cell shrinkage can possibly not be recovered or replenished rapidly enough, resulting in expansion-induced lysis (Steponkus, 1984).

In addition, recrystallization processes, particularly occurring during slow thawing of rapidly cooled cells, affect cell viability since the resulting larger, spherical ice crystals are more harmful to cells than the initial smaller, nonspherical ones. It has even been suggested that the most detrimental event for rapidly cooled cells might be the recrystallization of ice crystals rather than their initial formation (Mazur, 1970, 1977; McKown and Warren, 1991).

Intracellular freezing seems also associated with extensive gas bubble formation, as observed during warming of rapidly cooled yeast cells containing ice crystals (Morris *et al.*, 1998). Similar observations have been reported for other cell types (Steponkus and Dowgert, 1981; Morris and McGrath, 1981; Coulson *et al.*, 1986). These gas bubbles are believed to cause mechanical damage to organelles (Morris *et al.*, 1998).

Taken together, the cellular injury sustained during freezing and thawing is caused by a combination of effects due to the multiple types of stress imposed on the cells and cannot be attributed solely to one form of injury. The precise mechanisms of damage and inhibition caused by freeze–thaw stress are not well understood. The nature of the primary damage caused by slow and rapid freezing is different but both processes finally result in cellular injury.

IV. Microbial Freeze-Tolerance Mechanisms

A. Introduction

In general, the acquisition of yeast stress resistance involves aspects of growth control, sensing, and signal transduction as well as transcriptional, translational, and posttranslational control (Hohmann and Mager, 1997). As the precise mechanisms of injury caused by freezing and thawing are not completely understood, neither are the precise molecular and genetic mechanisms underlying freeze tolerance.

In microorganisms, several cellular factors have been correlated with improved freeze tolerance (Table I). Most of these freeze protection mechanisms (i.e., trehalose accumulation, synthesis of molecular chaperones, adaptation of plasma membrane composition, synthesis of antioxidant proteins, accumulation of compatible solutes, and expression of hydrophylins) overlap with tolerance mechanisms protecting against various other stress types. Different stress conditions indeed seem to cause injury through common mechanisms. On the other hand, more specific freeze-protection mechanisms (i.e., microbial antifreeze (glyco)proteins and ice nucleators) have also been identified. Recently, a novel and specific freeze-tolerance mechanism has been discovered—expression of aquaporin water channels (Tanghe *et al.*, 2002).

B. Trehalose

The unique stress-protection properties of trehalose and its role as a general stress protectant in various organisms such as fungi, bacteria, plants, and invertebrates have been extensively reviewed elsewhere

TABLE I

OVERVIEW OF MICROBIAL FREEZE STRESS-PROTECTION MECHANISMS

	Freeze stress protectants	Putative protection mechanism
General stress resistance mechanisms	Trehalose	Prevention of protein and membrane denaturation
	Molecular chaperones	Refolding of denatured proteins
Plasma membrane characteristics	Fluidity	Retain proper membrane function
	Permeability	Allow more efficient dehydration
	Aquaporins	Increase membrane permeability
Oxidative and osmotic stress mechanisms	Antioxidant proteins	Detoxification of ROS
	Compatible solutes	Retention of osmotic equilibrium
	Hydrophylins	Prevention of protein dehydration
Specific freeze stress protectants	Antifreeze proteins	Prevention of ice crystal formation
	Ice nucleators	Induction of ice crystal formation at higher temperatures below 0°C

(Van Laere, 1989; Wiemken, 1990). How trehalose provides protection to cells is not entirely clear. Both *in vitro* and *in vivo* evidence has been obtained for a dual mechanism: stabilization of membranes and proteins by replacing water and preservation of the intracellular water structure (Clegg, 1985; Burke, 1985; Singer and Lindquist, 1998a; Sano *et al.*, 1999).

Improved freeze tolerance in yeast cells, for instance, as a function of the growth condition, is often correlated with higher trehalose levels (Oda *et al.*, 1986; Hino *et al.*, 1990; Yokoigawa *et al.*, 1995; Almeida and Pais, 1996; Morris *et al.*, 1998; Diniz-Mendez *et al.*, 1999). Further evidence for the stress-protective role of trehalose has been provided with yeast strains genetically engineered in trehalose metabolism revealing a clear link between trehalose levels and freeze tolerance (Attfield *et al.*, 1992; Kim *et al.*, 1996; Shima *et al.*, 1999; Soto *et al.*, 1999). In line with the protective effect of exogenous trehalose on yeast cells during freezing (Diniz-Mendez *et al.*, 1999), it has been proposed

that the protection exerted by trehalose requires its presence at both sides of the plasma membrane (de Araujo, 1996; Diniz-Mendez *et al.*, 1999;).

In spite of the large amount of evidence pointing to an important role of trehalose as stress protectant in freezing conditions, there are also studies reporting inconsistencies between freeze tolerance and trehalose levels, apparently indicating the existence of other factors besides trehalose that are important or required for maintenance of viability under these conditions (Gélinas *et al.*, 1989; Hino *et al.*, 1990; Lewis *et al.*, 1993a; Van Dijck *et al.*, 1995; Almeida and Pais, 1996; Lewis *et al.*, 1997; Diniz-Mendez *et al.*, 1999; Teunissen *et al.*, 2002). A possible explanation is that trehalose is only able to postpone freeze damage and not to correct it, as opposed to molecular chaperone proteins which can refold partly denatured proteins.

C. Molecular Chaperones

Denaturation of proteins does not appear to be a major damaging factor during freezing and thawing (Mazur, 1970). However, the capacity to repair freeze-stress–induced damage to actin, and concomitantly actin association and activity of some glycolytic enzymes, has been demonstrated to be higher in strains displaying a better freeze tolerance and has suggested a contribution to improved freeze tolerance (Hatano *et al.*, 1996).

Molecular chaperone proteins stabilize macromolecules to prevent them from aggregating. They recognize, selectively bind, and reassemble proteins with an aberrant structure (Lindquist and Craig, 1988; Buchner, 1996). They might help to maintain a low degree of protein denaturation during freezing or, more likely, upon thawing by reassembling damaged proteins. A bacterial chaperone protein has been discovered which displays a high affinity for frozen denatured proteins and displays refolding activity with such proteins (Kawahara *et al.*, 2000). Yeast heat shock proteins, which are believed to exert a molecular chaperone function in both normal and stress conditions, have been suggested to protect yeast cells against freezing (Komatsu *et al.*, 1990).

During heat stress, trehalose has been shown to suppress the aggregation of denatured proteins in yeast, maintaining them in a partially folded state from which they can be activated by molecular chaperones (Singer and Lindquist, 1998a,b). A similar interplay between trehalose and molecular chaperones might contribute to freeze tolerance in microorganisms (Iwahashi *et al.*, 1995).

D. Plasma Membrane Characteristics

As the plasma membrane of unicellular organisms is in close contact with the surrounding medium, it is likely that its characteristics will influence the tolerance of the cells to all kinds of environmental challenges. Also for the determination of microbial freeze tolerance, the plasma membrane has been attributed a major role (Mazur, 1965, 1970; Steponkus, 1984; Steponkus and Lynch, 1989). Therefore, not surprisingly, virtually all cellular factors involved in freeze tolerance of microbial cells described so far have been demonstrated or suggested to help preserve membrane stability and/or integrity (Table I).

Whereas for bacteria, freeze tolerance has been correlated with specific fatty acid profiles (Fernandez Murga *et al.*, 2000; Zavaglia *et al.*, 2000), few studies have considered the importance of specific components of the yeast plasma membrane for freeze tolerance. Mainly, two plasma membrane characteristics have been studied in relation to freeze tolerance—fluidity and permeability.

1. *Membrane Fluidity*

Membrane fluidity is enhanced by the presence of (poly)unsaturated fatty acids and, to some extent, sterols. Freeze tolerance is usually higher in yeast and bacterial cells enriched in these membrane components (Calcott and Rose, 1982; Calcott *et al.*, 1984; Beal *et al.*, 2001; Rodriguez-Vargas *et al.*, 2002), indicating a positive correlation between membrane fluidity and freeze tolerance.

These findings suggest a protective role of proteins that are able to enhance membrane fluidity upon freezing. Overexpression in *S. cerevisiae* of a *Chlorella vulgaris* and an *Arabidopsis thaliana* gene, both encoding δ-12 fatty acid desaturases, has been reported but the effect on freeze tolerance has not been tested (Kajiwara *et al.*, 1996; Suga *et al.*, 2002). Interestingly, overexpression of *ERG10* was shown to improve freeze tolerance, whereas its deletion rendered yeast cells more freeze sensitive (Rodriguez-Vargas *et al.*, 2002). This finding might corroborate the putative importance of membrane composition for freeze tolerance. However, *ERG10* encodes acetoacetyl-CoA thiolase, an enzyme mediating the first step in the mevalonate pathway from acetyl-CoA to farnesyl pyrophosphate, and the latter leads to the synthesis not only of sterols but also of several other essential metabolic products including heme, quinones, and dolichols (Daum *et al.*, 1998).

On the other hand, the outcome of other studies contradicts the positive correlation between membrane fluidity and freeze tolerance.

An inverse correlation between yeast freeze tolerance and membrane fluidity was found under anaerobic conditions as opposed to aerobic conditions (Kruuv *et al.*, 1978). Moreover, freeze tolerance could not be correlated with differences in the degree of fatty acid unsaturation (Gélinas *et al.*, 1991a). No particular differences were observed in the unsaturation index of fatty acids between freeze-sensitive and freeze-tolerant strains (Murakami *et al.*, 1995, 1996; Teunissen *et al.*, 2002), and both a lower (Murakami *et al.*, 1996) and higher (Gélinas *et al.*, 1991a) sterol–phospholipid ratio were reported to correlate with freeze tolerance in yeast. Probably as a consequence of these highly contradictory results, lipid analysis alone was found to be of limited value in screening industrial yeast strains for improved freeze tolerance (Gélinas *et al.*, 1991a).

2. *Membrane Permeability*

Other studies have investigated the importance of membrane permeability for freeze tolerance in yeast. A relationship has been proposed between low residual water content in the cells and therefore high water permeability of the plasma membrane and a better survival of freeze stress (Mazur and Schmidt, 1968). The lower water permeability of batch-grown cells has been correlated with their lower freeze tolerance compared to fedbatch grown cells (Gélinas *et al.*, 1991b). The protective effect of EtOH and MetOH at rapid freezing rates has been shown to correlate with their stimulating effect on membrane permeability, presumably allowing faster water efflux during freezing (Lewis *et al.*, 1994). These findings suggest a protective role of proteins that are able to enhance plasma membrane permeability upon freezing.

3. *Aquaporin Function*

Recent genome-wide gene expression analyses and Northern analyses of freeze-tolerant and freeze-sensitive yeast strains have indeed revealed a correlation between freeze tolerance and expression of the aquaporin-encoding genes *AQY1*, and *AQY2*. This relationship was confirmed by deletion and overexpression of *AQY1* and *AQY2*, clearly reducing and enhancing yeast freeze tolerance, respectively (Tanghe *et al.*, 2002). Moreover, heterologous expression of the human aquaporin-encoding gene *hAQP1*, but not the poorly functional *hAQP1*-A73M allele, enhanced yeast freeze tolerance, supporting a role for plasma membrane water transport activity in determination of freeze tolerance in yeast (Tanghe *et al.*, 2002).

It is proposed that rapid osmotically driven efflux of water during the freezing process might reduce intracellular ice crystal formation and

resulting cell damage (Fig. 1). This notion has been supported by the finding that the artificial expression of an aquaporin has been found to improve the survival of mouse oocytes (Edashige *et al.*, 2003) and fish embryos (Hagedorn *et al.*, 2002) after cryopreservation.

E. Antioxidant Proteins

The formation of reactive oxygen species (ROS) has been associated with many types of stress. It produces mainly superoxide radicals, generated from oxygen, and electrons that have leaked from the mitochondrial electron transport chain. ROS cause cellular damage, such as protein inactivation, membrane damage due to lipid peroxidation, and damage to DNA (Santoro and Thiele, 1997). It has even been argued that the general robustness associated with industrial yeast strains is to a large extent related to their oxidative stress tolerance (Lewis *et al.*, 1997; Park *et al.*, 1998).

The involvement of an oxidative stress component in freeze stress has been studied using yeast mutants defective in different antioxidant functions. Mutants affected in the cytoplasmic Cu,Zn superoxide dismutase Sod1 and the mitochondrial Mn superoxide dismutase Sod2 showed impaired freeze tolerance levels (Park *et al.*, 1998). These findings correlated nicely with the higher levels of ROS in these strains, generated as a result of freezing and thawing. Moreover, the freeze- sensitive phenotype could be reversed by adding the superoxide anion scavenger $MnCl_2$ or by freezing in the absence of oxygen (Park *et al.*, 1998).

The activity of superoxide dismutase and other detoxifying ROS systems has likewise proven to be important for freeze tolerance in bacteria (Stead and Park, 2000) and other organisms, ranging from plants (Martinez *et al.*, 2001), to amphibia (Joanisse and Storey, 1996b), to insects (Joanisse and Storey, 1996a), to snakes (Hermes-Lima and Storey, 1993).

F. Compatible Solutes

In microorganisms and plants, the accumulation through uptake and synthesis of specific organic solutes in response to high osmolarity stress, so-called osmolytes, osmoprotectants, or compatible solutes, has been described (Kempf and Bremer, 1998; Nanjo *et al.*, 1999; Welsh, 2000). The nature of these solutes is diverse, ranging from amino acid–derived osmoprotectants such as proline and glycine betaine to sugar-related osmoprotectants such as trehalose, fructans,

and polyols. These osmolytes have been repeatedly shown to protect proteins and other macromolecules against different types of stresses, such as heat stress, desiccation stress, and freeze stress (Welsh, 2000). In view of the osmotic stress component inherent to freeze stress, a cryoprotective function of osmolytes seems likely.

In *S. cerevisiae*, glycerol has been demonstrated to serve as the major compatible solute (Albertyn *et al.*, 1994; Hohmann, 1997; Nevoigt and Stahl, 1997; Tamás *et al.*, 1999; Hohmann, 2002a,b). Given the cryoprotective role of glycerol under slow cooling conditions (Mazur, 1977; Farrant *et al.*, 1977), modification of the expression or activity of glycerol-producing enzymes and/or facilitators might improve freeze tolerance in yeast. In line with this assumption, incubation of baker's yeast in glycerol was reported to result in improved leavening capacity of sweet doughs after freeze storage (Myers and Attfield, 1999).

Notwithstanding its minor role as osmolyte in yeast cells, high levels of proline have been reported to protect mature yeast ascospores against desiccation (Ho and Miller, 1978). Using proline analogue-resistant mutants (Takagi *et al.*, 1997) and proline oxidase-deficient mutants (Takagi *et al.*, 2000), intracellular accumulation of proline has been shown to improve freeze tolerance (Morita *et al.*, 2003). In bacteria and plants, organisms in which proline is an important natural compatible solute during osmotic stress (Yoshiba *et al.*, 1997; Kempf and Bremer, 1998), freeze tolerance has also been correlated with high intracellular proline levels (Bulow and Mosbach, 1991; Xin and Browse, 1998; Nanjo *et al.*, 1999). This indicates that proline accumulation might be a general protective mechanism against freeze stress. In addition, intracellular accumulation of the charged amino acids arginine and glutamate also seems to enhance yeast freeze tolerance (Shima *et al.*, 2003).

The extracellular supplementation and intracellular accumulation of the osmolyte glycine betaine has been shown to confer enhanced freeze tolerance to *Listeria monocytogenes* (Ko *et al.*, 1994). Similar results were obtained for several plant species (Kishitani *et al.*, 1994; Allard *et al.*, 1998; Sakamoto *et al.*, 2000; Xing and Rajashekar, 2001).

G. Hydrophylins

At the onset of desiccation in maturing seeds, so-called late embryogenesis abundant (LEA) proteins are synthesized. They can be classified in a wider group called hydrophylins, which are characterized by a high percentage of glycines and a high hydrophilicity (Garay-Arroyo *et al.*, 2000). They are suggested to possess both water

and protein binding regions, enabling them to protect enzyme activities from water loss, as has been demonstrated for some enzymes upon dehydration and freezing *in vitro* (Covarrubias *et al.*, 2001). Proteins that meet the hydrophylin criteria have been found mainly in plants but also in nematodes (Browne *et al.*, 2002), bacteria, and fungi (Garay-Arroyo *et al.*, 2000; Covarrubias *et al.*, 2001). *In vitro*, some hydrophylins have been shown to protect enzymes under drying and/or freezing conditions (Honjoh *et al.*, 2000; Covarrubias *et al.*, 2001).

H. Antifreeze (Glyco)proteins

Proteins with antifreeze properties were first recognized in fishes (Davies and Hew, 1990) but have also been found in various other organisms that encounter freezing conditions in their surroundings, (e.g., insects, plants, and microorganisms) (Duman, 1982, 2001; Doucet *et al.*, 2002; Duman and Olsen, 1993; Liou *et al.*, 1999). These proteins have been classified as antifreeze glycoproteins (AFGPs) or antifreeze proteins (AFPs) type I, II, or III, according to their structural characteristics (Lillford and Holt, 1994; Cheng, 1998; Barrett, 2001; Davies *et al.*, 2002).

In spite of this structural diversity, all AFPs identified so far show similar functions. In relatively low concentrations, they depress the freezing point of water without affecting the melting point (so-called thermal hysteresis) and inhibit ice recrystallization (Chao *et al.*, 1996). Direct binding of antifreeze proteins to the ice surface, mainly through van der Waals and hydrophobic interactions, has been proposed (Jia and Davis, 2002). In this capacity, they inhibit further binding of water molecules and affect the shape of ice crystals, which occurs even at very low concentrations of the proteins. In addition, they have been ascribed a membrane-protecting function at low temperatures (Rubinsky *et al.*, 1990, 1991; Tablin *et al.*, 1996; Hays *et al.*, 1997; Wu, and Fletcher, 2001; Tomczak *et al.*, 2002).

Expression of natural fish AFPs as well as a chemically synthesized DNA fragment encoding an artificial antifreeze protein in *E. coli* has been demonstrated to improve both salt and freeze tolerance (Holmberg *et al.*, 1994; Meijer *et al.*, 1996).

I. Ice Nucleators

Ice-nucleating proteins or ice nucleators (INAs) limit supercooling and induce freezing at high subzero temperatures by mimicking the structure of an ice crystal surface. They impose an icelike arrangement

on the water molecules in contact with their surface and lower the energy necessary for the initiation of ice formation. Hereby, they either provide cold protection from the released heat of fusion or establish protective extracellular freezing instead of lethal intracellular freezing (Zachariassen and Kristiansen, 2000). Very potent ice nucleators, active at high subfreezing temperature, are produced by bacteria such as *Erwinia herbicola* (Kozloff *et al.*, 1983; Phelps *et al.*, 1986) and *Pseudomonas fluorescens* (Corotto *et al.*, 1986). In addition, ice-nucleating bacteria of Antarctic origin have been identified (Obata *et al.*, 1999). In addition to the microorganisms already described, other living organisms also produce ice nucleators (e.g., nematodes (Wharton *et al.*, 2003), insects (Duman, 1982, 2001), plants (Lundheim, 2002), and fungi (Pouleur *et al.*, 1992)).

J. Cross-protection, General Stress Response, and Growth Conditions

1. *Cross-protection*

Exposure of yeast cells to a mild dose of a particular type of stress results in the acquisition of resistance against a subsequent treatment with the same or another type of stress. Cross-protection seems to be a common mode of yeast and bacterial stress resistance, including tolerance to freeze stress. Freeze-induced cellular injury seems, to a certain extent, specific for freeze stress (for instance, injury due to ice crystal formation), but also to a certain extent common with other types of stress (such as osmotic stress and oxidative stress). This overlap renders it plausible that the mechanisms of protection and repair are likewise partially overlapping.

A mild heat pretreatment prior to freezing and thawing has been demonstrated to increase survival in yeast cells (Komatsu *et al.*, 1990) and bacterial cells (Chow and Tung, 1998; Walker *et al.*, 1999). Similar relationships between heat shock protein synthesis and acquisition of freeze tolerance have been reported in *E. coli* (Chow and Tung, 1998), *Lactococcus lactis* (Broadbent and Lin, 1999), and spores of *Neurospora crassa* (Guy *et al.*, 1986). Accordingly, other stress treatments resulting in heat shock protein synthesis (i.e., pretreatment with H_2O_2, cycloheximide or low NaCl concentrations (Komatsu *et al.*, 1990; Lewis *et al.*, 1995; Park *et al.*, 1997)) have also been shown to protect yeast cells against freeze stress (Lewis *et al.*, 1995; Park *et al.*, 1997). Likewise, the improvement of freeze–thaw tolerance by an osmotic stress applied prior to the freeze–thaw stress has been reported for the yeastlike fungus *Geotrichum candidum* (Dubernet *et al.*, 2002) and for *Lactobacillus delbrueckii ssp. Bulgaricus* (Panoff *et al.*, 2000a).

In *Bifidobacterium adolescentis*, a preconditioning treatment with sublethal bile-salt levels has been demonstrated to provide protection against subsequent freeze–thaw cycles (Schmidt and Zink, 2000).

2. *General Stress Response*

The phenomenon of cross-protection suggests that different stress conditions elicit common stress protection responses which protect against different types of stress (Mager and Moradas Ferreira, 1993; Siderius and Mager, 1997; Estruch, 2000). The proposed triggers for this so-called general stress response, such as the generation of aberrant proteins, intracellular acidification, the production of ROS, cytoskeletal alterations, and the modulation of second-messenger levels (Craig and Gross, 1991; Coote *et al.*, 1991; Chowdhury *et al.*, 1992; Moradas-Ferreira *et al.*, 1996; Siderius and Mager, 1997) have all been associated with freeze–thaw stress (Park *et al.*, 1997).

In *S. cerevisiae*, the general stress response consists mainly of the coordinated induction and repression of a variety of genes. The transcription factors Msn2 and Msn4 are required for activation of several yeast genes whose induction is mediated through stress-responsive promoter elements (STREs) (Martinez-Pastor *et al.*, 1996). The genes regulated through the general stress response pathway encode heat shock proteins, proteins involved in trehalose synthesis, catalase T, and a DNA damage repair factor (Mager and Moradas Ferreira, 1993; Siderius and Mager, 1997; Estruch, 2000). A disruption of both *MSN2*, and *MSN4* results in a higher sensitivity to different types of stress (Martinez-Pastor *et al.*, 1996). It is likely that these mechanisms act synergistically to protect yeast cells against freeze–thaw stress related damage, as has been described for trehalose and heat shock proteins in heat resistance (Elliott *et al.*, 1996; Singer and Lindquist, 1998a,b). From several studies it has been concluded that in addition to trehalose and heat shock proteins, still other factors must be involved in tolerance against freezing and various other stress types (Piper, 1993; Elliott *et al.*, 1996; Gross and Watson, 1996; Swan and Watson, 1998, 1999).

A general stress response has also been described in other microorganisms. In the model organism *Bacillus subtilis* as well as in other gram-positive bacteria, the sigmaB transcription factor induces the transcription of more than 150 genes after exposure to many different types of stress, providing the cells with nonspecific, diverse, and preventive stress protection (Volker *et al.*, 1999; Hecker and Volker, 2001; Petersohn *et al.*, 2001). However, the molecular bases of general stress responses seem to be, at least in part, species (or even subspecies)

specific. The absence of a sigmaB orthologue in most lactic acid bacteria represents a striking difference between the stress response mechanisms that *B. subtilis* and other bacteria have developed (van de Guchte *et al.*, 2002).

3. *Growth Conditions*

It is well recognized that the general stress resistance of yeast and other fungal cells strongly depends on the growth conditions. In general, slower growth is correlated with higher general stress resistance in yeast and many other fungi (Thevelein, 1996). Yeast cells growing on nonfermentable carbon sources show a strikingly higher general stress tolerance compared to cells growing on rapidly fermentable carbon sources (Schenberg-Frascino and Moustacchi, 1972; Plesset *et al.*, 1987; Elliott and Futcher, 1993; Gross and Watson, 1996; Park *et al.*, 1997). Likewise, the much higher general stress resistance of *S. cerevisiae* stationary phase cells as opposed to exponential phase cells has been extensively documented (Werner-Washburne *et al.*, 1993; Van Dijck *et al.*, 1995; Fuge, 1997; Park *et al.*, 1997; Morris *et al.*, 1998).

Slow growth and absence of growth is generally associated with high trehalose and heat shock protein levels. Besides differences in trehalose and heat shock protein levels, cells residing in different growth phases or cultured in different conditions are likely to differ also in other properties which might contribute to stress tolerance. The contribution of such other factors to (freeze) stress tolerance has not been studied intensively and is generally not clear. Next to higher trehalose and heat shock protein levels, stationary phase cells exhibit altered plasma membrane characteristics, which might also contribute to their higher resistance (Souzu, 1986a,b; Werner-Washburne *et al.*, 1993; Fuge, 1997). The higher freeze tolerance of respiring cells compared to fermenting cells has been related to the requirement of adequate mitochondrial function and respiratory ability for full freeze tolerance (Lewis *et al.*, 1993a). It has even been argued that the reported freeze tolerance of stationary phase cells is, in many cases, a manifestation of the correlation between freeze tolerance and respiration, since growth phases after diauxic shift are not always defined consistently (Lewis *et al.*, 1993b).

As in yeast cells, stress resistance in bacteria has also been reported to depend on the growth phase and culture conditions. For instance, *E. coli* stationary phase cells were shown to be more resistant to freeze–thaw damage compared to exponential phase cells (Souzu, 1982). Accordingly, cells of *G. candidum* showed a higher freeze

tolerance when harvested in stationary compared to exponential phase (Thammavongs *et al.*, 2000).

V. Physiological Importance of Microbial Freeze-tolerance Mechanisms

A. Acquired Freeze Tolerance by Cold Acclimation

Compared to heat shock, the response and adaptation to low temperatures has been less extensively studied. However, as for heat shock, a specific cold shock response has been documented in many cell types. The cold shock response in both prokaryotes and eukaryotes has been thoroughly reviewed elsewhere (Russell, 1990; Jones and Inouye, 1994; Thieringer *et al.*, 1998; Panoff *et al.*, 1998; Phadtare *et al.*, 1999; Hebraud and Potier, 1999; Los and Murata, 1999; Wouters *et al.*, 2000; Sahara *et al.*, 2002). The major problems that cells encounter when they are exposed to low temperatures seem to be low membrane fluidity and, at least in part as a result, impaired membrane function, stabilization of secondary DNA and RNA structures, low protein translation efficiency, and defective folding and functioning of proteins. Accordingly, major aspects of the cold shock response appear to be an alteration of membrane characteristics and an adaptation of the protein translation machinery, together with the increased synthesis and/or stability of so-called cold shock proteins.

In nature, freezing temperatures are established gradually, suggesting that cellular adaptations against low temperatures might provide protection against freezing. Whereas in plants the specific alterations associated with cold acclimation are believed to reflect the molecular basis of freeze tolerance (Thomashow, 1998), the significance of cold-induced proteins for the achievement of microbial freeze tolerance remains controversial (Park *et al.*, 1997; Panoff *et al.*, 2000b). Although the expression, stability, or production of many of the cellular factors involved in microbial freeze tolerance is induced by exposure to cold temperatures as part of a cold stress response, the relevance and relative contribution of the freeze tolerance mechanisms discussed in the first part of this chapter is largely unclear.

Both in bacteria (e.g., *Bacillus subtilis* (Willimsky *et al.*, 1992), *Enterococcus faecalis* (Thammavongs *et al.*, 1996), *Streptococcus mutans* (Tsien *et al.*, 1980), lactic acid bacteria (Kim and Dunn, 1997; Broadbent and Lin, 1999; Murga *et al.*, 2000), and in fungi (Thammavongs *et al.*, 2000)), an improved freeze tolerance has been demonstrated after a low temperature treatment. However, the correlation between cold treatment and freeze tolerance mechanisms is often unclear.

Conflicting data have been reported for *S. cerevisiae*, either confirming (Kaul *et al.*, 1992; Diniz-Mendez *et al.*, 1999) or denying (Park *et al.*, 1997) an acquired freeze tolerance of cold-shocked yeast cells or a correlation between expression of cold-shock–induced proteins and improved freeze tolerance. The absence of an observable effect of triple disruption of the cold-shock–induced genes *TIP1*, *TIR1*, and *TIR2* on the viability of yeast cells after freezing (Kondo and Inouye, 1991; Kowalski *et al.*, 1995) has brought into question the significance of these cold-shock–induced genes for freeze tolerance. Likewise, cold stress responses in lactic acid bacteria do not seem to be unequivocally correlated to subsequent cryotolerance (Panoff *et al.*, 2000b).

It has been speculated that bacterial INAs may induce frost damage to their hosts in order to make the nutrients in the plant or fruit tissues available to them. The frost susceptibility of plants and fruits that are infested by the ice-nucleating bacterium *Pseudomonas syringae* is caused by ice nucleators that are shed by the bacterial cells at temperatures just below 0°C (Maki *et al.*, 1974; Arny *et al.*, 1976; Lindow *et al.*, 1978; Lindow, 1983; Phelps *et al.*, 1986; Mueller *et al.*, 1990; Upper and Vali, 1995). Moreover, the presence of INA-producing bacteria in the normal flora of the gut of overwintering insects has been proposed as a means to control insect pests during winter (Lee *et al.*, 1996).

Hibernating yeast spores have been shown to be resistant to freezing temperatures due mainly to their high trehalose content, their firm cell walls, and their lower water content allowing only little, if any, ice crystal formation (Van Laere, 1986). Likewise, plant pathogens and ectomycorrhizal fungi are known to be able to survive severe winters, remaining viable as a resting stage or reproductive propagule (Tibett *et al.*, 2002). Whether these life forms are only intrinsically freeze-tolerant or whether they also show a cold-induced improvement in freeze tolerance is not entirely clear.

B. (Semi)Permanent Cold and Freezing Conditions

Various microbes have been described that are capable of surviving and even growing in an extremely harsh and cold environment such as the Arctic and Antarctic regions (Abyzov *et al.*, 1998; Price, 2000; Laybourn-Parry, 2002; Römisch and Matheson, 2003). Microorganisms that experience near and below 0°C temperatures for long periods of the year have evolved structural and physiological mechanisms to improve survival under these conditions. To maintain the fluidity of their membranes, they increase the proportion of polyunsaturated fatty acids

and/or the length of the fatty acid components of the lipids (Arthur and Watson, 1976; Watson, 1987; Suutari *et al.*, 1990; Nichols *et al.*, 1993; Rotert *et al.*, 1993). Furthermore, they have developed enzymes with a low-temperature optimum that are partially able to cope with the reduction in chemical reaction rates at low temperatures (Gerday *et al.*, 1997; Lonhienne *et al.*, 2000; D'Amico *et al.*, 2002). Moreover, psychrophilic yeast species have been shown to possess a higher content of cytochromes compared to mesophylic species (Arthur and Watson, 1976), and Antarctic species were found to contain giant mitochondria (Römisch and Matheson, 2003), probably to ensure adequate oxygen delivery at low temperature. Cold adaptations in microtubule assembly and dynamics, RNA, protein synthesis, and protein secretion have also been described (Römisch and Matheson, 2003). Finally, some Arctic and Antarctic microorganisms have been found to produce AFPs (Yamashita *et al.*, 2002) or INAs (Obata *et al.*, 1999; Wharton *et al.*, 2003).

VI. Biotechnological Applications of Microbial Freeze-tolerance Mechanisms and Freeze-tolerant Microorganisms

A. Introduction

A better understanding of microbial freeze tolerance is of interest not only from a fundamental point of view, but is also relevant to many commercial applications with components of biological origin and microorganisms. Microbial cryoprotectants and freeze-stress–related proteins as well as the freeze-tolerant microorganisms have a large potential for commercial application.

Trehalose, widely recognized for its stress-protective properties, has been used as a protectant for freeze storage and freeze-dried preservation of various biological molecules such as enzymes (Colaco *et al.*, 1992), antibodies (Hazen *et al.*, 1988), platelets (Wolkers *et al.*, 2001; Crowe *et al.*, 2003), vaccines (Gribbon *et al.*, 1996) as well as cell lines (Eroglu *et al.*, 2000), oocytes (Rayos *et al.*, 1994), sperm cells (An *et al.*, 2000), embryos (Honadel and Killian, 1988), and organs (Fukuse *et al.*, 1999). Trehalose has been proven superior to other cryoprotectants, particularly under suboptimal conditions of freezing, drying, and storage (Rudolph and Crowe, 1985; Crowe *et al.*, 2003). Moreover, the acceptance of trehalose as a safe food ingredient has broadened the range of its possible applications (Schiraldi *et al.*, 2002). Microbial AFPs and INAs have a great potential for use in industrial applications where control over the amount, size, shape,

and location of ice crystals would be beneficial (Gurian-Sherman and Lindow, 1993; Fletcher *et al.*, 1999; Lillford and Holt, 2002). Besides oocyte, embryo, cell, tissue, and organ cryopreservation (Grout *et al.*, 1990), examples are found in ice cream production and storage in frozen form of fruit, vegetables, and other foods (Li and Lee, 1995; Hwang *et al.*, 2001). In addition, the potential use of INAs to improve frozen dough quality by accelerating the freezing of the dough moisture at a higher temperature below 0°C has been suggested (Funaki *et al.*, 1996).

It can be foreseen that as the commercial applications of cryopreservation develop further, there will be an increasing demand for biological cryoprotectants (Lillford and Holt, 2002). The production by microorganisms of cryoprotectants from microbial and nonmicrobial origins provides a promising means to fulfill the increasing demand for these substances. Studies have been reported on the use of yeast cells to produce pure trehalose in a relatively inexpensive manner (Panek, 1995; Di Lernia *et al.*, 2002). AFPs can be isolated most easily from fish but only in relatively small quantities. Especially for future large-scale applications of AFPs, such as the storage and transportation of ice slurries and de-icing agents (Grandum and Nakagomi, 1997), the isolation from fish will prove to be inadequate (Fletcher *et al.*, 1999). Therefore, alternative ways of AFP production are currently under investigation, including production in prokaryotic and eukaryotic expression systems (Peters *et al.*, 1989; Loewen *et al.*, 1997; Solomon and Appels, 1999). Moreover, there will be a demand for better tailored AFPs. The different types of AFPs change ice crystal morphology each in a characteristic way (Chao *et al.*, 1995). Via genetic engineering, the properties of AFPs can be modified to arrive at a better understanding of their structure–function relationships (Mueller *et al.*, 1991; Li *et al.*, 1991; Doucet *et al.*, 2000). This information will allow the engineering of AFPs to suit particular requirements via chemical synthesis of artificial AFPs or via site-directed mutagenesis of existing AFPs (Lillford and Holt, 1994). As opposed to antifreeze proteins, the large-scale availability of INAs is not problematic (Lillford and Holt, 2002). Ice-nucleating bacteria are already used in applications where large amounts are needed, such as artificial snow production and cloud seeding (Margaritis and Bassi, 1991).

The development of transgenic plants with increased frost tolerance is another intriguing application. The introduction of genes from microorganisms or even whole microbial biosynthetic pathways in plants has already been shown to improve freeze tolerance. *A. thaliana* plants transformed with the *codA* gene from *Arthrobacter globiformis* encoding choline oxidase and accumulating glycine betaine in the

chloroplast showed a significant improvement in freeze tolerance (Sakamoto *et al.*, 2000).

B. Frozen and Freeze-dried Microorganisms

Freeze-tolerant microorganisms also have a great potential for use in industrial applications. The large-scale exploitation of microorganisms for industrial and scientific use often requires long-term preservation of the cells. Frozen preservation is the most widespread method serving that purpose. Theoretically spoken, for each cell type the optimal cooling rate can be determined at which both cellular dehydration and intracellular ice crystal formation are minimal (Mazur, 1970). However, a cooling rate slow enough to prevent intracellular freezing often causes unexpected injury due to solution effects. Moreover, from a practical point of view, this approach often takes too much time or is too laborious. It is usually circumvented by the use of cryoprotectants. Many types of cryoprotective agents, low, and high molecular weight molecules with different cell permeabilities, have been described, each providing protection in specific conditions (Farrant *et al.*, 1977; Carpenter *et al.*, 1992; Lewis *et al.*, 1993a, 1994). For instance, the standard osmoprotective substances glycerol, sucrose, and dimethylsulfoxide (DMSO) are only effective at low rates of cooling (Mazur, 1977; Farrant *et al.*, 1977). On the contrary, EtOH, and MetOH act as cryosensitizers at a cooling rate of 3°C/min but are excellent cryoprotectants at a cooling rate of 200°C/min (Lewis *et al.*, 1994). Not surprisingly, trehalose has been shown to be a superior protectant of yeast cells upon freezing compared to glycerol, sucrose, and DMSO (Coutinho *et al.*, 1988; Diniz-Mendez *et al.*, 1999). Given the low concentrations at which they exert their protective effects, AFPs provide an interesting alternative to small molecules which need to be added in high concentrations and might cause toxicity and side effects (Breton *et al.*, 2000).

Freeze-drying is an alternative method for the long-term preservation of microorganisms, allowing simple storage and distribution of the freeze-dried samples. Although both treatments involve removal of water from the cells, different injury mechanisms seem to be involved. The cellular damage brought about by freeze-drying has mostly been ascribed to dehydration and rehydration (Leslie *et al.*, 1995), although the freezing step is also detrimental for the cells (Cerrutti *et al.*, 2000). Most bacteria fairly withstand the freeze-drying process

(Miyamoto-Shinohara *et al.*, 2000). On the other hand, a significant drop in viability has been reported for baker's yeast cells (Mazur and Schmidt, 1968). The optimization of the freeze-drying process and the addition of protective substances such as trehalose can partially overcome this drop in viability (Leslie *et al.*, 1995; Diniz-Mendez *et al.*, 1999; Lodato *et al.*, 1999; Cerrutti *et al.*, 2000; Abadias *et al.*, 2001).

A particular problem with frozen and freeze-dried preservation is the fact that different microorganisms show a highly different survival rate. Even the outcome of freeze preservation of strains from the same species can be quite different. A better understanding of tolerance mechanisms protecting against freezing and freeze-drying will allow the optimization of these processes, allowing also a more appropriate and efficacious use of cryoprotective agents in order to maintain a better microbial stability and viability over time in the frozen state.

The use of frozen and freeze-dried starter cultures, in food biotechnology, in particular, cheese manufacture, meat fermentations, and the production of sourdoughs, would also benefit from this knowledge (Metz, 1993; Devaldez and Diekman, 1993; Panoff *et al.*, 2000b). The pure strains of microorganisms contained in starter cultures are grown on a large scale, and the resulting biomass is preserved by freeze-drying or freezing. The survival and activity of the frozen starter cultures is an important determinant of the quality of the end product. For *G. candidum*, a yeastlike fungus used as ripening starter in cheese making, a pretreatment with osmotic or cold stress has been shown to improve cryotolerance (Thammavongs *et al.*, 2000; Panoff *et al.*, 2000a; Dubernet *et al.*, 2002). In cheese ripening, promising results have been obtained with attenuated starter cultures of lactococci and lactobacilli, reducing ripening time and improving flavor (Klein and Lortal, 1999). However, the empirical character of the attenuation methods, including freeze-thawing and freeze-drying, hampers the widespread use of this technique.

The concept of probiotics is another recent application that involves frozen or freeze-dried microorganisms. These so-called ''functional food'' ingredients or supplements are intended to have a beneficial effect on the host through positive effects in the intestinal tract. They are used to prepare fermented dairy products such as frozen and nonfrozen yogurt, ice milk, and ice cream (Hekmat and McMahon, 1992; Sheu *et al.*, 1993; Davidson *et al.*, 2000), and they are also marketed as such as freeze-dried preparations of viable microorganisms.

C. The Concept and Problems of Frozen Dough Technology

The production and use of frozen doughs is steadily increasing in all industrialized countries because it offers economy of scale to manufacturers; convenient separation of production and use of dough, facilitating large-scale distribution; considerable savings in labor time and maintenance costs to bakers; and the availability of oven-fresh bakery products to private consumers. However, the frozen dough method suffers from several drawbacks: a lengthening of the dough proofing time, a diminished bread volume, and an unsatisfactory bread quality following freezing, freeze storage, and thawing. Compared to fresh doughs, the leavening capacity of frozen doughs is substantially lower and drops further with the duration of freeze storage. The frozen shelf-life during which relatively good performance is kept varies from a few weeks to several months at most, depending on the type of dough. These problems are caused mainly by the rapid loss of the yeast's intrinsic stress resistance during the preparation and prefermentation of the dough (Merritt, 1960; Lorenz, 1974) and further intensified by the damage and aggregation of gluten proteins in the dough (Ribotta *et al.*, 2001) (Fig. 2).

The production conditions for baker's yeast have been successfully optimized allowing the large-scale production of highly stress-tolerant yeast (Neyreneuf and Van Der Plaat, 1991) which is also resistant to freeze stress. However, when the yeast cells come into contact with the nutrients in the flour, they rapidly lose stress resistance (Fig. 2). The molecular reason for this effect is the activation of signal transduction pathways, in particular, activation of the cAMP-protein kinase A (PKA) pathway by fermentable sugar (Park *et al.*, 1997; Thevelein *et al.*, 2000). The drop in stress resistance is proportional to the prefermentation time and is due to the breakdown of trehalose, repression of heat shock protein encoding genes, and most probably disappearance of other unknown stress-protection factors. Consequently, the yeast in the dough is frozen at a moment when it has become considerably more susceptible to freeze injury, resulting in a significant loss of viability and reduction of fermentative capacity.

Much research has been carried out to find a solution for this problem in the frozen dough industry. The optimization of dough formulation and ingredients has proven inadequate. The supplementation with vital gluten, the use of high-gluten wheat or the use of certain emulsifiers increases the shelf life of frozen doughs but does not completely overcome the problem (Wang and Ponte, 1994). Likewise, the addition of more yeast, the use of active dry yeast instead of compressed yeast,

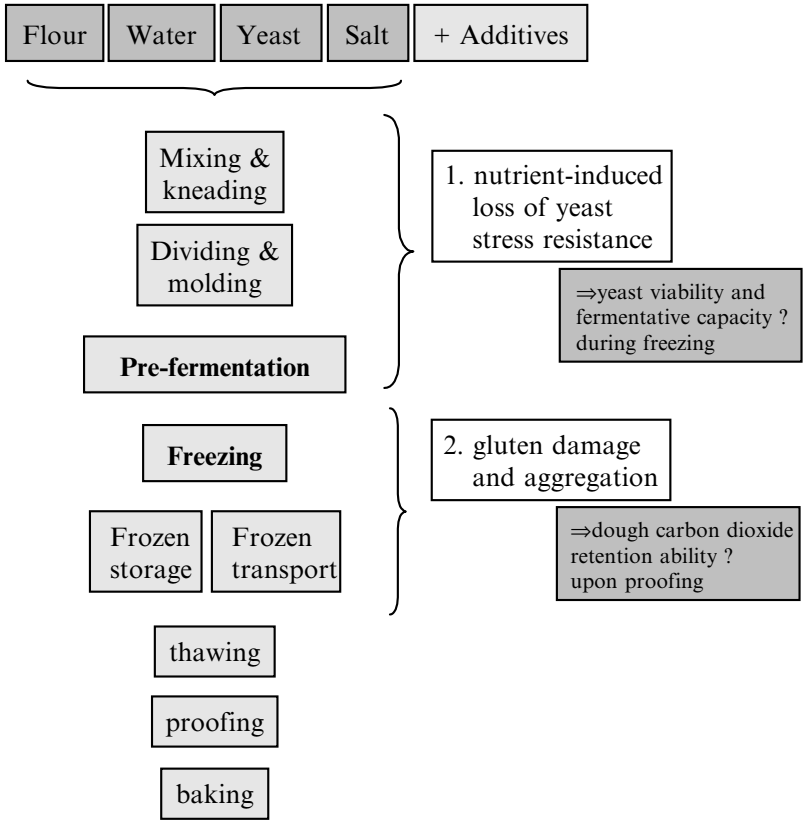

FIG. 2. Schematic outline of the frozen dough process. Two major problems are associated with this process: The nutrient-induced loss of yeast stress resistance during the preparation and prefermentation of the dough and the gluten damage and aggregation. Together, these events cause a longer dough-proofing time, a diminished bread volume, and an unsatisfactory bread quality.

precooling of the yeast, or the addition of yeast protective substances has not provided a satisfying solution (Kline and Sugihara, 1968). The use of specialized equipment for cold and rapid mixing of the dough, which is not available to artisanal bakers, minimizes but by no means overcomes the significant drop in yeast stress resistance at the onset of fermentation. Moreover, a short prefermentation period before freezing of the dough is indispensable to obtain an appropriate bread flavor, taste, and texture (Merritt, 1960). The major dough freezing system employed in the baker's industry is blast freezing, a process in which

extremely cold air is rapidly circulated over the products. Although cryogenic freezing, a process in which liquid carbon dioxide or liquid nitrogen is sprayed into the freezing chamber, has proven advantageous in terms of bread quality (Neyreneuf and Delpuech, 1993), frozen dough manufacturers are reluctant to adapt their freezing techniques, mainly for reasons of operational costs. Finally, neither the selection of suitable packaging material nor the determination of the most favorable storage conditions for frozen doughs has solved the problem (Lorenz, 1974).

Hence, although the problem of fermentation-induced loss of stress resistance was already recognized more than 40 years ago, it is still the main obstacle for the production of frozen doughs. It is generally believed that the only definite solution for this problem is the availability of industrial yeast strains specifically suited for use in frozen dough (i.e., strains which remain freeze-tolerant at the onset of fermentation and, in addition, display the same excellent growth capacity and gassing power as conventional baker's yeasts (Nagodawithana and Trivedi, 1990; Rose and Vijayalakshmi, 1993)). However, at present no such yeast strains are available (Attfield, 1997; Randez-Gil *et al.*, 1999).

D. Freeze-tolerant Strains Suited for Use in Frozen Dough

The concept of a yeast strain that remains highly (freeze)stress resistant during the onset of fermentation has for a long time been thought to go against biological design (Attfield, 1997). Both the physiological conditioning and selection of freeze-tolerant strains has proven inadequate to completely solve the fermentation-induced drop in stress resistance. This has given the impression that high stress resistance and high fermentation capacity are possibly incompatible biological properties (Meric *et al.*, 1995; Van Dijck *et al.*, 1995).

1. *Physiological Conditioning of Yeast Cells*

A true stress response probably does not exist for freeze stress conditions and, as a result, survival is mainly dependent on the physiological status of the cell prior to freezing. Indeed, no cellular adaptation has been observed following multiple freeze–thaw cycles with or without a recovery period in between the cycles, indicating that yeast cells do not adapt to freeze stress caused by repeated freezing and thawing (Park *et al.*, 1997). When yeast cells are frozen, the little amount of time available to adapt to this new situation renders it unlikely that cells could synthesize or modify stress proteins or stress metabolites, as has been described for responses to other stress treatments, including oxidative stress (Santoro and Thiele, 1997), osmotic stress (Hohmann,

1997), and salt stress (Serrano *et al.*, 1997). The improvement of freeze tolerance of commercial baker's yeasts in dough by heat treatment before freezing has been reported (Nakagawa and Ouchi, 1994b). However, in industrial practice, this effect seems to be counteracted by the accelerated loss of stress resistance due to heat-induced stimulation of fermentation.

The possibility to modulate stress tolerance of yeast cells by modification of the growth conditions is recognized and exploited in the baker's yeast industry, where baker's yeast is commercially produced by a fed-batch method, using molasses as a substrate. The fed-batch fermentation method consists of sequential propagation stages, each differing in fermenter size, aeration, and feeding conditions (Randez-Gil *et al.*, 1999). Fed-batch cultured cells were shown to be more freeze tolerant than cells in any batch culture phase, especially at lower growth rates and under strong aeration (Gélinas *et al.*, 1989). The higher stress tolerance of fed-batch cultured cells is most pronounced at the end of the production process, when trehalose constitutes up to 20% of the cells' dry weight (Attfield, 1997; Randez-Gil *et al.*, 1999;). Freeze tolerance of yeast cells can be improved by loading the cells with trehalose (Hirasawa *et al.*, 2001). A 4–5% trehalose content has been suggested to be sufficient to prevent yeast cells from freeze damage during freezing and freeze storage in dough (Meric *et al.*, 1995). A blend of cane and beet molasses is the most widely used substrate for baker's yeast production (Randez-Gil *et al.*, 1999). It is generally believed that growth-inhibitory substances present in the molasses evoke a stress response which contributes to the high stress resistance of the yeast cells at the end of the production process.

Although the present-day production conditions allow production of highly stress-tolerant yeast (Neyreneuf and Van Der Plaat, 1991), including high tolerance to freeze stress, this does not solve the basic problem of frozen dough technology—that the yeast cells rapidly lose stress resistance when they come into contact with nutrients in the flour. Hence, physiological conditioning does not seem to provide an answer to this fundamental problem.

2. *Strain Selection and Isolation*

Freeze-tolerant yeast strains have been selected from strain collections or have been isolated from natural sources such as soil, grains, fruits, and flowers (Oda *et al.*, 1986; Hino *et al.*, 1987; Hahn, and Kawai, 1990; Oda and Tonomura, 1993) as well as traditional corn and rye bread doughs (Almeida and Pais, 1996). Yeast strains with improved freeze tolerance have also been obtained by classic breeding

approaches in natural and commercial strains, including mutagenesis (Teunissen *et al.*, 2002), mating of spores (Nakagawa and Ouchi, 1994a), and protoplast fusion (Codon *et al.*, 2003) followed by selection for the desired trait.

Although usually strains of *S. cerevisiae* are used in breadmaking, studies on *Torulaspora delbrueckii, Torulaspora pretoriensis, Kluyveromyces thermotolerans*, and *Zygosaccharomyces rouxii* strains have also been reported (Hino *et al.*, 1987; Hahn and Kawai, 1990; Oda and Tonomura, 1993; Almeida and Pais, 1996). Strains of these species generally show a better freeze tolerance compared to conventional baker's yeast strains, but they are often compromised for important commercial features, such as maltose fermentation capacity. However, this is often also true for the freeze- tolerant *S. cerevisiae* strains that have been described (Oda *et al.*, 1986; Hino *et al.*, 1987).

In general, classic breeding approaches have proven inadequate for the development of industrial strains suited for frozen dough applications. A major problem associated with the modification of fundamental yeast characteristics, such as stress resistance, is the almost inevitable change of other, desirable properties. This happens so often that, for instance, rapid growth and fermentation have previously been considered to be incompatible with high stress resistance (Attfield, 1997; Randez-Gil *et al.*, 1999).

Yeast mutants deficient in ''fermentation-induced loss of stress resistance'' (*fil* mutants) have been isolated (Van Dijck *et al.*, 2000a). *Fil* mutants *fil1* and *fil2*, isolated in the laboratory strain M5 and W303, respectively, were affected in components of the cAMP–protein kinase A pathway, *in casu* adenylate cyclase and the putative glucose-sensing G-protein coupled receptor Gpr1, respectively (Kraakman *et al.*, 1999; Van Dijck *et al.*, 2000b). *Fil* mutant AT25 has been isolated in the industrial strain S47 (''Hirondelle Bleue'', Lesaffre) that is in commercial use worldwide (Colavizza *et al.*, 2000; Teunissen *et al.*, 2002). In addition to its higher freeze tolerance, the commercially important properties of AT25 are similar or improved compared to those of S47. Although the degree of improvement of freeze tolerance as well as other properties seems to be inadequate for the strain to be marketed as a freeze-tolerant strain, the approach that was used consisting of mutagenesis and selection in dough seems a promising tool for the isolation of strains better suited for use in frozen dough.

3. *Genetic Engineering*

a. *Strain improvement by genetic engineering*

Taking advantage of the wealth of genetic modification tools available for *S. cerevisiae*, its fairly well-known physiology, and the disclosure of its complete genome sequence, several attempts have been undertaken to generate strains better suited for frozen dough production by means of genetic engineering. However, the fermentation-induced loss of stress resistance inherent to frozen dough production and the lack of knowledge about the precise mechanisms and determinants of microbial freeze tolerance turned out to be major obstacles.

With respect to stress resistance in general and freeze tolerance in particular, the modification of intracellular trehalose levels has generally been regarded as one of the most promising avenues and has therefore been explored most intensively. Yeast strains in which the *NTH1* and/or *ATH1* gene encoding neutral and acid trehalase, respectively, have been disrupted show higher intracellular trehalose levels and also improved freeze tolerance (Kim *et al.*, 1996; Shima *et al.*, 1999). However, the correlation between trehalose content and stress resistance holds only in the absence of fermentation (Van Dijck *et al.*, 1995). Addition of glucose to cells of a $nth1^{\Delta}$ strain still caused a rapid loss of stress resistance in spite of a very high trehalose level and its very slow mobilization. Moreover, a survey of 14 industrial *S. cerevisiae* strains grown in batch culture to stationary phase and tested for their trehalose content and their tolerance to different types of stress indicated that trehalose may not be used as a general predictor of stress tolerance in true stationary phase yeast (Lewis *et al.*, 1997).

Freeze-tolerant strains have also been developed based on the correlation between freeze tolerance and other cellular factors. Mutants resistant to an analogue of L-proline displayed a high freeze tolerance, apparently due to their high intracellular proline level. Isolation of such mutants has been proposed as a practical method for breeding of novel freeze-tolerant yeast strains (Takagi *et al.*, 1997, 2000; Morita *et al.*, 2003). Likewise, enhanced freeze tolerance has been demonstrated upon disruption of the *CAR1* gene encoding arginase, resulting in accumulation of the charged amino acids arginine and glutamate (Shima *et al.*, 2003). A strain overexpressing the *ERG10* gene encoding acetoacetyl coenzyme A thiolase exhibited an increased freeze tolerance (Rodriguez-Vargas *et al.*, 2002). A baker's yeast strain to which mitochondria from highly EtOH-tolerant wine yeast were transferred showed improved freeze tolerance during a short period of frozen

storage (Codon *et al.*, 2003). The use of a baker's yeast mutant resistant to 2-deoxy-D-glucose in frozen dough resulted in bread with superior qualities (Codon *et al.*, 2003). Overexpression in *S. cerevisiae* of LEA proteins resulted in an improved tolerance to salt stress and osmotic stress (Imai *et al.*, 1996; Swire-Clark and Marcotte, 1999; Zhang *et al.*, 2000) as well as freeze stress (Honjoh *et al.*, 1999). A synthetic allele optimized for expression in yeast encoding a polar fish AFP was shown to be successfully expressed and secreted in *S. cerevisiae*, using the enzyme α-galactosidase as a carrier protein (Driedonks *et al.*, 1995). Cells expressing a chimeric protein, partly consisting of a functional AFP analogue, displayed a twofold increase in survival after rapid freezing and moderate rates of warming compared to cells lacking the fusion protein (McKown and Warren, 1991).

Recently, aquaporin expression has been identified as a determinant of yeast freeze tolerance (Tanghe *et al.*, 2002). Aquaporin overexpression improves maintenance of yeast viability of industrial strains, not only in liquid cultures but also in small doughs stored frozen or submitted to freeze/thaw cycles, without affecting commercially important characteristics (Tanghe *et al.*, 2002). Moreover, an aquaporin overexpression transformant could be selected based solely on its improved freeze–thaw tolerance without the need for a selectable marker gene (Tanghe *et al.*, 2002). These results open new perspectives for the successful development of freeze-tolerant baker's yeast strains for use in frozen dough. Unfortunately, the aquaporin-mediated improvement of freeze tolerance seems to be restricted to rapid freezing conditions and therefore limited to very small doughs (Tanghe, unpublished). Therefore, further research is required to evaluate the usefulness of aquaporin-mediated freeze tolerance for the development of new industrial strains for frozen dough applications.

b. *Problems associated with strain improvement by genetic engineering*

Industrial strain improvement is generally limited by lack of knowledge concerning the precise genetic and molecular mechanisms underlying commercially important multifactorial traits such as freeze tolerance. It is generally believed that freeze tolerance, like most important baker's yeast characteristics, is determined by the interaction of many genes (Attfield, 1997; Randez-Gil *et al.*, 1999). From this point of view, multiple modifications of genes or the modification of control genes with multiple downstream effects would be needed to substantially improve freeze tolerance (Attfield, 1997). Indeed, next to the aquaporin-encoding genes, no single gene has been identified that

allows reducing or enhancing baker's yeast freeze tolerance by manipulation of its expression level without affecting other commercially important properties.

Furthermore, there is also the large gap between theoretical knowledge and practical application: Results obtained with laboratory strains or in laboratory conditions do not always directly apply to industrial strains or industrial conditions. Industrial baker's and brewer's strains are usually polyploid (Rose and Vijayalakshmi, 1993; Benitez *et al.*, 1996). Little is known about the importance of gene copy number and allele variation for a specific trait such as freeze tolerance. In addition, it is well known that a yeast strain showing a high freeze tolerance when grown and tested in laboratory conditions often does not display the same features in industrial conditions.

Although there are many potential applications of genetically modified baker's yeasts in traditional industries, for instance, in frozen doughs, the effective introduction on the market of such strains is still stalled by negative public perception. Only a limited number of genetically modified organisms have received official approval for commercial use and, in spite of this permission, they are currently not exploited (Halford and Shewry, 2000; Dequin, 2001). Commercial freeze-tolerant yeast strains that are on the market now do not display satisfactory characteristics. If a superior strain becomes available via genetic engineering which clearly shows perceptible benefits, not only to frozen dough manufacturers and bakers but also to private consumers, such a strain might become more acceptable.

VII. Concluding Remarks

In conclusion, although much correlative evidence is available, little is known with certainty about the precise molecular and genetic mechanisms underlying freeze tolerance of yeast and microorganisms in general. Identification of freeze tolerance determinants, and in particular their interplay, remains an important challenge for the future. Once this knowledge is available, better targeted approaches for rational improvement of freeze tolerance by means of genetic engineering will become available.

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References

Abadias, M., Benabarre, A., Teixido, N., Usall, J., and Vina, I. (2001). Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake*. *Int. J. Food Microbiol.* **65,** 173–182.

Abyzov, S. S., Mitskevich, I. N., and Poglazova, M. N. (1998). Microflora of the deep glacier horizons of Central Antarctica. *Microbiology* **67,** 451–458.

Albertyn, J., Hohmann, S., and Prior, B. A. (1994). Characterization of the osmotic-stress response in *Saccharomyces cerevisiae*: Osmotic stress and glucose repression regulate glycerol-3-phosphate dehydrogenase independently. *Curr. Genet.* **25,** 12–18.

Allard, F., Houde, M., Krol, M., Ivanov, A., Huner, N. P. A., and Sarhan, F. (1998). Betaine improves freezing tolerance in wheat. *Plant Cell Physiol.* **39,** 1194–1202.

Almeida, M. J., and Pais, C. (1996). Leavening ability and freeze tolerance of yeasts isolated from traditional corn and rye bread doughs. *Appl. Environ. Microbiol.* **62,** 4401–4404.

An, T. Z., Iwakiri, M., Edashige, K., Sakurai, T., and Kasai, M. (2000). Factors affecting the survival of frozen-thawed mouse spermatozoa. *Cryobiology* **40,** 237–249.

Arny, D. C., Lindow, S. E., and Upper, C. D. (1976). Frost sensitivity of corn increased by application of *Pseudomonas syringae*. *Nature* **262,** 282–284.

Arthur, H., and Watson, K. (1976). Thermal adaptation in yeast: Growth temperatures, membrane lipid, and cytochrome composition of psychrophilic, mesophilic, and thermophilic yeasts. *J. Bacteriol.* **128,** 56–68.

Attfield, P. V. (1997). Stress tolerance: The key to effective strains of industrial baker's yeast. *Nature Biotechnol.* **15,** 1351–1357.

Attfield, P. V., Raman, A., and Northcott, C. J. (1992). Construction of *Saccharomyces cerevisiae* strains that accumulate relatively low concentrations of trehalose, and their application in testing the contribution of the disaccharide to stress tolerance. *FEMS Microbiol. Lett.* **94,** 271–276.

Barrett, J. (2001). Thermal hysteresis proteins. *Int. J. Biochem. Cell Biol.* **33,** 105–117.

Beal, C., Fonseca, F., and Corrieu, G. (2001). Resistance to freezing and frozen storage of *Streptococcus thermophilus* is related to membrane fatty acid composition. *J. Dairy Sci.* **84,** 2347–2356.

Benitez, T., Martinez, P., and Codon, A. C. (1996). Genetic constitution of industrial yeast. *Microbiol. Sem.* **12,** 371–384.

Breton, G., Danyluk, J., Ouellet, F., and Sarhan, F. (2000). Biotechnological applications of plant freezing associated proteins. *Biotechnol. Annu. Rev.* **6,** 59–101.

Broadbent, J. R., and Lin (1999). Effect of heat shock or cold shock treatment on the resistance of *Lactococcus lactis* to freezing and lyophilization. *Cryobiology* **39,** 88–102.

Browne, J., Tunnacliffe, A., and Burnell, A. (2002). Plant dessication gene found in a nematode. *Nature* **416,** 38.

Buchner, J. (1996). Supervising the fold: Functional principles of molecular chaperones. *FASEB J.* **10,** 10–19.

Bulow, L., and Mosbach, K. (1991). The oligopeptide (Gly-Pro)2-Ala-(Gly-Pro)2 increases the internal proline level and improves NaCl tolerance when produced in *Escherichia coli*. *Gene* **109,** 125–129.

Burke, M. J. (1985). The glassy state and survival of anhydrous biological systems. *In* ''Membranes, metabolism, and dry organisms'' (A. C. Leopold, ed.), pp. 358–363. Cornell Univ. Press, Ithaca, N.Y.

Calcott, P. H., and MacLeod, R. A. (1975). The survival of *Escherichia coli* from freeze–thaw damage: The relative importance of wall and membrane damage. *Can. J. Microbiol.* **21,** 1960–1968.

Calcott, P. H., Oliver, J. D., Dickey, K., and Calcott, K. (1984). Cryosensitivity of *Escherichia coli* and the involvement of cyclopropane fatty acids. *J. Appl. Bacteriol.* **56,** 165–172.

Calcott, P. H., and Rose, A. H. (1982). Freeze–thaw and cold-shock resistance of *Saccharomyces cerevisiae* as affected by plasma membrane lipid composition. *J. Gen. Microbiol.* **128,** 549–555.

Carpenter, J. F., Arakawa, T., and Crowe, J. H. (1992). Interactions of stabilizing additives with proteins during freeze–thawing and freeze–drying. *Dev. Biol. Stand.* **74,** 225–238.

Cerrutti, P., Segovia de Huergo, M., Schebor, C., and del Pilar Buera, M. (2000). Commercial baker's yeast stability as affected by intracellular content of trehalose, dehydration procedure, and the physical properties of external matrices. *Appl. Microbiol. Biotechnol.* **54,** 575–580.

Chao, H., Deluca, C. I., and Davies, P. L. (1995). Mixing antifreeze protein types changes ice crystal morphology without affecting antifreeze activity. *FEBS Lett.* **357,** 183–186.

Chao, H. M., Davies, P. L., and Carpenter, J. F. (1996). Effects of antifreeze proteins on red blood cell survival during cryopreservation. *J. Exp. Biol.* **199,** 2071–2076.

Cheng, C. H. (1998). Evolution of the diverse antifreeze proteins. *Curr. Opin. Genet. Dev.* **8,** 715–720.

Chow, K. C., and Tung, W. L. (1998). Overexpression of dnaK/dnaJ and groEL confers freeze tolerance to *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **253,** 502–505.

Chowdhury, S., Smith, K. W., and Gustin, M. C. (1992). Osmostress and the yeast cytoskeleton: Phenotype-specific suppression of an actin mutation. *J. Cell Biol.* **118,** 561–571.

Clegg, J. S. (1985). The physical properties and metallic status of *Artemia* cysts at low water contents: The ''water replacement hypothesis.''. *In* ''Membranes, metabolism, and dry organisms'' (A. C. Leopold, ed.), pp. 169–187. Cornell Univ. Press, Ithaca, N.Y.

Codon, A. C., Rincon, A. M., Moreno-Mateos, M. A., Delgado-Jarana, J., Rey, M., Limon, C., Rosado, I. V., Cubero, B., Penate, X., Castrejon, F., and Benitez, T. (2003). New *Saccharomyces cerevisiae* baker's yeast displaying enhanced resistance to freezing. *J. Agr. Food Chem.* **51,** 483–491.

Colaco, C., Sen, S., Thangavelu, M., Pinder, S., and Roser, B. (1992). Extraordinary stability of enzymes dried in trehalose: Simplified molecular biology. *Bio-Technology* **10,** 1007–1011.

Colavizza, D., Dumortier, F., Gorwa, M.-F., Lemaire, K., Loiez, A., Teunissen, A., Thevelein, J., Van Dijck, P., and Versele, M. (2000). New stress resistant eukaryotic strains in fermentation and/or growth phase, useful for production of frozen dough and alcoholic drinks. European patent application, Vol. EP99401444.

Coote, P. J., Cole, M. B., and Jones, M. V. (1991). Induction of increased thermotolerance in *Saccharomyces cerevisiae* may be triggered by a mechanism involving intracellular pH. *J. Gen. Microbiol.* **137,** 1701–1708.

Corotto, L. V., Wolber, P. K., and Warren, G. J. (1986). Ice nucleation activity of *Pseudomonas fluorescens:* Mutagenesis, complementation analysis, and identification of a gene product. *EMBO J.* **5,** 231–236.

Coulson, G. E., Morris, G. J., and Smith, D. (1986). A cryomicroscopic study of *Penicillium expansum* hyphae during freezing and thawing. *J. Gen. Microbiol.* **132,** 183–190.

Coutinho, C., Bernardes, E., Felix, D., and Panek, A. D. (1988). Trehalose as a cryoprotectant for preservation of yeast strains. *J. Biotechnol.* **7,** 23–32.

Covarrubias, A. A., Reyes, J. L., Rodrigo, M. J., Campos, F., and Bartels, D. (2001). Hydrophylins, study of their role in protecting enzymatic activities upon *in vitro* water limitation. *2nd International Meeting on Cellular Responses to Oxidative, and Osmotic Stress, Povoa de Varzim, Portugal.*

Craig, E. A., and Gross, C. A. (1991). Is hsp70 the cellular thermometer?. *Trends Biochem. Sci.* **16,** 135–140.

Crowe, J. H., Tablin, F., Wolkers, W. F., Gousset, K., Tsvetkova, N. M., and Ricker, J. (2003). Stabilization of membranes in human platelets freeze-dried with trehalose. *Chem. Phys. Lipids* **122,** 41–52.

D'Amico, S., Claverie, P., Collins, T., Georlette, D., Gratia, E., Hoyoux, A., Meuwis, M.-A., Feller, G., and Gerday, C. (2002). Molecular basis of cold adaptation. *Phil. Trans. R. Soc. Lond.* **2002,** 917–925.

Daum, G., Lees, N. D., Bard, M., and Dickson, R. (1998). Biochemistry, cell biology, and molecular biology of lipids of *Saccharomyces cerevisiae. Yeast* **14,** 1471–1510.

Davidson, R. H., Duncan, S. E., Hackney, C. R., Eigel, W. N., and Boling, J. W. (2000). Probiotic culture survival and implications in fermented frozen yogurt characteristics. *J. Dairy Sci.* **83,** 666–673.

Davies, P. L., and Hew, C. L. (1990). Biochemistry of fish antifreeze proteins. *FASEB J.* **4,** 2460–2468.

Davies, P. L., Baardsnes, J., Kuiper, M. J., and Walker, V. K. (2002). Structure and function of antifreeze proteins. *Phil. Trans. R. Soc. Lond.* **2002,** 927–935.

de Araujo, P. S. (1996). The role of trehalose in stress. *Braz. J. Med. Biol. Res.* **29,** 873–875.

Dequin, S. (2001). The potential of genetic engineering for improving brewing, wine making, and baking yeast. *Appl. Microbiol. Biotechnol.* **56,** 577–588.

Devaldez, G. F., and Diekman, H. (1993). Freeze-drying conditions of starter cultures for sourdoughs. *Cryobiology* **30,** 185–190.

Di Lernia, I., Schiraldi, C., Generoso, M., and De Rosa, M. (2002). Trehalose production at high temperature exploiting an immobilized cell bioreactor. *Extremophiles* **6,** 341–347.

Diniz-Mendez, L., Bernardes, E., de Araujo, P. S., Panek, A. D., and Paschoalin, V. M. F. (1999). Preservation of frozen yeast cells by trehalose. *Biotechn. Bioeng.* **65,** 572–578.

Doucet, D., Tyshenko, M. G., Davies, P. L., and Walker, V. K. (2002). A family of expressed antifreeze protein genes from the moth *Choristoneura fumiferana. Eur. J. Biochem.* **269,** 38–46.

Doucet, D., Tyshenko, M. G., Kuiper, M. J., Graether, S. P., Sykes, B. D., Daugulis, A. J., Davies, P. L., and Walker, V. K. (2000). Structure–function relationships in spruce budworm antifreeze protein revealed by isoform diversity. *Eur. J. Biochem.* **267,** 6082–6088.

Douzou, P. (1982). Developments in low-temperature biochemistry and biology. *Proc. R. Soc. Lond. B. Biol. Sci.* **217,** 1–28.

Driedonks, R. A., Toschka, H. Y., Van Almker, J. W., Schaffers, I. M., and Verbrakel, J. M. A. (1995). Expression and secretion of antifreeze peptides in the yeast *Saccharomyces cerevisiae*. *Yeast* **11,** 849–864.

Dubernet, S., Panoff, J. M., Thammavongs, G., and Gueguen, M. (2002). Nystatin and osmotica as chemical enhancers of the phenotypic adaptation to freeze–thaw stress in *Geotrichum candidum* ATCC 204307. *Int. J. Food Microbiol.* **76,** 215–221.

Duman, J. G. (1982). Insect antifreezes and ice-nucleating agents. *Cryobiology* **19,** 613–627.

Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annu. Rev. Physiol.* **63,** 327–357.

Duman, J. G., and Olsen, T. M. (1993). Thermal hysteresis protein activity in bacteria, fungi, and phylogenetically diverse plants. *Cryobiology* **30,** 322–328.

Edashige, K., Yamaji, Y., Kleinhans, F. W., and Kasai, M. (2003). Artificial expression of aquaporin-3 improves the survival of mouse oocytes after cryopreservation. *Biol. Reprod.* **68,** 87–94.

Elliott, B., and Futcher, B. (1993). Stress resistance of yeast cells is largely independent of cell cycle phase. *Yeast* **9,** 33–42.

Elliott, B., Haltiwanger, R. S., and Futcher, B. (1996). Synergy between trehalose and Hsp104 for thermotolerance in *Saccharomyces cerevisiae*. *Genetics* **144,** 923–933.

Eroglu, A., Russo, M. J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H., and Toner, M. (2000). Intracellular trehalose improves the survival of cryopreserved mammalian cells. *Nat. Biotechnol.* **18,** 163–167.

Estruch, F. (2000). Stress-controlled transcription factors, stress-induced genes, and stress tolerance in budding yeast. *FEMS Microbiol. Rev.* **24,** 469–486.

Farrant, J., Walter, C. A., Lee, H., Morris, G. J., and Clarke, K. J. (1977). Structural and functional aspects of biological freezing techniques. *J. of Microbiol.* **111,** 17–34.

Fernandez Murga, M. L., Cabrera, G. M., Font de Valdez, G., Disalvo, A., and Seldes, A. M. (2000). Influence of growth temperature on cryotolerance and lipid composition of *Lactobacillus acidophilus*. *J. Appl. Microbiol.* **88,** 342–348.

Fletcher, G. L., Goddartd, S. V., and Wu, Y. (1999). Antifreeze proteins and their genes: From basic research to business opportunity. *ChemTech* **30,** 17–28.

Fonseca, F., Beal, C., and Corrieu, G. (2002). Operating conditions that affect the resistance of lactic acid bacteria to freezing and frozen storage. *Cryobiology* **43,** 189–198.

Franks, F., Mathias, S. F., Galfre, P., Webster, S. D., and Brown, D. (1983). Ice nucleation and freezing in undercooled cells. *Cryobiology* **20,** 298–309.

Fuge, E. K. (1997). Stationary phase in the yeast *Saccharomyces cerevisiae*. *In* ''Yeast stress responses'' (S. Hohmann and W. H. Mager, eds.). R. G. Landes Co., Austin, TX.

Fukuse, T., Hirata, T., Nakamura, T., Ueda, M., Kawashima, M., Hitomi, S., and Wada, H. (1999). Role of saccharides on lung preservation. *Transplantation* **68,** 110–117.

Funaki, J., Michigami, Y., Yano, M., Shiraishi, A., Abe, K., and Arai, S. (1996). Applying a bacterial ice nucleation-active protein to preparation of frozen bread dough. *J. Jap. Soc. Food Sci. Technol.* **43,** 835–838.

Garay-Arroyo, A., Colmenero-Flores, J. M., Garciarrubio, A., and Covarrubias, A. A. (2000). Highly hydrophylic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. *J. of Biol. Chem.* **275,** 5668–5674.

Gélinas, P., Fiset, G., LeDuy, A., and Goulet, J. (1989). Effect of growth conditions and trehalose content on cryotolerance of bakers' yeast in frozen doughs. *Appl. Environ. Microbiol* **55,** 2453–2459.

Gélinas, P., Fiset, G., Willemot, C., and Goulet, J. (1991a). Lipid content and cryotolerance of bakers' yeast in frozen doughs. *Appl. Environ. Microbiol.* **57,** 463–468.

Gélinas, P., Toupin, C. J., and Goulet, J. (1991b). Cell water permeability, and cryotolerance of *Saccharomyces cerevisiae*. *Lett. Appl. Microbiol.* **12,** 236–240.

Gerday, C., Aittaleb, M., Arpigny, J. L., Baise, E., Chessa, J. P., Garsoux, G., Petrescu, I., and Feller, G. (1997). Psychrophilic enzymes: A thermodynamic challenge. *Biochim. Biophys. Acta* **1342,** 119–131.

Grandum, S., and Nakagomi, K. (1997). Characteristics of ice slurry containing antifreeze protein for ice storage applications. *J. Thermophys. and Heat Transf.* **11,** 461–466.

Gribbon, E. M., Sen, S. D., Roser, B. J., and Kampinga, J. (1996). Stabilisation of vaccines using trehalose (W-T4) technology. *Dev. Biol. Stand.* **87,** 193–199.

Gross, C., and Watson, K. (1996). Heat shock protein synthesis and trehalose accumulation are not required for induced thermotolerance in depressed *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **220,** 766–772.

Grout, B., Morris, J., and McLellan, M. (1990). Cryopreservation and the maintenance of cell lines. *TIBTECH* **8**.

Gurian-Sherman, D., and Lindow, S. E. (1993). Bacterial ice nucleation: Significance and molecular basis. *FASEB J.* **7,** 1338–1343.

Guy, C. L., Plesofsky-Vig, N., and Brambl, R. (1986). Heat shock protects germinating conidiospores of *Neurospora crassa* against freezing injury. *J. Bacteriol.* **167,** 124–129.

Hagedorn, M., Lance, S. L., Fonseca, D. M., Kleinhans, F. W., Artimov, D., Fleischer, R., Hoque, A. T., Hamilton, M. B., and Pukazhenthi, B. S. (2002). Altering fish embryos with aquaporin-3: An essential step towards successful cryopreservation. *Biol. Reprod.* **67,** 961–966.

Hahn, Y. S., and Kawai, H. (1990). Isolation and characterization of freeze-tolerant yeasts from nature available for the frozen-dough method. *Agric. Biol. Chem.* **54,** 829–831.

Halford, N. G., and Shewry, P. R. (2000). Genetically modified crops: Methodology, benefits, regulation, and public concerns. *Br. Med. Bull.* **56,** 62–73.

Hatano, S., Udou, M., Koga, N., Honjoh, K., and Miyamoto, T. (1996). Impairment of the glycolytic system and actin in baker's yeast during frozen storage. *Biosci. Biotechnol. Biochem.* **60,** 61–64.

Hays, L. M., Feeney, R. E., Tablin, F., Oliver, A. E., Walker, N. J., Crowe, L. M., and Crowe, J. H. (1997). Fish antifreeze glycoproteins protect cellular membranes during lipid-phase transitions. *News Physiol. Sci.* **12,** 189–194.

Hazen, K. C., Bourgeois, L. D., and Carpenter, J. F. (1988). Cryoprotection of antibody by organic solutes and organic solute/divalent cation mixtures. *Arch. Biochem. Biophys.* **267,** 363–371.

Hebraud, M., and Potier, P. (1999). Cold shock response and low temperature adaptation in psychotrophic bacteria. *J. Mol. Microbiol. Biotechnol.* **1,** 211–219.

Hecker, M., and Volker, U. (2001). General stress response of *Bacillus subtilis* and other bacteria. *Adv. Microb. Physiol.* **44,** 35–91.

Hekmat, S., and McMahon, D. J. (1992). Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in ice cream for use as a probiotic food. *J. Dairy Sci.* **75,** 1415–1422.

Hermes-Lima, M., and Storey, K. B. (1993). Antioxidant defenses in the tolerance of freezing and anoxia by garter snakes. *Am. J. Physiol.* **265**.

Hino, A., Mihara, K., Nakashima, K., and Takano, H. (1990). Trehalose levels and survival ratio of freeze-tolerant versus freeze-sensitive yeasts. *Appl. Environ. Microbiol.* **56,** 1386–1391.

Hino, A., Takano, H., and Tanaka, Y. (1987). New freeze-tolerant yeast for frozen dough preparations. *Cereal Chem.* **64,** 269–275.

Hirasawa, R., Yokoigawa, K., Isobe, Y., and Kawai, H. (2001). Improving the freeze tolerance of bakers' yeast by loading with trehalose. *Biosc. Biotech. Biochem.* **65,** 522–526.

Ho, K. H., and Miller, J. J. (1978). Free proline content and sensitivity to dessication and heat during yeast sporulation and spore germination. *Can. J. Microbiol.* **24,** 312–320.

Hohmann, S. (1997). Shaping up: The response of yeast to osmotic stress. *In* ''Yeast stress responses'' (S. Hohmann and W. H. Mager, eds.). R.G. Landes Co., Austin, TX.

Hohmann, S. (2002a). Osmotic adaptation in yeast—Control of the yeast osmolyte system. *Int. Rev. Cytol.* **215,** 149–187.

Hohmann, S. (2002b). Osmotic stress signalling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* **66,** 300–372.

Hohmann, S., and Mager, W. H. (1997). ''Yeast stress responses,'' R. G. Landes Co., Austin, TX.

Holmberg, N., and Bülow, L. (1998). Improving stress tolerance in plants by gene transfer. *Trends Plant Sci.* **3,** 61–66.

Holmberg, N., Lilius, G., and Bulow, L. (1994). Artificial antifreeze proteins can improve NaCl tolerance when expressed in *E. coli*. *FEBS Lett.* **349,** 354–358.

Honadel, T. E., and Killian, G. J. (1988). Cryopreservation of murine embryos with trehalose and glycerol. *Cryobiology* **25,** 331–337.

Honjoh, K., Matsumoto, H., Shimizu, H., Ooyama, K., Tanaka, K., Oda, Y., Takata, R., Joh, T., Suga, K., Miyamoto, T., Lio, M., and Hatano, S. (2000). Cryoprotective activities of group 3 late embryogenesis abundant proteins from *Chlorella vulgaris* C-27. *Biosci. Biotechnol. Biochem.* **64,** 1656–1663.

Honjoh, K., Oda, Y., Takata, R., Miyamoto, T., and Hatano, S. (1999). Introduction of the hiC6 gene, which encodes a homologue of a late embryogenesis abundant (LEA) protein, enhances freezing tolerance of yeast. *J. Plant Physiol.* **155,** 509–512.

Hwang, W. Z., Coetzer, C., Turner, N. E., and Lee, T. C. (2001). Expression of a bacterial ice nucleation gene in a yeast *Saccharomyces cerevisiae* and its possible application in food freezing processes. *J. Agr. Food Chem.* **49,** 4662–4666.

Imai, R., Chang, L., Ohta, A., Bray, E. A., and Takagi, M. (1996). A lea-class gene of tomato confers salt and freezing tolerance when expressed in *Saccharomyces cerevisiae*. *Gene* **170,** 243–248.

Iwahashi, H., Obuchi, K., Fujii, S., and Komatsu, Y. (1995). The correlative evidence suggesting that trehalose stabilizes membrane structure in the yeast *Saccharomyces cerevisiae*. *Cell. Mol. Biol.* **41,** 763–769.

Jia, Z., and Davis, P. L. (2002). Antifreeze proteins, an unusual receptor–ligand interaction. *TIBS* **27,** 101–106.

Joanisse, D., and Storey, K. (1996a). Oxidative stress and antioxidants in overwintering larvae of cold-hardy goldenrod gall insects. *J. Exp. Biol.* **199,** 1483–1491.

Joanisse, D. R., and Storey, K. B. (1996b). Oxidative damage and antioxidants in *Rana sylvatica*, the freeze-tolerant wood frog. *Am. J. Physiol.–Regul. Integr. Comp. Physiol.* **40,** 545–553.

Jones, P. J., and Inouye, M. (1994). The cold-shock response—A hot topic. *Mol. Microbiol.* **11,** 811–818.

Kajiwara, S., Shirai, A., Fujii, T., Toguri, T., Nakamura, K., and Ohtaguchi, K. (1996). Polyunsaturated fatty acid biosynthesis in *Saccharomyces cerevisiae*: Expression of ethanol tolerance and the *FAD2* gene from *Arabidopsis thaliana*. *Appl. Environ. Microbiol.* **62,** 4309–4313.

Kaul, S. C., Obuchi, K., and Komatsu, Y. (1992). Cold shock response of yeast cells: Induction of a 33 kDa protein and protection against freezing injury. *Cell. Mol. Biol.* **38,** 553–559.

Kawahara, H., Koda, N., Oshio, M., and Obata, H. (2000). A cold acclimation protein with refolding activity on frozen denatured enzymes. *Biosci. Biotechnol. Biochem.* **64,** 2668–2674.

Kempf, B., and Bremer, E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol.* **170,** 319–330.

Kim, J., Alizadeh, P., Harding, T., Hefnergravink, A., and Klionsky, D. J. (1996). Disruption of the yeast *ATH1* gene confers better survival after dehydration, freezing, and ethanol shock: Potential commercial applications. *Appl. Environ. Microbiol.* **62,** 1563–1569.

Kim, W. S., and Dunn, N. W. (1997). Identification of a cold shock gene in lactic acid bacteria and the effect of cold shock on cryotolerance. *Curr. Microbiol.* **35,** 59–63.

Kishitani, S., Watanabe, K., Yasuda, S., Arakawa, K., and Takabe, T. (1994). Accumulation of glycine betaine during cold-acclimation and freezing tolerance in leaves of winter and spring barley plants. *Plant Cell and Environ.* **17,** 89–95.

Klein, N., and Lortal, S. (1999). Attenuated starters: An efficient means to influence cheese ripening—A review. *Int. Dairy J.* **9,** 751–762.

Kline, L., and Sugihara, T. F. (1968). Factors affecting the stability of frozen bread doughs. I. Prepared by the straight dough method. *The Bakers Digest* **42,** 44–50.

Ko, R., Smith, L. T., and Smith, G. M. (1994). Glycine betaine confers enhanced osmotolerance and cryotolerance on *Listeria monocytogenes*. *J. Bacteriol.* **176,** 426–431.

Komatsu, Y., Kaul, S. C., Iwahashi, H., and Obuchi, K. (1990). Do heat shock proteins provide protection against freezing? *FEMS Microbiol. Lett.* **72,** 159–162.

Kondo, K., and Inouye, M. (1991). *TIP1*, a cold shock-inducible gene of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266,** 17537–17544.

Kowalski, L. R. Z., Kondo, K., and Inouye, M. (1995). Cold-shock induction of a family of *TIP1*-related proteins associated with the membrane in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **15,** 341–353.

Kozloff, L. M., Schofield, M. A., and Lute, M. (1983). Ice nucleating activity of *Pseudomonas syringae* and *Erwinia herbicola*. *J. Bacteriol.* **153,** 222–231.

Kraakman, L., Lemaire, K., Ma, P., Teunissen, A. W. R. H., Donaton, M. C. V., Van Dijck, P., Winderickx, J., de Winde, J. H., and Thevelein, J. M. (1999). A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Mol. Microbiol.* **32,** 1002–1012.

Kruuv, J., Brailsford, L. L., Glofcheski, D. J., and Lepock, J. R. (1985). Effects of dissolved gasses on freeze–thaw survival of mammalian cells. *Cryo-Lett.* **6,** 233–238.

Kruuv, J., Lepock, J. R., and Keith, A. D. (1978). The effect of fluidity of membrane lipids on freeze–thaw survival of yeast. *Cryobiology* **15,** 73–79.

Laybourn-Parry, J. (2002). Survival mechanisms in Antarctic lakes. *Phil. Trans. R. Soc. Lond.* **2002,** 863–869.

Lee, R. E., Costanzo, J. P., and Mugnano, J. A. (1996). Regulation of supercooling and ice nucleation in insects. *Eur. J. Entomol.* **93,** 405–418.

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., and Crowe, L. M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Appl. Environ. Microbiol.* **61,** 3592–3597.

Lewis, J. G., Learmonth, R. P., Attfield, P. V., and Watson, K. (1997). Stress co-tolerance and trehalose content in baking strains of *Saccharomyces cerevisiae*. *J. Ind. Microbiol. Biotechnol.* **18,** 30–36.

Lewis, J. G., Learmonth, R. P., and Watson, K. (1993a). Role of growth phase and ethanol in freeze–thaw stress resistance of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **59,** 1065–1071.

Lewis, J. G., Learmonth, R. P., and Watson, K. (1994). Cryoprotection of yeast by alcohols during rapid freezing. *Cryobiology* **31,** 193–198.

Lewis, J. G., Learmonth, R. P., and Watson, K. (1995). Induction of heat, freezing, and salt tolerance by heat and salt shock in *Saccharomyces cerevisiae*. *Microbiology* **141,** 687–694.

Lewis, J. G., Northcott, C. J., Learmonth, R. P., Attfield, P. V., and Watson, K. (1993b). The need for consistent nomenclature and assessment of growth phases in diauxic cultures of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **139,** 835–839.

Li, J. K., and Lee, T. C. (1995). Bacterial ice nucleation and its potential application in the food industry. *Trends in Food Sci. Technol.* **6,** 259–265.

Li, X. M., Trinh, K. Y., and Hew, C. L. (1991). Expression and characterization of an active and thermally more stable recombinant antifreeze polypeptide from ocean pout, *Macrozoarces americanus*, in *Escherichia coli:* Improved expression by the modification of the secondary structure of the mRNA. *Protein Eng.* **4,** 995–1002.

Lillford, P. J., and Holt, C. B. (1994). Antifreeze proteins. *J. Food Eng.* **22,** 475–482.

Lillford, P. J., and Holt, C. B. (2002). *In vitro* uses of biological cryoprotectants. *Phil. Trans. R. Soc. Lond.* **2002,** 945–951.

Lindow, S. E. (1983). The role of bacterial ice nucleation in frost injury to plants. *A. Rev. Phytopathol.* **21,** 363–384.

Lindow, S. E., Arny, D. C., and Upper, C. D. (1978). Distribution of ice nucleation- active bacteria on plants in nature. *Appl. Environ. Microbiol.* **36,** 831–838.

Lindquist, S., and Craig, E. A. (1988). The heat-shock proteins. *Annu. Rev. Genet.* **22,** 631–677.

Liou, Y. C., Thibault, P., Walker, V. K., Davies, P. L., and Graham, L. A. (1999). A complex family of highly heterogeneous and internally repetitive hyperactive antifreeze proteins from the beetle *Tenebrio molitor*. *Biochemistry* **38,** 11415–11424.

Lodato, P., de Huergo, M. S., and Buera, M. P. (1999). Viability and thermal stability of a strain of *Saccharomyces cerevisiae* freeze-dried in different sugar and polymer matrices. *Appl. Microbiol. Biotechnol.* **52,** 215–220.

Loewen, M. C., Liu, X., Davies, P. L., and Daugulis, A. J. (1997). Biosynthetic production of type II fish antifreeze protein: Fermentation by *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* **48,** 480–486.

Lonhienne, T., Gerday, C., and Feller, G. (2000). Psychrophilic enzymes: Revisiting the thermodynamic parameters of activation may explain local flexibility. *Biochim. Biophys. Acta* **1543,** 1–10.

Lorenz, K. (1974). Frozen dough, present trend, and future outlook. *The bakers digest* **48,** 14–30.

Los, D. A., and Murata, N. (1999). Responses to cold shock in cyanobacteria. *J. Mol. Microbiol. Biotechnol.* **1,** 221–230.

Lundheim, R. (2002). Physiological and ecological significance of biological ice nucleators. *Phil. Trans. R. Soc. Lond.* **2002,** 937–943.

Mager, W. H., and Moradas Ferreira, P. (1993). Stress response of yeast. *Biochem. J.* **290,** 1–13.

Maki, L. R., Gaylan, E. L., Chang-Chien, M., and Caldwell, D. R. (1974). Ice nucleation induced by *Pseudomonas syringae. Appl. Microbiol.* **28,** 456–459.

Margaritis, A., and Bassi, A. S. (1991). Principles and biotechnological applications of bacterial ice nucleation. *Crit. Rev. Biotechnol.* **11,** 277–295.

Martinez, C. A., Loureiro, M. E., Oliva, M. A., and Maestri, M. (2001). Differential responses of superoxide dismutase in freezing resistant *Solanum curtilobum* and freezing sensitive *Solanum tuberosum* subjected to oxidative and water stress. *Plant Sci.* **160,** 505–515.

Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996). The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *EMBO J.* **15,** 2227–2235.

Mazur, P. (1965). The role of cell membranes in the freezing of yeast and other single cells. *Ann. NY Acad. Sci.* **125,** 658–676.

Mazur, P. (1970). Cryobiology: The freezing of biological systems. *Science* **168,** 939–949.

Mazur, P. (1977). The role of intracellular freezing in the death of cells cooled at supraoptimal rates. *Cryobiology* **14,** 251–272.

Mazur, P., and Schmidt, J. J. (1968). Interactions of cooling velocity, temperature, and warming velocity on the survival of frozen and thawed yeast. *Cryobiology* **5,** 1–17.

McGann, L. E., Yang, H. Y., and Walterson, M. (1988). Manifestations of cell damage after freezing and thawing. *Cryobiology* **25,** 178–185.

McKown, R. L., and Warren, G. J. (1991). Enhanced survival of yeast expressing an antifreeze gene analogue after freezing. *Cryobiology* **28,** 474–482.

Meijer, P. J., Holmberg, N., Grundstrom, G., and Bulow, L. (1996). Directed evolution of a type I antifreeze protein expressed in *Escherichia coli* with sodium chloride as selective pressure and its effect on antifreeze tolerance. *Protein Eng.* **9,** 1051–1054.

Meric, L., Lambertguilois, S., Neyreneuf, O., and Richardmolard, D. (1995). Cryoresistance of baker's yeast *Saccharomyces cerevisiae* in frozen dough—Contribution of cellular trehalose. *Cereal Chem.* **72,** 609–615.

Merritt, P. (1960). The effect of preparation on the stability and performance of frozen, unbaked, yeast-leavened doughs. *The bakers digest* **34,** 57–58.

Metz, M. (1993). Starter cultures—Their industrial manufacture for the meat industry. *Fleischwirtschaft* **73,** 1394–1396.

Miyamoto-Shinohara, Y., Imaizumi, T., Sukenobe, J., Murakami, Y., Kawamura, S., and Komatsu, Y. (2000). Survival rate of microbes after freeze-drying and long-term storage. *Cryobiology* **41,** 251–255.

Moradas-Ferreira, P., Costa, V., Piper, P., and Mager, W. (1996). The molecular defences against reactive oxygen species in yeast. *Mol. Microbiol.* **19,** 651–658.

Mori, Y., Suzuki, H., and Nei, T. (1986). Freezing injury in the yeast respiratory system. *Cryobiology* **23,** 64–71.

Morita, Y., Nakamori, S., and Takagi, H. (2003). L-proline accumulation and freeze tolerance in *Saccharomyces cerevisiae* are caused by a mutation in the *PRO1* gene encoding *gamma*-glutamyl kinase. *Appl. Environ. Microbiol.* **69,** 212–219.

Morris, G. J., Coulson, G. E., and Clarke, K. J. (1998). Freezing injury in *Saccharomyces cerevisiae:* The effect of growth conditions. *Cryobiology* **25,** 471–482.

Morris, G. J., and McGrath, J. J. (1981). Intracellular ice nucleation and gas bubble formation in *Spirogyra*. *Cryo-Lett.* **2,** 341–352.

Mueller, G. M., McKown, R. L., Corotto, L. V., Hague, C., and Warren, G. J. (1991). Inhibition of recrystallization in ice by chimeric proteins containing antifreeze domains. *J. Biol. Chem.* **266,** 7339–7344.

Mueller, G. M., Wolber, P. K., and Warren, G. J. (1990). Clustering of ice nucleation protein correlates with ice nucleation activity. *Cryobiology* **27,** 416–422.

Murakami, Y., Yokoigawa, K., Kawai, F., and Kawai, H. (1996). Lipid composition of commercial bakers' yeasts having different freeze-tolerance in frozen dough. *Biosci. Biotechnol. Biochem.* **60,** 1874–1876.

Murakami, Y., Yokoigawa, K., and Kawai, H. (1995). Lipid composition of freeze-tolerant *Torulaspora delbrueckii* and its freeze-sensitive mutant. *Appl. Microbiol. Biotechnol.* **44,** 167–171.

Murga, M. L., Cabrera, G. M., DeValdez G. F., Disalvo, A., and Seldes, A. M. (2000). Influence of growth temperature on cryotolerance and lipid composition of *Lactobacillus acidophilus*. *J. Appl. Microbiol.* **88,** 342–348.

Myers, D. K., and Attfield, P. V. (1999). Intracellular concentration of exogenous glycerol in *Saccharomyces cerevisiae* provides for improved leavening of frozen sweet doughs. *Food Microbiol.* **16,** 45–51.

Nagodawithana, T. W., and Trivedi, N. B. (1990). Yeast selection for baking. *In* ''Yeast strain selection'' (C. J. Panchal, ed.), pp. 139–184. Marcel Dekker, Inc., New York.

Nakagawa, S., and Ouchi, K. (1994a). Construction from a single parent of baker's yeast strains with high freeze tolerance and fermentative activity in both lean and sweet doughs. *Appl. Environ. Microbiol.* **60,** 3499–3502.

Nakagawa, S., and Ouchi, K. (1994b). Improvement of freeze tolerance of commercial baker's yeast in dough by heat-treatment before freezing. *Biosci. Biotechnol. Biochem.* **58,** 2077–2079.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999). Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. *FEBS Lett.* **461,** 205–210.

Nevoigt, E., and Stahl, U. (1997). Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **21,** 231–241.

Neyreneuf, O., and Delpuech, B. (1993). Freezing experiments on yeasted dough slabs—Effects of cryogenic temperatures on the baking performance. *J. Food Engin.* **45,** 139–145.

Neyreneuf, O., and Van Der Plaat, J. B. (1991). Preparation of frozen French bread dough with improved stability. *Cereal Chem.* **68,** 60–66.

Nichols, D. S., Nichols, P. D., and McMeekin, T. A. (1993). Polyunsaturated fatty-acids in Antarctic bacteria. *Antarctic Sci.* **5,** 149–160.

Obata, H., Muryoi, N., Kawahara, H., Yamade, K., and Nishikawa, J. (1999). Identification of a novel ice-nucleating bacterium of Antarctic origin and its ice nucleation properties. *Cryobiology* **38,** 131–139.

Oda, Y., Kazuo, U., and Ohta, S. (1986). Selection of yeasts for breadmaking by the frozen-dough method. *Appl. Environ. Microbiol.* **52,** 941–943.

Oda, Y., and Tonomura, K. (1993). Selection of a novel baking strain from the *Torulaspora* yeasts. *Biosc. Biotechn. Biochem.* **57,** 1320–1322.

Panek, A. D. (1995). Trehalose metabolism—New horizons in technological applications. *Braz. J. Med. Biol. Res.* **28,** 169–181.

Panoff, J. M., Thammavongs, B., and Gueguen, M. (2000a). Cryoprotectants lead to phenotypic adaptation of freeze–thaw stress in *Lactobacillus delbrueckii ssp. bulgaricus* CIP 101027T. *Cryobiology* **40,** 264–269.

Panoff, J. M., Thammavongs, B., and Gueguen, M. (2000b). Cryotolerance and cold stress in lactic acid bacteria. *Sciences des Aliments* **20,** 105–110.

Panoff, J. M., Thammavongs, B., Gueguen, M., and Boutibonnes, P. (1998). Cold stress responses in mesophilic bacteria. *Cryobiology* **36,** 75–83.

Park, J., Grant, C. M., Davies, M. J., and Dawes, I. W. (1998). The cytoplasmatic Cu, Zn superoxide dismutase of *Saccharomyces cerevisiae* is required for resistance to freeze–thaw stress. *J. Biol. Chem.* **36,** 22921–22928.

Park, J. I., Grant, C. M., Attfield, P. V., and Dawes, I. W. (1997). The freeze–thaw stress response of the yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the RAS-cyclic AMP signal. *Appl. Environ. Microbiol.* **63,** 3818–3824.

Peters, I. D., Hew, C. L., and Davies, P. L. (1989). Biosynthesis of winter flounder antifreeze proprotein in *E. coli*. *Protein Eng.* **3,** 145–151.

Petersohn, A., Brigulla, M., Haas, S., Hoheisel, J. D., Volker, U., and Hecker, M. (2001). Global analysis of the general stress response of *Bacillus subtilis*. *J. Bacteriol.* **183,** 5617–5631.

Phadtare, S., Alsina, J., and Inouye, M. (1999). Cold-shock response and cold-shock proteins. *Curr. Opin. Microbiol.* **2,** 175–180.

Phelps, P., Giddings, T. H., Prochoda, M., and Fall, R. (1986). Release of cell-free ice nuclei by *Erwinia herbicola*. *J. Bacteriol.* **167,** 496–502.

Piper, P. W. (1993). Molecular events associated with acquisition of heat tolerance by the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **11,** 339–355.

Plesset, J., Ludwig, J. R., Cox, B. S., and McLaughlin, C. S. (1987). Effect of cell cycle position on thermotolerance in *Saccharomyces cerevisiae*. *J. Bacteriol.* **169,** 779–784.

Pouleur, S., Richard, C., Martin, J. G., and Antoun, H. (1992). Ice nucleation activity in *Fusarium acuminatum* and *Fusarium avenaceum*. *Appl. Environ. Microbiol.* **58,** 2960–2964.

Price, P. B. (2000). A habitat for psychrophiles in deep Antarctic ice. *Proc. Natl. Acad. Sci. USA* **97,** 1247–1251.

Randez-Gil, F., Sanz, P., and Prieto, J. A. (1999). Engineering baker's yeast: Room for improvement. *Trends Biotechnol.* **17,** 237–244.

Rayos, A. A., Takahashi, Y., Hishinuma, M., and Kanagawa, H. (1994). Quick freezing of unfertilized mouse oocytes using ethylene glycol with sucrose or trehalose. *J. Reprod. Fertil.* **100,** 123–129.

Ribotta, P. D., Leon, A. E., and Anon, M. C. (2001). Effect of freezing and frozen storage of doughs on bread quality. *J. Agric. Food Chem.* **49,** 913–918.

Rodriguez-Vargas, S., Estruch, F., and Randez-gil, F. (2002). Gene expression analysis of cold and freeze stress in baker's yeast. *Appl. Environ. Microbiol.* **68,** 3024–3030.

Römisch, K., and Matheson, T. (2003). Cell biology in the Antarctic: Studying life in the freezer. *Nature Cell Biol.* **4,** 3–6.

Rose, A. H., and Vijayalakshmi, G. (1993). Baker's yeasts. *In* ''Yeast technology'' Vol.5. (A. H. Rose and J. S.Harrison, Eds.), pp. 357–397. Academic Press, New York.

Rotert, K. R., Toste, A. P., and Steiert, J. G. (1993). Membrane fatty acid analysis of Antarctic bacteria. *FEMS Microbiol. Lett.* **114,** 253–257.

Rubinsky, B., Arav, A., and Fletcher, G. L. (1991). Hypothermic protection—A fundamental property of ''antifreeze'' proteins. *Biochem. Biophys. Res. Comm.* **180,** 566–571.

Rubinsky, B., Arav, A., Mattioli, M., and Devries, A. L. (1990). The effect of antifreeze glycopeptides on membrane potential changes at hypothermic temperatures. *Biochem. Biophys. Res. Comm.* **173,** 1369–1374.

Rudolph, A. S., and Crowe, J. H. (1985). Membrane stabilization during freezing: The role of two natural cryoprotectants, trehalose and proline. *Cryobiology* **22,** 367–377.

Russell, N. J. (1990). Cold adaptation of microorganisms. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **326,** 595–608.

Sahara, T., Goda, T., and Ohgiya, S. (2002). Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. *J. Biol. Chem.* in press.

Sakamoto, A., Valverde, R., Alia, Chen, T. H., and Murata, N. (2000). Transformation of *Arabidopsis* with the *codA* gene for choline oxidase enhances freezing tolerance of plants. *Plant J.* **22,** 449–453.

Sano, F., Asakawa, N., Inoue, Y., and Sakurai, M. (1999). A dual role for intracellular trehalose in the resistance of yeast cells to water stress. *Cryobiology* **39,** 80–87.

Santoro, N., and Thiele, D. J. (1997). Oxidative stress responses in the yeast *Saccharomyces cerevisiae*. *In* ''Yeast stress responses'' (S. Hohmann and W. H. Mager, eds.). R. G. Landes Co., Austin TX.

Schenberg-Frascino, A., and Moustacchi, E. (1972). Lethal and mutagenic effects of elevated temperature on haploid yeast. *Mol. Gen. Genet.* **115,** 243–257.

Schiraldi, C., Di Lernia, I., and De Rosa, M. (2002). Trehalose production: Exploiting novel approaches. *Trends in Biotechnol.* **20,** 420–425.

Schmidt, G., and Zink, R. (2000). Basic features of the stress response in three species of bifidobacteria: *B. longum, B. adolescentis*, and *B. breve*. *Int. J. Food Microbiol.* **55,** 41–45.

Schwartz, G. J., and Diller, K. R. (1983a). Osmotic response of individual cells during freezing. I. Experimental volume measurements. *Cryobiology* **20,** 61–77.

Schwartz, G. J., and Diller, K. R. (1983b). Osmotic response of individual cells during freezing. II. Membrane permeability analysis. *Cryobiology* **20,** 542–552.

Serrano, R., Marquez, J. A., and Rios, G. (1997). Crucial factors in salt stress tolerance. *In* ''Yeast stress responses'' (S. Hohmann and W. H. Mager, eds.). R. G. Landes Co., Austin TX.

Sheu, T. Y., Marshall, R. T., and Heymann, H. (1993). Improving survival of culture bacteria in frozen desserts by microentrapment. *J. Dairy Sci.* **76,** 1902–1907.

Shima, J., Hino, A., Yamada-Yyo, C., Suzuki, Y., Nakajima, R., Watanabe, H., Mori, K., and Takano, H. (1999). Stress tolerance in doughs of *Saccharomyces cerevisiae* trehalase mutants derived from commercial baker's yeast. *Appl. Environ. Microbiol.* **65,** 2841–2846.

Shima, J., Sakata-Tsuda, Y., Suzuki, Y., Nakajima, R., Watanabe, H., Kawamoto, S., and Takano, H. (2003). Disruption of the *CARI* gene encoding arginase enhances freeze tolerance of the commercial baker's yeast *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **69,** 715–718.

Siderius, M., and Mager, W. H. (1997). General stress response: In search of a common denominator. *In* ''Yeast stress responses'' (S. Hohmann and W. H. Mager, eds.). R. G. Landes Co., Austin TX.

Singer, M. A., and Lindquist, S. (1998a). Multiple effects of trehalose on protein folding *in vitro* and *in vivo*. *Mol. Cell* **1,** 639–648.

Singer, M. A., and Lindquist, S. (1998b). Thermotolerance in *Saccharomyces cerevisiae*: The Yin and Yang of trehalose. *TIB Tech.* **16,** 460–467.

Solomon, R. G., and Appels, R. (1999). Stable, high-level expression of a type I antifreeze protein in *Escherichia coli*. *Protein Expr. Purif.* **16,** 53–62.

Soto, T., Fernandez, J., Vicente-Soler, J., Cansado, J., and Gacto, M. (1999). Accumulation of trehalose by overexpression of *tps1*, coding for trehalose-6-phosphate synthase, causes increased resistance to multiple stresses in the fission yeast *Schizosaccharomyces pombe*. *Appl. Environ. Microbiol.* **65,** 2020–2024.

Souzu, H. (1982). Escherichia coli B membrane stability related to growth phase. Measurement of temperature-dependent physical state change of the membrane over a wide range. *Biochim. Biophys. Acta* **691,** 161–170.

Souzu, H. (1986a). Fluorescence polarization studies on *Escherichia coli* membrane stability and its relation to the resistance of the cell to freeze–thawing. I. Membrane stability in cells of different growth phase. *Biochim. Biophys. Acta* **861,** 353–360.

Souzu, H. (1986b). Fluorescence polarization studies on *Escherichia coli* membrane stability and its relation to the resistance of the cell to freeze–thawing. II. Stabilization of the membranes by polyamines. *Biochim. Biophys. Acta.* **861,** 361–367.

Souzu, H. (1989a). Changes in chemical structure and function in *Escherichia coli* cell membranes caused by freeze–thawing. I. Change of lipid state in bilayer vesicles and in the original membrane fragments depending on rate of freezing. *Biochim. Biophys. Acta* **978,** 105–111.

Souzu, H. (1989b). Changes in chemical structure and function in *Escherichia coli* cell membranes caused by freeze–thawing. II. Membrane lipid state and response of cells to dehydration. *Biochim. Biophys. Acta* **978,** 112–118.

Spencer, J. F. T., and Spencer, D. M. (1996). Isolation and identification of yeasts from natural habitats. *In* ''Yeast protocols'' (I. H. Evans, ed.), pp. 1–4. Humana Press, Totowa, NJ.

Spencer, J. F. T., and Spencer, D. M. (1997). Ecology: Where yeasts live. *In* ''Yeasts in natural and artificial habitats'' (J. F. T. Spencer and D. M. Spencer, eds.), pp. 33–58. Springer-Verlag, Berlin Heidelberg, Germany.

Stead, D., and Park, S. F. (2000). Roles of Fe superoxide dismutase and catalase in resistance of *Campylobacter coli* to freeze–thaw stress. *Appl. Environ. Microbiol.* **66,** 3110–3112.

Steponkus, P. L. (1984). Role of the plasma membrane in freezing injury and cold acclimation. *Ann. Rev. Plant Physiol.* **35,** 543–584.

Steponkus, P. L., and Dowgert, M. F. (1981). Gas bubble formation during intracellular ice formation. *Cryo-Lett.* **2,** 42–47.

Steponkus, P. L., and Lynch, D. V. (1989). Freeze/thaw-induced destabilization of the plasma membrane and the effects of cold acclimation. *J. Bioenerg. Biomembr.* **21,** 21–41.

Suga, K., Honjoh, K., Furuya, N., Shimizu, H., Nishi, K., Shinohara, F., Hirabaru, Y., Maruyama, I., Miyamoto, T., Hatano, S., and Lio, M. (2002). Two low-temperature-inducile *Chlorella* genes from delta12 and omega-3 fatty acid desaturase (FAD): Isolation of delta12 and omega-3 fad cDNA clones, expression of delta12 fad in *Saccharomyces cerevisiae*, and expression of omega-3 fad in *Nicotiana tabacum*. *Biosci. Biotechnol. Biochem.* **66,** 1314–1327.

Suutari, M., Liukkonen, K., and Laakso, S. (1990). Temperature adaptation in yeasts: The role of fatty acids. *J. Gen. Microbiol.* **136,** 1469–1474.

Swan, T. M., and Watson, K. (1998). Stress tolerance in a yeast sterol auxotroph: Role of ergosterol, heat shock proteins, and trehalose. *FEMS Microbiol. Lett.* **169,** 191–197.

Swan, T. M., and Watson, K. (1999). Stress tolerance in a yeast lipid mutant: Membrane lipids influence tolerance to heat and ethanol independently of heat shock proteins and trehalose. *Can. J. Microbiol.* **45,** 472–479.

Swire-Clark, G. A., and Marcotte, W. R. J. (1999). The wheat LEA protein Em functions as an osmoprotective molecule in *Saccharomyces cerevisiae*. *Plant Mol. Biol.* **39,** 117–128.

Tablin, F., Oliver, A. E., Walker, N. J., Crowe, L. M., and Crowe, J. H. (1996). Membrane phase transition of intact human platelets: Correlation with cold-induced activation. *J. Cell. Physiol.* **168,** 305–313.

Takagi, H., Iwamoto, F., and Nakamori, S. (1997). Isolation of freeze-tolerant laboratory strains of *Saccharomyces cerevisiae* from proline-analogue-resistant mutants. *Appl. Microbiol. Biotechnol.* **47,** 405–411.

Takagi, H., Sakai, K., Morida, K., and Nakamori, S. (2000). Proline accumulation by mutation or disruption of the proline oxidase gene improves resistance to freezing and dessication stresses in *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **184,** 103–108.

Tamás, M. J., Luyten, K., Sutherland, F. C. W., Hernandez, A., Albertyn, J., Valadi, H., Li, H., Prior, B. A., Killan, S. G., Ramos, J., Gustafsson, L., Thevelein, J. M., and Hohmann, S. (1999). Fpslp controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol. Microbiol.* **31,** 1087–1104.

Tanghe, A., Van Dijck, P., Dumortier, F., Teunissen, A., Hohman, S., and Thevelein, J. M. (2002). Aquaporin expression correlates with freeze tolerance in yeast and overexpression improves freeze tolerance in industrial yeast. *Appl. Environ. Microbiol.* **68,** 5981–5989.

Teunissen, A., Dumortier, F., Gorwa, M.-F., Bauer, J., Tanghe, A., Smet, P., Van Dijck, P., and Thevelein, J. M. (2002). A freeze-tolerant diploid derivative of an industrial baker's yeast strain: Isolation, characterization, and use in frozen doughs. *Appl. Environ. Microbiol.* **68,** 4780–4787.

Thammavongs, B., Corroler, D., Panoff, J. M., Auffray, Y., and Boutibonnes, P. (1996). Physiological response of *Enterococcus faecalis* JH2-2 to cold shock: Growth at low temperatures and freezing/thawing challenge. *Lett. Appl. Microbiol.* **23,** 398–402.

Thammavongs, B., Panoff, J. M., and Gueguen, M. (2000). Phenotypic adaptation to freeze–thaw stress of the yeast-like fungus *Geotrichum candidum*. *Int. J. Food Microbiol.* **60,** 99–105.

Thevelein, J. M. (1996). Regulation of trehalose metabolism and its relevance to cell growth and function. *In* ''The Mycota III: Biochemistry, and molecular biology'' (Branbl/Marzluf, ed.), pp. 395–420. Springer-Verlag, Berlin-Heidelberg, Germany.

Thevelein, J. M., Cauwenberg, L., Colombo, S., de Winde, J. H., Donaton, M., Dumortier, F., Kraakman, L., Lemaire, K., Ma, P., Nauwelaers, D., Rolland, F., Teunissen, A., Van Dijck, P., Versele, M., Wera, S., and Winderickx, J. (2000). Nutrient-induced signal transduction through the protein kinase A pathway and its role in the control of metabolism, stress resistance, and growth in yeast. *Enzyme Microbial Technol.* **26,** 819–825.

Thieringer, H. A., Jones, P. G., and Inouye, M. (1998). Cold shock and adaptation. *BioEssays* **20,** 49–57.

Thomashow, M. F. (1998). Role of cold-responsive genes in plant freezing tolerance. *Plant Physiol.* **118,** 1–7.

Tibett, M., Sanders, F. E., and Cairney, J. W. (2002). Low-temperature-induced changes in trehalose, mannitol, and arabitol associated with enhanced tolerance to freezing in ectomycorrhizal basidiomycetes (*Hebeloma* spp.). *Mycorrhiza* **12,** 249–255.

Tomczak, M. M., Hincha, D. K., Estrada, S. D., Wolkers, W. F., Crowe, L. M., Feeney, R. E., Tablin, F., and Crowe, J. H. (2002). A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. *Biophys. J.* **82,** 874–881.

Toner, M., Cravalho, E. G., and Karel, M. (1993). Cellular response of mouse oocytes to freezing stress: Prediction of intracellular ice formation. *J. Biochem. Eng.* **115,** 169–174.

Tsien, H., Panos, C., Shockman, G. D., and Higgins, M. L. (1980). Evidence that *Streptococcus mutans* constructs its membrane with excess fluidity for survival at suboptimal temperatures. *J. Gen. Microbiol.* **121,** 105–111.

Upper, C. D., and Vali, G. (1995). The discovery of bacterial ice nucleation and its role in the injury of plants by frost. *In* ''Biological ice nucleation and its applications'' (R. E. Lee. Jr., G. J. Warren, and L. V. Gusta, eds.), pp. 29–39. APS Press, St. Paul, MN.

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S. D., and Maguin, E. (2002). Stress responses in lactic acid bacteria. *Antonie Van Leeuwenhoek* **82,** 187–216.

Van Dijck, P., Colavizza, D., Smet, P., and Thevelein, J. M. (1995). Differential importance of trehalose in stress resistance in fermenting and nonfermenting *Saccharomyces cerevisiae* cells. *Appl. Environ. Microbiol.* **61,** 109–115.

Van Dijck, P., Gorwa, M.-F., Lemaire, K., Teunissen, A., Versele, M., Colombo, S., Dumortier, F., Ma, P., Tanghe, A., Loïez, A., and Thevelein, J. M. (2000a). Characterization of a new set of mutants deficient in fermentation-induced loss of stress resistance for use in frozen dough applications. *Int. J. Food Microbiol.* **55,** 187–192.

Van Dijck, P., Ma, P., Versele, M., Gorwa, M.-F., Colombo, S., Lemaire, K., Bossi, D., Loïez, A., and Thevelein, J. M. (2000b). A baker's yeast mutant (*fill*) with a specific, partially inactivating mutation in adenylate cyclase maintains a high stress resistance during active fermentation and growth. *J. Mol. Microbiol. Biotechnol.* **2,** 521–530.

Van Laere, A. (1986). Resistance of germinating *Phycomyces* spores to dessication, freezing, and acids. *FEMS Microbiol. Ecol.* **38,** 251–256.

Van Laere, A. (1989). Trehalose, reserve and/or stress metabolite? *FEMS Microbiol. Rev.* **63,** 201–210.

Volker, U., Maul, B., and Hecker, M. (1999). Expression of the sigmaB-dependent general stress regulon confers multiple stress resistance in *Bacillus subtilis*. *J. Bacteriol.* **181,** 3942–3948.

Walker, D. C., Girgis, H. S., and Klaenhammer, T. R. (1999). The groESL chaperone operon of *Lactobacillus johnsonii*. *Appl. Environ. Microbiol.* **65,** 3033–3041.

Wang, Z. J., and Ponte, J. G. (1994). Improving frozen dough qualities with the addition of vital wheat gluten. *Cereal Foods World* **39,** 500–503.

Watson, K. (1987). Temperature relations. *In* ''The yeasts'', Vol. 2, pp. 41–71. Academic Press Inc., London, UK.

Welsh, D. T. (2000). Ecological significance of compatible solute accumulation by microorganisms: From single cells to global climate. *FEMS Microbiol. Rev.* **24,** 263–290.

Werner-Washburne, M., Braun, E., Johnston, G. C., and Singer, R. A. (1993). Stationary phase in the yeast *Saccharomyces cerevisiae*. *Microbiol. Rev.* **57,** 383–401.

Wharton, D. A., Goodall, G., and Marshall, C. J. (2003). Freezing survival and cryoprotective dehydration as cold tolerance mechanisms in the Antarctic nematode *Panagrolaimus davidi*. *J. Exp. Biol.* **206,** 215–221.

Wiemken, A. (1990). Trehalose in yeast, stress protectant rather than reserve carbohydrate? *Antonie van Leeuwenhoek* **58**, 209–217.

Willimsky, G., Bang, H., Fischer, G., and Marahiel, M. A. (1992). Characterization of *cspB*, a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperatures. *J. Bacteriol.* **174,** 6326–6335.

Wolfe, J., Dowgert, M. F., and Steponkus, P. L. (1985). Dynamics of membrane exchange of the plasma membrane and the lysis of isolated protoplasts during rapid expansion in area. *J. Membr. Biol.* **86,** 127–138.

Wolkers, W. F., Walker, N. J., Tablin, F., and Crowe, J. H. (2001). Human platelets loaded with trehalose survive freeze-drying. *Cryobiology* **42,** 79–87.

Wouters, J. A., Rombouts, F. M., Kuipers, O. P., de Vos, W. M., and Abee, T. (2000). The role of cold-shock proteins in low-temperature adaptation of food-related bacteria. *Syst. Appl. Microbiol.* **23,** 165–173.

Wu, Y., and Fletcher, G. L. (2001). Efficacy of antifreeze protein types in protecting liposome membrane integrity depends on phospholipid class. *Biochim. Biophys. Acta* **1524,** 11–16.

Xin, Z., and Browse, J. (1998). Eskimol mutants of *Arabidopsis* are constitutively freezing-tolerant. *Proc. Natl. Acad. Sci. USA* **95,** 7799–7804.

Xing, W., and Rajashekar, C. B. (2001). Glycine betaine involvement in freezing tolerance and water stress in *Arabidopsis thaliana*. *Environ. Exp. Bot.* **46,** 21–28.

Yamashita, Y., Nakamura, N., Omiya, K., Nishikawa, J., Kawahara, H., and Obata, H. (2002). Identification of an antifreeze lipoprotein from *Moraxella* sp. of Antarctic origin. *Biosc. Biotechnol. Biochem.* **66,** 239–247.

Yokoigawa, K., Murakami, Y., and Kawai, H. (1995). Trehalase activity and trehalose content in a freeze-tolerant yeast, *Torulaspora delbrueckii*, and its freeze-sensitive mutant. *Biosci. Biotechnol. Biochem.* **59,** 2143–2145.

Yoshiba, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1997). Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol.* **38,** 1095–1102.

Zachariassen, K. E., and Kristiansen, E. (2000). Ice nucleation and antinucleation in nature. *Cryobiology* **41,** 257–279.

Zavaglia, G., Disalvo, E. A., and De Antoni, G. L. (2000). Fatty acid composition and freeze–thaw resistance in *lactobacilli*. *J. Dairy Res.* **67,** 241–247.

Zhang, L., Ohta, A., Takagi, M., and Imai, R. (2000). Expression of plant group 2 *lea* genes in *Saccharomyces cerevisiae* revealed functional divergence among LEA proteins. *J. Biochem.* **127,** 611–616.

Fungal Osmotolerance

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I. Introduction

Osmotolerant fungi are those fungal species adapted to environments of low water activity, being found in environments where water is limited or where water availability is lowered owing to the presence of high concentrations of environmental solutes. Solutes which determine the distribution of osmotolerant fungi fall into two groupings. First, ionic solutes are the most widespread environmentally and include most commonly the cations sodium, potassium, calcium, and magnesium with the counteranions chloride, sulfate, carbonate, and bicarbonate (Flowers *et al.*, 1986). Ionically dominated environments include marine ecosystems, which cover perhaps 70% of global surface area and are dominated by sodium and chloride at around 500 mM, and a range of terrestrial environments including inland seas, salt marshes, salt pans, and salt flats, where K^+, Mg^{2+}, Ca^{2+}, $CO_3{}^{2-}$, $HCO_3{}^-$, , and $SO_4{}^{2-}$ salts can also be common (Flowers *et al.*, 1986). Within such environments, specialized communities have grown up composed of organisms with adaptations to high ionic concentrations. Second, high concentrations of organic solutes also present problems for the growth of many organisms. Such situations are frequent where

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carbohydrate concentrations are high, particularly of sugars or carbohydrates, for example, in foodstuffs such as fruit products. Yeasts and filamentous fungi are one of the few groups able to grow at low water activity in the presence of high sugar concentrations (Hocking, 1993). Areas characterized as lacking water also present problems to fungal growth; these include arid zones which comprise perhaps 30% of terrestrial land surface area (Flowers *et al.*, 1986) and polar regions where water is locked away as ice.

Osmotolerance refers to mechanisms that have evolved for survival in osmotically challenging environments, and a number of terms have been used to describe organisms living in such environments. ''Halophile'' is used to describe a fungal organism adapted to highly saline or ionic conditions, while ''xerophile'' is used for a fungus adapted to low water activity (Hocking, 1993). In each case, adaptation has occurred giving tolerance to highly negative external water potentials. The term ''osmophile'' is used as a general term for any organism adapted to negative water potential/low water activity. Frequently, the term ''stress'' is used to describe any environmental parameter which might be problematic to biological function, with the expression ''salt stress'' widely used. Some caution must be expressed in the appropriateness of such terminology; a fungus adapted to and successfully growing in a highly osmotic environment will not exhibit the stress physiologies that a nonadapted species would and therefore is unlikely to be stressed.

Fungi can be divided into five osmotic response groupings, based upon their ability to survive at different medium water potentials. The first two groupings include most mesophilic fungi, including coprophiles, wood decay fungi, and fungi of the phylloplane, able perhaps to survive down to external water potentials of around −5 Mpa. Many soil fungi and marine fungi can survive external water potentials down to about −20 Mpa, while many aspergilli, penicillia, and some osmophilic yeasts (e.g., *Zygosaccharomyces rouxii*) may survive down to −40 Mpa (Griffin, 1994). In the late 1990s, there have been reports of a number of fungal species being isolated from the Dead Sea, a hypersaline environment dominated by the cations sodium (0.67M), magnesium (1.67M), calcium (0.42M), and potassium (0.17M), together with chloride (5.97M) giving an A_w of 0.67. Species isolated from the Dead Sea include the ascomycete *Gymnascella marismortui* and the deuteromycetes *Ulocladium chlamydosporum* and *Penicillium westlingii* (Buchalo *et al.*, 1998).

The most systematically studied group of osmophiles at an ecological level is the marine fungi (Moss, 1986; Clipson and Jennings, 1992; Jones and Mitchell, 1996). These are fungi defined as being able

to complete their life cycles in marine ecosystems (Kohlmeyer and Kohlmeyer, 1979), implying that tolerance has to be manifested at all life cycle stages for such a species to be successful in the marine environment. This does not appear to preclude transient terrestrial species being found in marine ecosystems, although it is unclear whether populations are maintained by sporulation or vegetatively. An interesting example is a strain of *Aspergillus sydowii* which causes mortality of the Caribbean sea fan *Gorgonia ventalina*, with disease-causing populations maintained by continual influx of vegetative material from neighboring terrestrial environments (Geiser *et al*., 1998).

At present, it is not clear whether osmotolerance is more common within particular fungal phyla or whether it is distributed widely through the fungal kingdom. In a survey of European marine fungal species, Clipson *et al*. (2001) found that 67% of species were Ascomycetes and 26% were Deuteromycetes, suggesting that salt tolerance may be particularly prevalent in the Ascomycotina. Although the marine habit appears to be rarely found among the terrestrially diverse basidiomycetes (Jones and Mitchell, 1996), 4% of marine species were Zygomycetes. Ecologically, caution must be expressed in terms of the extent of osmotolerant fungal diversity because there have been few systematic fungal surveys of osmotically challenging environments. Furthermore, total fungal diversity remains greatly underestimated (Hawksworth, 2001).

The focus of this chapter is the molecular mechanisms which confer osmotolerance in fungi. The basic physiologies involved will be briefly overviewed, but the main emphasis will revolve around evaluating recent advances in our understanding of fungal osmotic responses at the genetic level.

II. Physiological Mechanisms of Osmotolerance

Physiological mechanisms conferring fungal osmotolerance have been extensively reviewed (see Clipson and Jennings, 1992; Blomberg and Adler, 1993; Jennings, 1995). Detailed studies have largely been restricted to a few representative fungal model species, including the ascomycetous yeast *Saccharomyces cerevisiae*, the marine yeast *Debaryomyces hansenii*, the marine hyphomycete *Dendryphiella salina*, and the ascomycete *Aspergillus nidulans*, together with less studied species such as *Z. rouxii*. *S. cerevisiae* forms the most complete model for fungal salt tolerance at both physiological and genetic levels, based upon its ease of physiological and genetic manipulation. Nevertheless, *S. cerevisiae* is not a particularly osmotolerant species, and

caution must be exercised in using it as a proxy for species showing moderate or high levels of halo- and osmotolerance.

In fungi not adapted to osmotic environments, growth inhibition occurs at quite moderate solute concentrations. For example, Magan *et al.* (1995) found that in the basidiomycete *Agaricus bisporus* grown at different KCl concentrations, mycelial growth was optimal at osmotic potentials between −0.5 and −1.0 Mpa (equivalent to approximately 100 to 200 mM). In contrast, for the xerophile *Aspergillus ochraceus*, growth was maximal at around −10 Mpa, regardless of whether the external solute was NaCl or glycerol, although growth was higher when the osmoticum was glycerol (Ramos *et al.*, 1999). In the marine fungus *D. salina*, mycelial dry weight was maximal at 200 mM NaCl (Clipson and Jennings, 1990).

Central to cellular osmotolerance is the maintenance of osmotic gradients across the fungal cell membrane to maintain inwardly directed water transport, so that cellular water potentials are more negative than those in the external environment (Clipson and Jennings, 1992). Fungal cells are generally walled and turgor maintenance is essential for growth, turgor being a driving force for apical growth and expansion (Money, 1994, 1997). Cellular water potentials are generated through the accumulation and/or synthesis of osmotically active solutes by the cell. In natural environments, external water potentials are rarely stable, so that fungi adapted to these conditions presumably are able to undergo osmotic adjustment processes, either through changes in solute uptake or in intracellular synthesis rates of osmotically active solutes, in order to survive such situations. There have been some studies that have measured fungal water potential in response to osmotic agents. In the marine hyphomycete *D. salina*, Clipson and Jennings (1990) measured water and osmotic potentials in mycelium growing at different NaCl concentrations, with representative data included in Table I.

Although there have been few studies, fungal cellular metabolism appears to be inhibited by ionic agents. In *D. salina*, malate dehydrogenase and NAD-linked glycerol dehydrogenase both showed substantial inhibition *in vitro* at quite low NaCl (100 mM) concentrations (Gibb *et al.*, 1986; Paton and Jennings, 1988), while cell-free extracts of the halotolerant yeast *Pichia miso* were completely inhibited by NaCl for glucose consumption and glycerol production (Onishi, 1963). Some caution is necessary in interpreting *in vitro* enzyme assays since conditions may be radically different from *in vivo* in terms of protein concentration, pH, and counteranion. Additionally, protein production may possibly be up-regulated to overcome ion inhibitory effects. For example, Nilsson and Adler (1990) found that although

TABLE I

OSMOTIC ADJUSTMENT IN THE MARINE HYPHOMYCETE *DENDRYPHIELLA SALINA* GROWING IN THE ABSENCE OF SALT (0 mM NaCl) AND AT SEAWATER CONCENTRATION (500 mM NaCl). INTRACELLULAR ION CONCENTRATIONS, COMPARTMENTAL VOLUME FRACTIONS, AND CELLULAR WATER POTENTIALS

$[NaCl]_o$	Ψ_w	Cytoplasm	Vacuole	Wall
0 mM	−0.25			
Na		21	14	10
K		164	89	150
Cl		33	24	37
V_f		88%	12%	
500 mM	−2.3			
Na		173	199	172
K		110	86	74
Cl		199	173	210
V_f		83%	17%	

Compartmental concentrations are expressed as mM on a tissue water basis. Volume fractions (V_f) are expressed as % protoplasmic volume. Water potential (Ψ_w) is expressed as Mpa. Adapted from Clipson and Jennings (1990) and Clipson *et al.* (1989).

NAD-dependent glycerol 3-phosphate dehydrogenase from *D. hansenii* was strongly inhibited by NaCl or KCl, when ions were added as glutamates, enzyme activity increased. They concluded that if intracellular ion concentrations increased, cellular metabolism may well be protected by cellular counteranion environment.

Key to fungal osmotic adaptation is the generation and maintenance of intracellular osmotic and water potentials through accumulation or synthesis of solutes. Ionic solutes are readily available in external media but are cytotoxic to metabolic processes. Most studies measuring intracellular cation concentrations have not accounted for extracellular cation content in hyphal walls or have not discriminated between cytoplasmic and vacuolar contents intracellularly. This is important as most metabolism is centered upon the cytoplasm, which is most vulnerable to inhibition by excessive ionic concentrations. Table I details intracellular ion concentrations in *D. salina*, as measured by x-ray microanalysis allowing precise ultrastructural regions to be assessed. At seawater salinities, cytoplasmic sodium concentration was 173 mM, probably still compatible with cytosolic enzyme activity albeit with perhaps some inhibition of more salt-sensitive enzymes (Clipson and Jennings, 1990). Surprisingly, there was no evidence of preferential ion localization in vacuoles. These represented a low

cellular volume fraction (12–17%) and would not quantitatively have a substantial impact on overall cellular osmotic adjustment. In fungal species where vacuoles occupy a much larger fraction of the cell, vacuolar influence on localization of ions and osmotic tolerance may be more significant. Intracellular ionic solute concentrations are maintained by a range of uptake systems at the plasma membrane, which will be detailed in the ensuing sections.

The principal organic solutes used in the generation of cellular osmotic potential in fungi are the polyols, including glycerol, mannitol, arabinitol, and erythritol (Blomberg and Adler, 1993). These are compatible solutes in that they have little or no detrimental effect on cellular metabolism and contribute to enzyme stability (discussed by Jennings and Burke, 1990; Blomberg and Adler, 1993). Many studies have shown that glycerol is the primary osmo-responsive polyol in growing fungal cells (e.g., Beever and Laracy, 1986; Hocking, 1986; Pfyffer *et al.*, 1990), but declines as cells approach stationary phase. Other polyols appear to be less osmo-responsive, although they do increase as glycerol content declines with cell age, although not sufficiently to compensate for the loss of glycerol (Adler and Gustaffson, 1980; Van Eyk *et al.*, 1989). Blomberg and Adler (1993) calculated the contribution of polyols to the generation of sufficient cellular osmotic potential to counteract the external water potential. For a number of fungal species, including *Dendryphiella salina*, *Debaryomyces hansenii*, and *Z. rouxii* growing between 0.5 and 1.4M NaCl, polyols contributed between 36 and 75% of the balancing osmotic potential. Other osmolytes have been shown also to accumulate in fungi in response to osmotic environments. Proline accumulation has been described in oomycetes (Luard, 1982) and in *A. nidulans*, *Neurospora crassa*, and *S. cerevisiae* (Jennings and Burke, 1990), and trehalose accumulation has also been widely reported (Davis *et al.*, 2000). Davis *et al.* (2000) also discuss the implications of accumulating mixtures of osmolytes in fungal cellular osmotic adjustment. Although there is little advantage in the generation of osmotic potential by accumulating mixtures of osmolytes, mixtures may reduce possible toxic effects of individual osmolytes or reduce feedback mechanisms regulating their synthesis or accumulation at high concentrations.

Critical to cellular osmotolerance is its regulation, in terms of both the expression of genes conferring osmotolerance and the regulation of those genes. This will be the focus of the ensuing sections. The approaches used for the study of fungal osmoadaptation at the genetic level have moved through three distinct stages. Initially, *in vivo* studies concentrated upon the production of mutants together with

the physiological characterization of the phenotypes that their respective genes controlled. Second, the advent of *in vitro* molecular approaches has allowed the cloning (often by the complementation of mutant phenotypes) and characterization of individual genes, facilitating the testing of the precise role of a gene in an osmoregulatory pathway (e.g., Brewster *et al.*, 1993). Lastly, with the completion of the first fungal genome projects, *in silico* genetics begins to allow an appreciation of the full range of genes and gene products associated with adaptation to osmotic stress (e.g., Yale and Bohnert, 2001).

III. Control of Osmotic Responses at the Molecular Level

A. Osmotic Adjustment in Yeasts

Yeasts have proved extremely amenable for genetic analysis of osmotic responses. A very large body of information is now available for the ascomycetous yeast *S. cerevisiae*, based upon an extensive and easily derived mutant library, straightforward and efficient molecular protocols, and its ease of cultivation. Some caution must be exercised in applying such data to other fungal osmophiles, as *S. cerevisiae* is poorly osmotolerant, and its nonfilamentous nature reduces the value of comparisons with filamentous fungi. The other yeast which is relatively well characterized is the extreme osmophile *D. hansenii*.

1. Saccharomyces cerevisiae

a. Regulation of osmotic responses at the molecular level. Among fungi, *S. cerevisiae* provides the most sophisticated molecular model of osmotic adaptation, albeit of an organism of relatively limited osmotic tolerance. Osmotic adjustment in yeast has been shown to include increased production and accumulation of glycerol, enhanced Na^+/K^+ discrimination by a K^+ uptake system, and Na^+ efflux controlled by an ATPase (Blomberg and Adler, 1993). Using 2D-PAGE, Blomberg (1995) identified several proteins encoded by potentially salinity-responsive genes, with some 200 or so genes and their products being responsive to osmotic challenge in *S. cerevisiae*, although a number of these appeared to have no obvious role in the alleviation of osmotic challenge (Blomberg, 1995). Nevertheless, this approach is likely to underestimate the number of responsive genes as some changes may occur transiently at the onset of osmotic challenge, some may be very specific to precise induction conditions, and some highly induced proteins may still be present at levels below the threshold of detection.

Many proteins characteristic of other general stress responses, such as heat shock proteins and DNA damage inducible proteins, may also be apparent after exposure to salinity. A number of osmotically responsive genes in *S. cerevisiae* with known phenotype are listed in Table 2.

Many components of the yeast osmotic regulatory cascade are now well understood, particularly for aspects of the HOG (high osmolarity glycerol response) regulatory cascade (outlined in Fig. 1) (Brewster *et al.*, 1993). The HOG regulatory apparatus relies upon specific protein phosphorylation events mediated by MAP kinases, with the Sln1p and Sho1p membrane spanning proteins being important osmosensors (Maeda *et al.*, 1994, 1995). Via a relay of MAP kinases, the stress signal is transmitted to Pbs2p which phosphorylates Hog1p, so facilitating its import by the nucleus (reviewed by Rep *et al.*, 2000). Activated Hog1p directly or indirectly influences the activity of one or more transcriptional regulators within the nucleus. Experiments with Hog1p/green fluorescent protein fusions reveal that the rapid concentration in the nucleus upon osmotic challenge is reversed on return to an iso-osmotic environment or after a period of adaptation to high osmolarity. The intracellular distribution of Hog1p, and hence its ability to

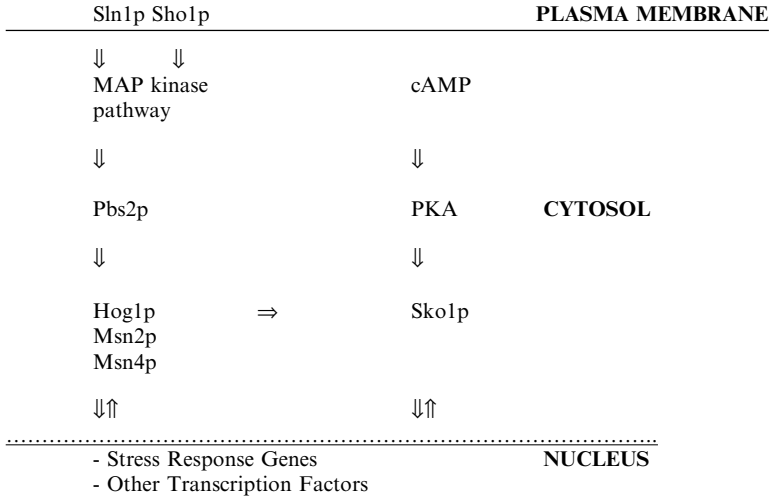

FIG. 1. A simplified summary of the regulation of osmotic stress response genes by the HOG pathway in *S. cerevisiae*. Hog1p exerts its effects through at least five different transcription factors, including Msn2p, Msn4p, and Sko1p. There is evidence for cross talk between these and other stress response pathways, for example, the PKA pathway may directly act upon Msn2p and Msn4p (Hohmann, 2002).

TABLE II

EXAMPLES OF OSMOTICALLY RESPONSIVE GENES IN *SACCHAROMYCES CEREVISIAE*

HAL1	Na^+/K^+ selectivity
ENA1	ATP-ase/Na^+ efflux
GPD1	Glycerol production
TPS2	Trehalose synthase complex subunit
CTT1	Catalase
HSP 26, 12, 104	Heat shock proteins
DDR2	DNA damage inducible
GAC1	Protein phosphatase

After Blomberg, 1995

influence transcription, depends upon phosphorylation-linked accumulation in the nucleus, binding and retention by targets in the nucleus, and kinase-controlled export to the cytosol (Reiser *et al.*, 1999).

The cAMP-activated protein kinase A pathway (PKA) also modulates stress gene expression by controlling the movements of transcription factors between the nucleus and the cytoplasm. For example, the Sko1p repressor regulates a subset of osmotically inducible genes. Under conditions favorable for growth, it represses transcription of genes such as *ENA1* and *HAL1*. When osmotically challenged, Hog1p phosphorylates Sko1p near the amino terminus, resulting in the export of Sko1p from the nucleus and the derepression of osmotic stress defense genes under Sko1p control. PKA activity can also phosphorylate Sko1p in the central region of the protein. Favorable growth conditions lead to high PKA activity, accumulation of Sko1p in the nucleus, and the subsequent repression of stress response genes. High PKA activity also prevents the nuclear import of the stress response transcription factors Msn2p and Msn4p (Pascual-Ahuir *et al.*, 2001).

Hog1p is activated as an early response to salinity challenge and may act in concert with another factor also involved in osmotic stress-induced transcription, Hot1p. The response may then diverge into separate pathways, with one controlled by the general stress response transcription factors Msn2p and Msn4p. When activated, Msn2p and Msn4p move from a cytosolic location to the nucleus (Gorner *et al.*, 1998). The protein phosphatase calcineurin is also thought to interact with Hog1-based signaling (reviewed by Yale and Bohnert, 2001). The accumulation by the nucleus of these activated transcription factors then alters the transcription of a range of structural genes (e.g.,

TABLE III

EXAMPLES OF OSMOTIC MUTATIONS IN *ASPERGILLUS NIDULANS*

Mutation	Phenotype/Gene product	Author
bimG	Osmotic remedial phosphatase	Borgia and Dodge, 1992
ch1A/dafA/dic	Osmo-sensitivity/dicarboximide resistance	Chabani and Grindle, 1990
chsD,E	Chitin synthase	Specht *et al.*, 1996
gfdA	Glycerol-3-phosphate dehydrogenase	Fillinger *et al.*, 2001
hogA	MAP kinase (signaling)	Han and Prade, 2002
myoA	Myosin 1	McGoldrick *et al.*, 1995
nfsA	Sodium fluoride sensitivity	Shawcross *et al.*, 1994
orlA,B	Osmotic remedial chitin formation	Borgia and Dodge, 1992
orl C,D	Csmotic remedial B 1,3 glucan development	Borgia and Dodge, 1992
scy	Cycloheximide resistant	de Souza *et al.*, 1998
sltA1	NaCl/KCl sensitivity	Spathas, 1978
sorA,B	Sorbose resistance	Elorza and Arst, 1971
tcsA	Osmotic remedial sporulation	Virginia *et al.*, 2000
tsE	Temperature sensitive glucosamine synthesis	Katz & Rosenberger, 1970

Updated from Clement *et al.*, 1999, and reproduced from the *Mycologist* with permission of the British Mycological Society.

Table II) involved in the osmotic response. In addition to membrane sensors, the actin cytoskeleton is also one of the first cellular components to sense osmotic challenge and can rapidly disassemble in response to osmotic challenge (Blomberg, 1995).

b. Transcriptional responses. There have been a number of studies which have examined the global response of the yeast genome to saline or osmotic challenge. Dependent upon the precise induction conditions or the threshold of expression set, each reaches a different estimate of the proportion of the genome that is responsive to osmotic challenge. Rep *et al.* (2000) applied 0.5 or 0.7M NaCl, or 0.95M sorbitol, and suggested that the transcriptional response to osmotic stress involved around 5% of *S. cerevisiae* genes. Posas *et al.* (2000) estimated that about 7% of *S. cerevisiae* genes were induced in excess of fivefold after a 0.4M NaCl challenge for 10 min (equivalent to more than 400 genes). Yale and Bohnert (2001), using a more extreme saline challenge

of 1M NaCl, observed transcriptional changes over 90 min following upshock and suggested that expression levels were affected in around 10% of genes. However, each of these studies recognized that adaptation to a saline challenge involved the regulation of many aspects of cell function. The HOG pathway, described in the previous section, is necessary for the induction of many or most of these genes and can thus be categorized as a global regulator of the overall response to osmotic challenge. More specific aspects of the response are then controlled by the Hot1p transcription factor and the general stress response proteins Msn2p and Msn4p (Rep *et al.*, 2000).

In a *hog1* mutant, some genes became more strongly induced by salt than in the otherwise isogenic wild type, with most being related to mating functions via a shared pheromone response MAP kinase pathway (Rep *et al.*, 2000). Posas *et al.* (2000) also examined the response of a *hog1* loss of function mutant and, while noting many differences in gene expression compared to the wild type, emphasized that most changes were relatively small quantitative changes in the level of transcription. They concluded that Hog1, while playing a key role in regulating the response to osmotic challenge, did not control the only signaling pathway mediating osmotic adaptation.

The earliest responses to severe saline challenge have been found to be transcripts involved in nucleotide and amino acid metabolism. These were subsequently followed by transcripts related to intracellular transport, protein synthesis, and targetting. After 90 min of a 1M NaCl osmotic challenge, genes involved in detoxification, metabolism of energy reserves, and lipid biosynthesis were highly expressed (Yale and Bohnert, 2001). While a similar set of genes up-regulated by salt and osmotic (sorbitol) challenge were apparent, some 10 transcripts were more strongly up-regulated by saline rather than osmotic effects, while just over double this number were stimulated more by sorbitol than salt (Rep *et al.*, 2000). This further demonstrates differences based upon the specific effects of ionic and nonionic osmotically active solutes. Nevertheless, there is a correlation between those genes identified by earlier methods as being up-regulated by osmotic challenge (see Table II) and the induction patterns indicated by microarray studies. For example, the prolonged high salinity treatments of Yale and Bohnert (2001) induced *GPD1* 39-fold, *HSP12* 62-fold, *CTT1* 9-fold, and *TPS2* 7-fold.

Bohnert *et al.* (2001) have collated the genomics-based approaches that use microarray data together with ESTs (expressed sequence tags) which represent partial clones of transcribed genes, to explore the parallels between lower and higher eukaryote stress responses. For

example, salt challenge treatments of *S. cerevisiae* and *Oryza sativa* (rice) induce a large number of ribosomal proteins as an early response in both species. Ribosome synthesis and the elevation of transcripts required for protein turnover may then be ubiquitous stress responses. However, perhaps 20% of stress-induced transcripts identified as ESTs in higher organisms cannot be identified by comparison with previously characterized genes from yeast or other models.

2. Debaryomyces hansenii

In contrast to *S. cerevisiae*, the genetics of the marine yeast *D. hansenii* have been relatively poorly studied, but potentially *D. hansenii* forms a more appropriate model for fungi tolerant to moderate and high concentrations of osmotically active solutes. Most strains of *S. cerevisiae* cease growth at around 10% NaCl (Lepingle *et al.*, 2000), while *D. hansenii* can tolerate salinity levels of up to 24% (Hernandez-Saavedra *et al.*, 1995) and has been isolated from a number of highly ionic environments such as concentrated brine lakes and the deep sea. Systems for the genetic analysis of *D. hansenii* remain underdeveloped, and our understanding of its molecular responses to bring about osmotic adaptation are less advanced than for *S. cerevisiae*. Nevertheless, the *S. cerevisiae* model has been helpful in increasing the understanding of *D. hansenii* osmotic tolerance.

A number of genes involved in stress responses in *D. hansenii* have been characterized. Hernandez-Saavedro and Romero-Geraldo (2001) described the cloning of the oxidative stress response enzyme superoxide dismutase (SOD) from marine strains of *D. hansenii*. Thome and Trench (1999) used Northern blots to show increases in glycerol-3-phosphate dehydrogenase mRNA 15 min after salt challenge and immunoblots demonstrated increases in the quantity of the enzyme itself 30 min after addition of NaCl. Hence, as with *S. cerevisiae*, there is clearly rapid transcriptional up-regulation of GPD in response to salt challenge. Calderon-Torres and Thome (2001) studied electrophoretic profiles of polypeptides from various cell compartments of *D. hansenii*. They showed that different osmotically active solutes, such as NaCl, KCl, and sorbitol, produced different responses. For example, while sorbitol caused significant declines in the concentration of some mitochondrial and cytosolic polypeptides, KCl did not have a major effect on any of the subcellular compartments.

Other elements of the osmotic stress response also appear to mirror the *S. cerevisiae* model. Bansal and Mondal (2000) described the cloning of a *HOG1* homologue encoding a MAP kinase involved in osmotic signaling. Thome-Ortiz *et al.* (1998) identified a number of

ion transporters, including a proton-pumping ATPase responsible for the generation of a membrane potential which drives K^+ movement through a uniporter. They also identified a K^+/H^+ exchange system and a rapid cation/cation exchange system. Almagro *et al.* (2001) cloned two genes encoding Na^+ATPases from *D. hansenii* by using degenerate PCR primers designed for previously characterized Na^+ and Ca^+-ATPase genes. *DhENA1* was induced by high NaCl concentrations while *DhENA2* required alkali pH shock. These cloned genes also complemented the corresponding Na^+ efflux defect in *S. cerevisiae* mutants and showed homology with previously reported *ENA* genes from *S. cerevisiae*, *S. occidentalis*, and *Z. rouxii*.

The key question is why is *D. hansenii* so much more osmotically tolerant than *S. cerevisiae*? Prista *et al.* (1997) recorded intracellular concentrations of Na^+ at 800 mM in *D. hansenii* growing at 2M NaCl. This might suggest that the metabolism of this species is less sensitive to Na^+ than is *S. cerevisiae*, although the measurements made do not take into account any possible localization in vacuoles which might lead to a low salt cytoplasmic environment. Comparisons of the ability to extrude Na^+ in each species revealed that the efficiency of this process was similar in both yeasts. Hence, Na^+ extrusion alone cannot explain the enhanced salt tolerance of *D. hansenii*. Some clues to the molecular basis of this enhanced salt tolerance are provided by Lepingle *et al.* (2000). Using the completed genome project for *S. cerevisiae* as a comparative reference, analysis of 2830 random sequence tags (RSTs) from *D. hansenii* showed that particular functions, especially those related to transport facilitation, were underrepresented in *S. cerevisiae* when compared to *D. hansenii*. For example, amino acid and carbohydrate transporters were nearly twice as common in *D. hansenii* than in *S. cerevisiae* and there was also a small increase in the number of genes involved in osmosensing.

3. *Other Yeasts*

A number of components of osmotic adaptation in *S. cerevisiae* have also been shown to operate in other yeasts. For example, Hahnenberger *et al.* (1996) demonstrated that the Na^+/H^+ antiporter gene from *Schizosaccharomyces pombe* can confer Na^+ and Li^+ tolerance upon *S. cerevisiae*. However, while this is the major Na^+ extrusion system in *S. pombe*, its equivalent in *S. cerevisiae* (*NHA1*) is less important for Na^+ tolerance than the Ena-ATPase (Prior *et al.*, 1996). Watanabe *et al.* (1995) isolated a Na^+/H^+ antiporter gene (*Z-SOD2*) from the salt-tolerant yeast *Z. rouxii* whose expression was constitutive and independent of salt treatments as in the *S. pombe* homologue. Gene

disruptions of *Z-SOD2* depressed salt tolerance but still allowed growth in the presence of 50% sorbitol. However, a total loss of function mutation of *Z-SOD2* did not depress salt sensitivity to the levels found in halosensitive yeasts, suggesting other mechanisms of salt tolerance were important. Two MAP kinase genes (*ZrHOG1* and *ZrHOG2*) homologous in function to *S. cerevisiae HOG1* have been cloned from *Z. rouxii* (Iwaki *et al.*, 1999). Each could restore salt and osmotolerance upon an *S. cerevisiae hog1* loss of function mutant. The presence of two HOG MAP kinases rather than one was suggested by these workers as a possible contributor to salt tolerance.

Even though glycerol appears to be the main salt-induced osmolyte accumulated in more than 20 different fungi, important differences in strategy may exist even among different yeast species. For example, Garcia *et al.* (1997) compared the relative salt tolerance of *Candida tropicalis* with *S. cerevisiae* in glucose media. While *C. tropicalis* was better adapted to Na^+ and Li^+ stress than *S. cerevisiae*, it showed greater inhibition during osmotic challenge by KCl and sorbitol. General carbon catabolite control based on the preferential use of favored C sources like glucose, in *S. cerevisiae* but not *C. tropicalis*, correlated with enhanced glycerol synthesis and osmotolerance. In the absence of glucose repression, trehalose was a key osmoresponsive solute in *S. cerevisiae* but was not accumulated in *C. tropicalis* under any conditions.

Several yeast species including *S. cerevisiae* have been shown to demonstrate dimorphism. In *Candida albicans* at alkali pH (8.0), Na^+ ions can inhibit germ tube outgrowth, as can gluconate. There are several sites at which Na^+ could act but gluconate is proposed to block the putative CI^- channels or exchangers required to allow plasma membrane ATPases to increase internal pH (Northrop *et al.*, 1997). Pseudohyphal growth in *S. cerevisiae* is considered to be a response not just to starvation but also to a stressful environment which can include osmotic stress (Zaragoza and Gancedo, 2000).

In conclusion, salt-tolerant yeasts may differ from their more sensitive relatives in a number of key functions not necessarily related to the efficiency of extrusion of Na^+ ions. A number of transport and uptake functions coupled to alterations in sensing the osmotic and nutritional environment may be important features in enhanced halotolerance.

B. Osmotic Adjustment in Filamentous Fungal Species

There is a considerable legacy of genetic and physiological studies of filamentous fungal osmotic relations, although the molecular details of mechanisms lag well behind that of the model established for

S. cerevisiae. At a physiological level, the marine hyphomycete *Dendryphiella salina* and the ascomycete *Aspergillus nidulans* are relatively well characterized. *A. nidulans* is one of the best characterized filamentous fungal species at the genetic level, with well-developed mutant libraries and techniques for genetic analysis (Clement *et al.*, 1999a). Nevertheless, the complex mycelial morphology reflecting complex life cycles presents much greater difficulty for the elucidation of osmotic relations than in unicellular organisms.

1. *Genes with ''Osmotic'' Phenotypes*

The classic approach of fungal genetics is to create mutant strains with a particular phenotype and to characterize such strains to reveal insights into processes controlling such a phenotype. The ease with which mutant libraries can be screened for changes in osmotic adaptation has identified a substantial number of osmotic mutants, although their underlying osmotic physiology has rarely been followed up in detail. For example, one of the earliest fungal transformation systems used germinating conidia of *Neurospora crassa*–osmosensitive mutants as recipients of DNA (Wooton *et al.*, 1980), although the nature of the osmosensitivity was not then fully understood.

Examples of genes causing osmotic phenotypes in *A. nidulans* are summarized in Table II, with several mutants exhibiting pleiotropy. *A. nidulans* is very osmotolerant, being able to grow vegetatively in NaCl concentrations in excess of 3M (Beever and Laracy, 1986). Two types of mutant are common: osmosensitive mutants which are more susceptible to negative medium water potential than the wild type and osmotic remedial mutants which show a mutant phenotype under one set of conditions but are wild type when the external osmotic environment changes, usually by an increase in the solute concentration of the medium (for example, the mutants *chsD* and *E* confer osmotic remedial rather than osmosensitive phenotypes; Specht *et al.*, 1996).

Osmotic mutants of *A. nidulans* include strains showing defects in cytoskeleton components such as *myoA* (McGoldrick *et al.*, 1995). Null mutations of *myoA* are lethal—the conidia of such strains can swell and undergo nuclear division but do not produce hyphae. Myosin appears to have multiple roles in defining cell polarity, septal wall formation, hyphal branching patterns, and in establishing hyphal size and shape (Liu *et al.*, 2001). Some vacuolar biogenesis mutations such as *scy* confer osmosensitivity (de Souza *et al.*, 1998) but not others such as *vps* (Tarutani *et al.*, 2001). An *A. nidulans scy* cycloheximide-resistant mutant has a pleiotropic phenotype that includes a fragmented vacuolar system as well as osmosensitivity (de Souza

et al., 1998; Tarutani *et al.*, 2001). Similarly, while glycerol dehydrogenase activity is salt inducible, not all glycerol biosynthesis mutants are osmosensitive (Hondmann and Visser, 1994; Redkar *et al.*, 1995, 1998). In a further example of pleiotropy, Fillinger *et al.* (2001b) showed that a glycerol processing mutation also influenced cell wall features. There are several other examples of osmotic mutations that affect cell wall characteristics, such as *orl A-D* (Borgia and Dodge, 1992), *chsD* and *E* (Specht *et al.*, 1996), and *tsE* (Katz and Rosenberger, 1970). Sorbose-resistant mutants show a very specific resistance phenotype to a toxic sugar based upon a defect in an uptake system (Elorza and Arst, 1971). Other osmotic mutations, such as *bimG*, produce an osmosensitive phenotype because of an alteration in the sensitivity of a specific enzyme (Borgia and Dodge, 1992). Interestingly, particular mutations in glucose transporters of yeast can convert these membrane transporters into nonspecific ion channels which are then capable of mediating movement of cations such as K^+ (Kruckeberg, 1996; Liang *et al.*, 1998).

The incomplete nature of the collection of mutant phenotypes is unlikely to give a comprehensive view of the variety of cellular processes that may influence osmotic relations. Genes identified by conventional mutation analysis number an order of magnitude lower than the estimated 8000 or so in the genome of a filamentous ascomycete (Kupfer *et al.*, 1997). A gene encoding a plasma membrane H^+ ATPase (*pmaA*) encodes a product essential for the maintenance of a proton gradient and for driving antiporters dependent upon such gradients. This is an essential gene producing a lethal phenotype if deleted in *A. nidulans*, although in *Z. rouxii* some mutations in the equivalent gene do produce osmosensitivity (Reoyo *et al.*, 1998). Nevertheless, the mutation approach suggests that the cytoskeleton, the cell wall, relative enzyme tolerance/sensitivity to osmotic environments, polyol biosynthesis, and vacuole biogenesis are all intimately involved in the osmotolerance response.

Other relevant processes shown by mutation analysis in filamentous fungi, but not yet in *A. nidulans*, include G protein signaling, with mutations in the G alpha subunit of *Magnaporthe grisea* giving increased sensitivity to repression of conidiation by osmotic stress (Fang and Dean, 2000). In *Coprinus cinereus*, mutations in the *hyt1* gene, which encodes a novel protein, produce osmotic remedial swelling of hyphal tips (which can be corrected by osmotic stabilizers in the medium). This gene is required for cell wall function but intriguingly the composition of wall polysaccharides remains unchanged (Maida *et al.*, 1997).

2. *Molecular Approaches*

Some modest progress has also been made in understanding the molecular basis of osmotolerance in filamentous species such as *A. nidulans*. Redkar *et al.* (1996a) used 2D SDS PAGE to estimate that around 49 novel or induced proteins were produced by salt-adapted cultures of *A. nidulans* with 26 further *de novo* synthesized or upregulated proteins being identified in salt-shocked cultures. They showed that salinity increased the copy number of rRNA genes in *A. nidulans*, presumably reflecting a requirement for increased total protein synthesis under stressful conditions where many proteins and the ribosomes themselves have short life expectancies. Salt-inducible genes identified via subtractive hybridization include those encoding two ATPase subunits, a mitochondrial transport protein, and a ubiquitin extension protein (Redkar *et al.*, 1996b). The recognition that the *gpdA* promoter, widely used in *A. nidulans* expression vectors, is inducible by osmotic treatments opens the prospect of identifying and exploiting specific motifs in osmotically responsive genes (Redkar *et al.*, 1998).

As a prelude to complete genome projects, EST libraries of several filamentous species, including *A. nidulans, Aspergillus fumigatus, Magnaporthe grisea*, and *N. crassa*, are now available. *A. nidulans* libraries have been screened electronically to identify likely homologues of *S. cerevisiae* genes known to be active in osmotic responses (Bohnert *et al.*, 2001). In some cases, this has allowed the design of PCR primers for the isolation of complete clones of target genes such as *hogA* (Han and Prade, 2002).

The cloning and sequencing of wild-type DNA that complemented the *N. crassa os-1* allele confirmed the gene product as a likely osmosensing, two-component (fused sensor and response regulator) histidine kinase. DNA sequencing of some mutant alleles has identified one group of null mutations which conferred a high level of resistance to fungicides such as iprodione and moderate sensitivity to osmotic agents. A second group of mutants with more moderate fungicide resistance but high sensitivity to osmotic stress showed alterations in single amino acids. These changes tended to occur in a common ''linker'' region of tandem repeats, which may represent a regulatory site for kinases and phosphatases (Ochiai *et al.*, 2001). However, a deletion mutant of a homologous gene (*fos1*) from *A. fumigatus*, while showing dicarboximide resistance, was not osmosensitive (Pott *et al.*, 2000). Hence, the roles of homologous genes may differ subtly among the different species and particular mutations can confer quite precise phenotypes. It is noteworthy that in *Ustilago maydis*, mutations that

lead to dicarboximide resistance also confer osmosensitivity. However, one class has been described based on disruptions in the *ubc1* gene encoding a regulatory subunit of protein kinase A (PKA). This suggests a role for cAMP signaling in determining such phenotypes. By analogy with the yeast model, this perhaps occurs via crosstalk with a HOG pathway and/or the control of the subcellular locations of Msn2p/Msn4p homologs (Ramesh *et al.*, 2001). A common phenotype such as dicarboximide resistance may thus have more than one molecular basis.

3. A. nidulans *as a Model Expression System*

Isolation and characterization of mutants provides the opportunity to attempt to clone corresponding wild-type, functional genes by complementation and restoration of a normal phenotype. The subsequent characterization by DNA sequencing of complementing sequences facilitates a molecular study of the process controlled by a particular gene. Figure 2 (see color insert) demonstrates the complementation of the *sltA1* salt sensitivity mutation in *A. nidulans* by DNA sequences made from a strain with wild-type responses to salt (O'Neil *et al.*, 2002). Such an approach need not be limited to the cloning of native genes from the same species but may be extended to the isolation of heterologous genes from other species. This may be particularly useful where a favored physiological model, such as *D. salina*, has not provided a library of mutant strains for complementation or a transformation system.

Wernars *et al.* (1987) demonstrated that protoplasts of *A. nidulans* would frequently take up a DNA molecule without a conventional selectable marker along with a typical transformation vector carrying a suitable marker gene. This phenomenon of cotransformation may allow the rapid screening of gene libraries from other species without the need to construct gene libraries in *A. nidulans* vectors. Stanley *et al.* (1995) described the cotransformation of a salt-sensitive *A. nidulans* host carrying the *sltA1* mutation with a lambda EMBL3 genomic DNA library of the marine hyphomycete *D. salina* and the plasmid vector pDJB3 (see Fig. 3). Using this approach, a number of *D. salina* clones were isolated that conferred enhanced salt tolerance upon the alternative host and several of these showed an increased polyol content under osmotic challenge (Clement *et al.*, 1999b).

In the case of the *sltA1* mutation, Attwell *et al.* (1995) and Clement *et al.* (1996) proposed that arginine may act as a signal transducer modulating Na^+/H^+ antiporter function. The fact that the arginase gene, *agaA*, mapped to the same region of chromosome linkage group VI as *sltA1* (Clutterbuck, 1993) and heterokaryons formed from parental strains carrying both mutations remained mutant in character

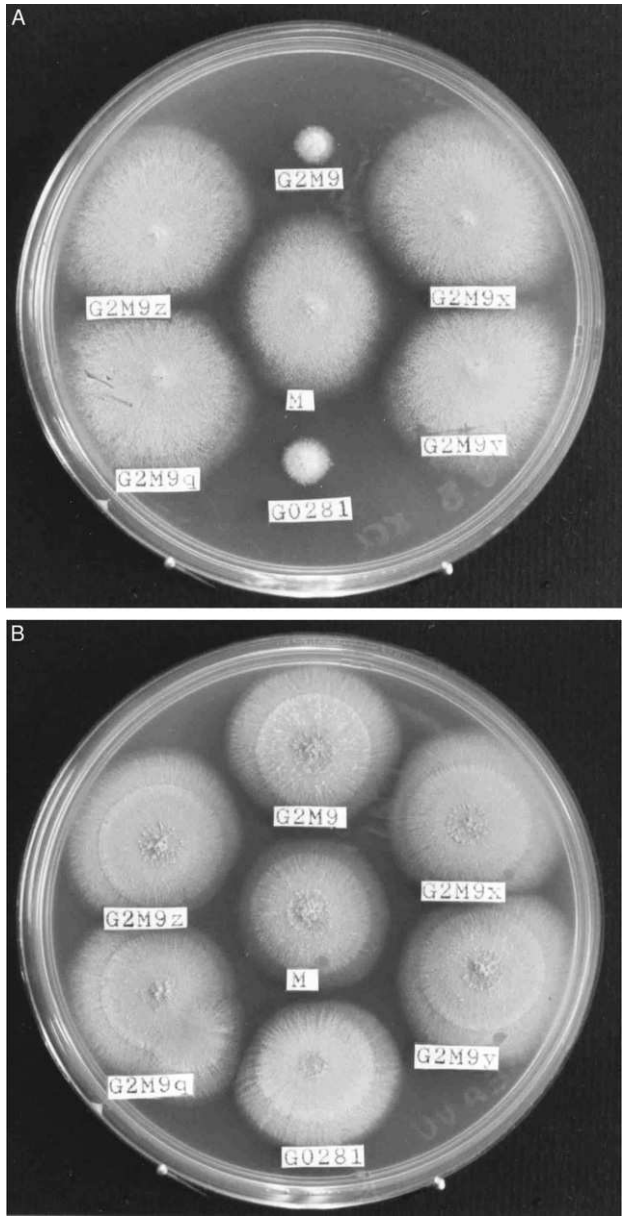

FIG. 2. Strains of *A. nidulans* growing in the presence and absence of 0.8M KCl. Strains GO281 and G2M9 carry the *sltA1* salt sensitivity mutation while M is a control strain showing a wild type salt response. G2M9q,x,y and z are selected transformants of G2M9 that carry cosmid genomic DNA clones which complement the salt sensitivity conferred by *sltA1* (O'Neil *et al.*, 2002).

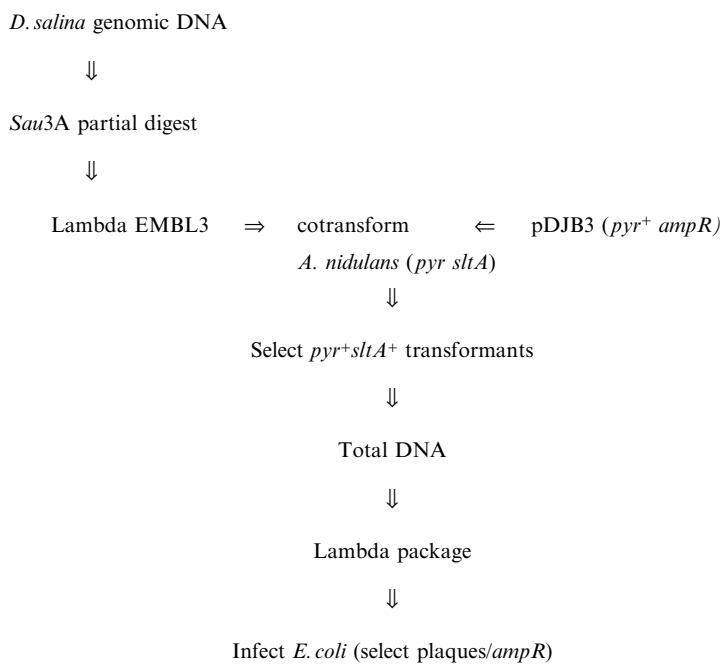

FIG. 3. A cotransformation strategy for the complementation cloning of *Dendryphiella salina* osmotolerance determinants in *A. nidulans*. *pyr* = pyrimidine requirement, *ampR* = ampicillin resistance, *sltA* = salt sensitivity (after Clement *et al.*, 1999b).

(O'Mahony *et al.*, 2002) suggested that *sltA1* is an allelic variant of *agaA* that confers salt sensitivity (Clement *et al.*, 1996). Alternatively, *sltA1* and *agaA* may represent alleles of a gene encoding a transcription factor that controls several genes and phenotypes, including salt tolerance and arginine catabolism (O'Neil *et al.*, 2002).

4. *Life Cycle Stage Sensitivity*

Simple plate growth tests and more sophisticated physiological analyses are routinely performed upon vegetative hyphae, particularly as such material provides sufficient biomass for biochemical or molecular approaches. The properties of spores in responding to osmotic stress may become overlooked in the drive to acquire amenable experimental material. Many terrestrial fungi, though markedly salt-tolerant (Tresner and Hayes, 1971), may nevertheless not be considered as marine species in that they are unable to complete their life cycles within the sea. Cox *et al.* (1995) demonstrated that *A. nidulans*, while showing

considerable salt tolerance in terms of radial growth as a vegetative colony, was measurably more salt sensitive at the stage of elongation of germ tubes from conidia. This may relate to the mobilization of trehalose and glycerol as energy sources during spore germination (d'Enfert, 1997) thus compromising the ability of the germling to provide such compatible solutes for osmoprotection.

Other compatible solutes such as trehalose may have more general roles in tolerance of other environmental challenges such as heat shock at this stage (d'Enfert *et al.*, 1999; Fillinger *et al.*, 2001a). Transient accumulation of glycerol in germinating conidia of *A. nidulans* is based upon enhanced trehalase activity. This is not essential for the completion of germination but is required to achieve optimal germination rates under conditions of carbon limitation (d'Enfert *et al.*, 1999). Controlled spore swelling (isotropic growth) may be regulated by a number of factors. O'Mahony *et al.* (2002) implicated the role of a plasma membrane Na^+/H^+ antiporter in the isotropic growth of *A. nidulans* at acidic pH values, based upon sensitivity of the process to the inhibitor amiloride. Specht *et al.* (1996) reported that disruption of the *chsD* gene in the same species resulted in a reduction of chitin content in the wall. This mutation also produced conidia that commonly swelled excessively and burst when germinated on media of low osmotic strength.

Ecologically, poor adaptation to existing environmental conditions at key life cycle stages may explain why vegetatively osmotolerant species are infrequently found in some osmotic environments, such as the sea. Smith *et al.* (1996) and Geiser *et al.* (1998) suggested that Caribbean sea fan mortalities resulted from the vegetative growth of a terrestrial *Aspergillus* species upon this important component of the coral reef, with the source of this pathogen probably subject to a continual replenishment of vegetative inoculum, rather than spores, from freshwater runoff from nearby land. This would suggest that the ability to establish a colony in the marine environment (or indeed in other osmotically challenging environments) may extend beyond mere osmotolerance. For example, large sigmoidal-shaped spores such as found in *D. salina* are easily arrested when tumbling across a solid surface (Webster and Davey, 1984), which may contrast with the behavior of spherical spore shapes found in species such as *A. nidulans*. This property, coupled to the rapid germination and production of mucilage by settled spores of some aquatic species (Au *et al.*, 1996), may be an important element in ensuring rapid attachment in a tidal regime. Hence, the osmotic relations of a fungus may be variable at different life

cycle stages, but other factors affecting colony establishment may also have a major influence upon ecological distribution.

5. *Regulation of Osmotic Responses in* A. nidulans

a. Introduction to transcription factors. Osmoadaptation clearly involves a coordinated response of large numbers of genes and their products. At one level, regulation may be achieved by protein/protein interactions controlled by molecules like Hog1p. However, the ultimate control of gene expression will depend upon specific proteins, transcription factors, that influence gene transcription by binding to DNA sequences immediately upstream of target structural genes. Transcription factors fall into a limited number of well-defined categories classified upon their distinctive DNA binding motifs. The commonest motif is the C_2H_2 zinc finger which produces the characteristic secondary structure for which it is named by almost adjacent pairs of cysteine and histidine residues binding a zinc ion. Intervening amino acid residues then form the ''finger,'' which interacts with the DNA molecule.

O'Neil *et al.* (2002) described a novel gene which encoded a C_2H_2 zinc finger protein (StzA) involved in mediating salt tolerance in *A. nidulans*. This showed a ubiquitous motif conserved in a variety of regulatory proteins from fungi and animals. For example, the StzA protein from *A. nidulans* contains three C_2H_2 zinc fingers and shows significant homology across the DNA binding region with the corresponding second, third, and fourth zinc fingers of the human transcription factor WT-1 associated with Wilm's tumor. Direct homologues of *A. nidulans* StzA (O'Neil *et al.*, 2002) can be found in a number of filamentous fungi, notably the Ace1 protein of *Hypocrea jecorina* (38% of 437 amino acids identical) which has a role in regulating cellulase expression (Saloheimo *et al.*, 2000). However, putative protein matches to gene products of other organisms, although representing a diverse group of species including yeasts, are restricted to relatively small sections of StzA, normally to the highly conserved DNA binding region. Hence, while StzA appears able to control a wide range of stress responses, including sensitivity to salt and DNA-damaging agents, this may be restricted to filamentous fungi alone (O'Neil *et al.*, 2002).

Other genes might also be expected to play a role in responding to changing osmotic environment. For example, during carbon catabolite repression, a fungus will selectively utilize favored carbon sources, such as glucose, over other less energetically attractive compounds. This is achieved by the induction of particular permeases and catabolic enzymes and the repression of the genes encoding proteins required for

the less favored carbon sources. In *A. nidulans*, the wide domain transcription factor CreA is responsible for the control of these events. An extreme mutation in the encoding gene (*creA^{d}-30*) leads to dramatic changes in the individual constituents of the hyphal pool of polyols. Changes in a number of enzyme activities in this mutant lead to an altered pattern of glycolysis and a more rapid synthesis of polyols when the host strain is grown on D–glucose (van der Green *et al.*, 1995).

IV. Conclusions

A. Comparisons of Yeast and Filamentous Models

Han and Prade (2002) searched EST libraries of *A. nidulans* for homologues of genes involved in the HOG pathway of *Saccharomyces cerevisiae*. Thirteen of 17 signal integrating MAP kinases and enzymes involved in compatible solute synthesis in *S. cerevisiae* were identified. For example, significant matches were found to the translated sensors *SHO1* and *SLN1*, signal integration genes such as *STE20*, *HOG1* itself, and the transcription factor *MSN2*. The deduced product of the *A. nidulans hogA* gene showed 86% identity with *HOG1* from *S. cerevisiae*. As the EST libraries by no means represent a complete collection of all *A. nidulans* genes and each EST may itself be an incomplete reflection of a structural gene, there is good reason to believe that most if not all of the components of the HOG response pathway exist in filamentous fungi. The *A. nidulans* HOG pathway genes were not activated by stresses such as hydrogen peroxide, heat shock, or asexual or sexual development, although *pbsA* (homologue of *S. cerevisiae PBS2*, MAPKK high osmolarity growth) and *hogA* did accumulate in advanced developmental stages. In *A. nidulans, hogA* interfered directly with apical growth as a deletion mutant could not produce complete septa and accumulated nuclei at the hyphal tip. Hence, *hogA* mutations may confer additional phenotypes in *A. nidulans* which the *HOG1* homologue of *S. cerevisiae* could not control. A novel gene *lsdA* (late sexual development) has recently been identified in *A. nidulans* which inhibits sexual development in the presence of salt (Lee *et al.*, 2001).

It has been estimated that multicellular ascomycetes have more than 8000 genes, while *S. cerevisiae* only has around 75% of this number (Kupfer *et al.*, 1997). Braun *et al.* (2000) compared EST libraries of *N. crassa* with *S. cerevisiae* and inevitably a large number of genes from the filamentous fungus were found to have no homologues in the yeast. Such genes have been termed ''orphans.'' Conceivably, such orphans

may have arisen since the divergence of the two lines from the gain of function by the filamentous relative and/or the ''streamlining'' or loss of genes in the unicellular species. Candidate sequences for gene losses from the yeast may be found in other fungi. Interestingly, the most dramatic examples of genes that have been lost or are very divergent from other fungi are those involved in ion homeostasis, in particular for Ca^{2+}. These authors suggest that the complex developmental pathways found in filamentous fungi but not in yeast are mediated by novel genes and proteins. Hence, it is reasonable to expect that there will be important differences in the molecular basis of even quite basic cellular processes between unicellular yeasts and filamentous species. Nonetheless, tests of gene function in diploid species such as *C. albicans* will be complicated by the difficulty in generating gene knockouts where recessive mutations will be phenotypically silent in heterozygotes.

B. Sporulation and Development

The most obvious differences between yeasts and filamentous fungi lie in the ability of the latter to form complex, differentiating, multicellular colonies. Allied to this is the ability of some strains to form secondary metabolites, not found in yeasts. Clearly, the responsibility for these additional properties will largely be found in novel genes which are absent from yeasts and perhaps, in some cases, missing from other eukaryotes too. Prade *et al.* (2001) examined EST libraries and showed that during asexual development of *A. nidulans*, a significant number of stress response transcripts were also induced, perhaps to enhance the survival properties of the spore with heat shock, DNA repair, trehalose cycling, and starvation response genes being highly expressed during *A. nidulans* spore development. Interestingly, this was much more prevalent than in the equivalent *N. crassa* library. A large proportion of *A. nidulans* mutants initially isolated as showing defects in sporulation gave more normal phenotypes when grown on media of higher osmolarity (Martinelli and Clutterbuck, 1971; Timberlake and Clutterbuck, 1994). Strains carrying loss of function mutations in *tcsA*, encoding a histidine kinase and regulatory domain, cannot produce conidia on normal media. However, they are able to sporulate in the presence of 1M sorbitol, indicating that an osmotic signal can cancel or override a blocked developmental pathway (Virginia *et al.*, 2000). This would suggest that molecular responses to osmotic conditions may be rather different in the developmentally more complex filamentous fungi than in unicellular yeasts.

C. Origins of Fungal Stress Response Genes

Benito *et al.*, (2002) searched databases of fungal genes and cloned partial sequences of fungal *ENA* type genes from Zygomycetes and Basidiomycetes, so concluding that all fungi have such genes. As K^+ is the most abundant cation in cells, they proposed that fungal K^+ or Na^+ ATPases evolved from ancestral K^+ ATPases driven by the selective pressure of the need to exploit living or dead plant or animal material. The diversification of an ancestral sequence to produce genes of different function may have relied upon gene duplication. Some ATPases retain a dual function as in yeasts where gene duplication is proposed to be recent, whereas duplications in *N. crassa ENA* sequences earlier in evolution may have allowed the development of more specialized ATPases.

S. cerevisiae has been widely and successfully exploited as a model for plant cells. For example, the expression of some *Pisum sativum* or *Arabidopsis thaliana* sequences can complement mutational defects in the yeast affecting the HOG1/MAP kinase cascade (e.g., Hasegawa *et al.*, 2000). Components of the signaling process in the stress response pathway are thus similar between plants and fungi. Other aspects of salt tolerance physiology are quite different. While ENA type ATPases are widespread in fungi (Benito *et al.*, (2002), they are apparently absent from plants (Garciadeblas *et al.*, 2001). Benito *et al.* (2002) proposed that this results from the specific exposure of fungi to the high concentrations of K^+ found in their common substrate—plant material. Animal cells were exposed to seawater or a similar medium for a longer portion of their evolution and so separately evolved a Na^+/K^+ ATPase. Conversely, fungi and plants exploited a H^+ ATPase to produce a proton gradient and similar K^+ and Na^+ transporters in terrestrial habitats with lower Na^+ concentrations.

Braun *et al.* (2000) identified 13 cases of *N. crassa* DNA sequences where the best match was to a prokaryotic gene with there being no closely related eukaryotic homologue. However, they estimated that less than 2% of *N. crassa* genes result from horizontal transfer of prokaryotic genes after the divergence of the yeast and filamentous fungal lines. Bentley *et al.* (2002) recently reported the completion of the genome project for *Streptomyces coelicolor* A3(2). The 8000 or so predicted genes represent the largest number yet recorded for a prokaryote. These genes include 20 clusters encoding secondary metabolites and a large number of regulatory genes involved in responses to environmental stimuli and stresses. A number of duplicated gene sets are required for the complex procedure of colony development, including sporulation. Streptomycetes are filamentous, soil-inhabiting

bacteria and so would be expected to be exposed to similar osmotic challenges as other soil dwellers including fungi. Indeed, the thiostrepton-inducible promoter *ptipA*, controlling the expression of two transcriptional activators, TipAL and TipAS, has also been shown to respond to changes in the osmolarity of the medium. Increases in osmolarity enhance negative DNA supercoiling and stimulate *ptipA* expression. A direct consequence of this is that high medium osmolarity then induces mycelial autolysis. Programmed autolysis of the mycelium would be a normal event in the development of a colony to fuel upward growth of aerial mycelium and eventually spore formation. There appears therefore to be an intimate association between osmotic responses, sporulation, and antibiotic production in streptomycetes, where thiostrepton acts not only as an antibiotic but as a physiological signal for cell death to trigger sporulation (Ali *et al.*, 2002).

It would be reasonable to suggest that a system of stress sensing and response would be required to coordinate sporulation and antibiotic production in soil-dwelling fungi, too. Transcription factors involved in mediating stress responses, such as StzA, then become interesting candidates in the identification of regulatory genes that influence secondary metabolites.

D. Specificity of Stress Responses

A key finding of the microarray work on stress responses in yeast is that global changes in gene expression patterns are common in response even to quite specific imposed stresses. For example, Jelinsky and Samson (1999) studied changes in gene expression in *S. cerevisiae* resulting from exposure to the alkylating agent methyl methane sulphonate (MMS). Around 5% of gene transcripts increased above the $> 4\times$ above control threshold set while about 1% decreased. In addition to the induction of expected DNA repair genes such as 3-methyladenine DNA glycosylase, a range of genes with unexpected functions were induced, such as those associated with carbohydrate metabolism, cell wall biogenesis, and membrane transport. Induced transcripts included those encoding functions required for eliminating and replacing alkylated proteins. For example, a large number of ribosomal proteins such as *RPS11B* were induced by both salt (Posas *et al.*, 2000) and alkylation (Jelinsky and Samson, 1999) treatments. Similarly, *DDR48*, a DNA damage–inducible heat shock protein and a neutral trehalase *NTH1* were also induced by each treatment. Among the salt-induced transcripts of *S. cerevisiae* identified by Posas *et al.* (2000) are listed several unidentified transcripts also induced by

MMS. Alkylation damage therefore induces a full stress response (Gasch *et al.*, 2000; Goldman *et al.*, 2002). Hence, the assumption that there is a specific stress response involving a small subset of genes and particular to a certain stimulus is questioned.

There is increasing evidence in plants that there is ''crosstalk'' between different abiotic stress signaling pathways in a network of responses, so questioning whether there are any truly specific stress responses (Knight and Knight, 2001). The microarray work for *S. cerevisiae* outlined previously suggests a similar situation exists in fungi. Under some conditions, it may be that two different stresses are indistinguishable, so a general response would be appropriate. On the other hand, the production of a specific metabolite may enhance tolerance to more than one stress. For example, trehalose appears to be required for resistance to several stresses in *A. nidulans* and is induced in response to heat or oxidative shock (Fillinger *et al.*, 2001). In other cases, a specific stress may require a number of physiological responses to correct diverse changes, as in the alkylation damage response of yeast. Spore germination in *A. nidulans* is also controlled by cAMP via a protein kinase A (PKA) pathway and *ras* signaling to regulate early activation in response to carbon source availability (Fillinger *et al.*, 2002).

The development of microarray technology now means that it is technically feasible to identify all of the osmotically inducible genes in an organism (Hasegawa *et al.*, 2000). However, the vast range of changes in gene expression induced by osmotic stress emphasizes the continued requirement for physiological analysis in carefully defined mutants. Where strains carrying specific gene knockouts can be compared with wild-type controls using microarrays, the goal of precisely identifying those processes key to adapting to osmotic challenge will be realized (e.g., Liu *et al.*, 2001).

E. Concluding Remarks

The mechanisms by which fungi can survive and flourish in osmotically challenging environments are starting to be unraveled. The physiological approach has produced a wealth of data, but often it has allowed us only a glimpse at isolated biochemical activites. The same can be argued of the traditional genetics approach of gene cloning and mutant analysis. The availability of full genomic sequences means much of our knowledge can now come from the study of networks of genes and pathways. As the number of completed genome projects extends from the yeasts *S. cerevisiae*, *S. pombe*, and *C. albicans* to filamentous fungi

such as *A. fumigatus*, *Neurospora crassa*, and *Ashbya gossypii*, then comparative genomics approaches become a reality (e.g., Lepingle *et al.*, 2000). Entire fungal genomes may be compared for the presence or absence of homologous osmotically responsive genes. Commerically produced microarrays are being developed for species such as *C. albicans* (Eurogentec), which will facilitate detailed comparisons of the transcriptional responses of related organisms to osmotic environments. Nevertheless, this approach will need to be treated with caution as exemplified by the high number of homologues in the HOG pathways of *A. nidulans* and *S. cerevisiae*, and yet these ascomycetes are greatly different in their responses to osmotic challenge. The true challenge and adventure of the postgenomics era will be the comparison of the metabolic pathways of osmoadaptation and the understanding of the precise interactions of individual proteins in fungal species adapted to quite different habitats.

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References

Adler, L., and Gustaffson, L. (1980). Polyhydric alcohol production and intracellular amino acid pool in relation to halotolerance of the yeast *Debaryomyces hansenii*. *Arch. Microbiol.* **124,** 123–130.

Ali, N., Herron, P. R., Evans, M. C., and Dyson, P. J. (2002). Osmotic regulation of the *Streptomyces lividans* thiostrepton-inducible promoter, *ptipA*. *Microbiol.* **148,** 381–390.

Almagro, A., Prista, C., Benito, B., Loureiro-Dias, M. C., and Ramos, J. (2001). Cloning and expression of two genes coding for sodium pumps in the salt-tolerant yeast *Debaryomyces hansenii*. *J. Bact.* **183,** 3251–3255.

Attwell, N. A., Clement, D. J., Stanley, M. S., Clipson, N. J. W., Hooley, P., and Fincham, D. J. (1995). Arginine interactions with the Na^+/H^+ antiporter in protoplasts from *Aspergillus nidulans*. *J. Physiol.* **489,** 104–105.

Au, D. W. T., Jones, E. B. G., and Moss, S. T. (1996). Spore attachment and extracellular mucilage of aquatic hyphomycetes. *Biofouling* **10,** 123–140.

Bansal, P. K., and Mondal, A. K. (2000). Isolation and sequence of the HOG1 homologue from *Debaryomyces hansenii* by complementation of the *hog1 Delta* strain of *Saccharomyces cerevisiae*. *Yeast* **16,** 81–88.

Beever, R. E., and Laracy, E. P. (1986). Osmotic adjustment in the filamentous fungus *Aspergillus nidulans*. *J. Bact.* **168,** 1358–1365.

Benito, B., Garciadeblas, B., and Rodriguez-Navarro, A. (2002). Potassium- or sodium-efflux ATPase, a key enzyme in the evolution of fungi. *Microbiology* **148,** 933–941.

Bentley, S. D., and 42 others (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417,** 141–147.

Blomberg, A. (1995). Global changes in protein synthesis during adaptation of the yeast *Saccharomyces cerevisiae* to 0.7 M NaCl. *J. Bact.* **177,** 3563–3572.

Blomberg, A., and Adler, L. (1993). Tolerance of fungi to NaCl. *In* ''Stress Tolerance of Fungi'' (D. H. Jennings, ed.), pp. 233–256. Marcel Dekker, New York, Basel, Hong Kong.

Bohnert, H. J., Ayoubi, P., Borchert, C., Bressan, R., Burnap, R. L., Cushman, J. C., Cushman, M. A., Deyholos, M., Fischer, R., Galbraith, D. W., Hasegawa, P. M., Jenks, M., Kawasaki, S., Koiwa, H., Koreeda, S., Lee, B. H., Michalowski, C. B., Misawa, E., Nomura, M., Ozturk, N., Postier, B., Prade, R., Song, C. P., Tanaka, Y., Wang, H., and Zhu, J. K. (2001). A genomics approach towards salt stress tolerance. *Plant Physiol. Biochem.* **39,** 295–311.

Borgia, P. T., and Dodge, C. L. (1992). Characterization of *Aspergillus nidulans* mutants deficient in cell wall chitin or glucan. *J. Bact.* **174,** 377–383.

Braun, E. L., Halpern, A. L., Nelson, M. A., and Natvig, D. O. (2000). Large-scale comparison of fungal sequence information: Mechanisms of innovation in *Neurospora crassa* and gene loss in *Saccharomyces cerevisiae*. *Genome Res.* **10,** 416–430.

Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993). An osmosensing signal transduction pathway in yeast. *Science* **259,** 1760–1763.

Buchalo, A. S., Nevo, E., Wasser, S. P., Oren, A., and Molitoris, H. P. (1998). Fungal life in the extremely hypersaline Dead Sea: First records. *Proc. R. Soc. Lond. B* **265,** 1461–1465.

Calderon-Torres, M., and Thome, P. E. (2001). Subcellular level variation in protein accumulation of the halophylic yeast *Debaryomyces hansenii* grown under conditions of high osmolarity. *J. Basic Microbiol.* **41,** 231–240.

Chabani, A. W., and Grindle, M. (1990). Isolation, characterization and genetic analysis of mutants of *Aspergillus nidulans* resistant to the herbicide dichlobenil. *Mycol. Res.* **94,** 523–528.

Clement, D. J., Stanley, M. S., Attwell, N. A., Clipson, N. J. W., Fincham, D. A., and Hooley, P. (1996). Evidence for *sltA1* as a salt sensitive allele of the arginase gene (*agaA*) in the ascomycete *Aspergillus nidulans*. *Curr. Genet.* **29,** 462–467.

Clement, D. J., Clipson, N. J. W., and Hooley, P. (1999a). The genetical control of osmotolerance in fungi: A mutation analysis in the ascomycete *Aspergillus nidulans*. *Mycologist* **13,** 79–82.

Clement, D. J., Stanley, M. S., O'Neil, J., Woodcock, N. A., Fincham, D. A., Clipson, N. J. W., and Hooley, P. (1999b). Complementation cloning of salt tolerance determinants from the marine hyphomycete *Dendryphiella salina* in *Aspergillus nidulans*. *Mycol. Res.* **103,** 1252–1258.

Clipson, N. J. W., Jennings, D. H., and Smith, J. L. (1989). The response to salinity at the microscopic level of the marine fungus *Dendryphiella salina* Nicot. and Pugh as investigated stereologically. *New Phytol.* **113,** 21–27.

Clipson, N. J. W., Hajibagheri, M. A., and Jennings, D. H. (1990). Ion compartmentation in the marine fungus *Dendryphiella salina* in response to salinity: X-ray microanalysis. *J. Exp. Bot.* **41,** 199–202.

Clipson, N. J. W., and Jennings, D. H. (1990). Role of potassium and sodium in the generation of osmotic potential of the marine fungus *Dendryphiella salina*. *Mycol. Res.* **94,** 378–388.

Clipson, N. J. W., and Jennings, D. H. (1992). *Dendryphiella salina* and *Debaryomyces hansenii*: Models for ecophysiological adaptation to salinity by fungi which grow in the sea. *Can. J. Bot.* **70,** 2097–2105.

Clipson, N., Landy, E., and Otte, M. (2001). Fungi. *In* ''European register of marine species. A check-list of the marine species in Europe and a bibliography of guides to their identification'' (M. J. Costello C. S. Emblow, and R. White, eds.), *Patrimoines naturels*, **50**, 15–19.

Clutterbuck, A. J. (1993). Aspergillus nidulans. *In* ''Genetic Maps'' (S. J. O'Brien, ed.). Cold Spring Harbor, New York.

Cox, P. W., Clipson, N. J. W., Hooley, P., and Thomas, C. R. (1995). Effects of growth medium solute concentration upon the spore germination and colony growth characteristics of *Aspergillus nidulans*. *Proceedings of the Institution of Chemical Engineers Research Event, Edinburgh* **2,** 986–988.

Davis, D. J., Burlak, C., and Money, N. P. (2000). Osmotic pressure of fungal compatible osmolytes. *Mycol. Res.* **104,** 800–804.

d'Enfert, C. (1997). Fungal spore germination: Insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. *Fung. Gen. Biol.* **21,** 163–172.

d'Enfert, C., Bonini, B. M., Zapella, P. D., Fontaine, T., da Silva, A. M., and Terenzi, H. F. (1999). Neutral trehalases catalyze intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. *Mol. Microbiol.* **32,** 471–483.

de Souza, C. C., Pellizzon, C. H., Hiraishi, M., Goldman, M. H., and Goldman, G. H. (1998). Isolation and characterization of cycloheximide—Sensitive mutants of *Aspergillus nidulans*. *Curr. Genet.* **33,** 60–69.

Elorza, M. V., and Arst, Jr., H. N. A., (1971). Sorbose resistant mutants of *Aspergillus nidulans*. *Mol. Gen. Genet.* **111,** 185–193.

Fang, E. G., and Dean, R. A. (2000). Site-directed mutagenesis of the *magB* gene affects growth and development in *Magnaporthe griseaz*. *Mol. Plant Microbe Interact.* **13,** 1214–1227.

Fillinger, S., Chaveroche, M. K., van Dijck, P., de Vries, R., Ruijter, G., Thevelein, J., and d'Enfert, C. (2001a). Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. *Microbiology* **147,** 1851–1862.

Fillinger, S., Ruijter, G., Tamas, M. J., Visser, J., Thevelein, J. M., and d'Enfert, C. (2001b). Molecular and physiological characterization of the NAD-dependent glycerol 3-phosphate dehydrogenase in the filamentous fungus *Aspergillus nidulans*. *Microbiology* **147,** 1851–1862.

Fillinger, S., Chaveroche, M. K., Shimizu, K., Keller, N., and d'Enfert, C. (2002). cAMP and *ras* signalling independently control spore germination in the filamentous fungus *Aspergillus nidulans*. *Mol. Microbiol.* **44,** 1001–1016.

Flowers, T. J., Hajibagheri, M. A., and Clipson, N. J. W. (1986). Halophytes. *Quart. Rev. Biol.* **61,** 313–337.

Garcia, M. J., Rios, G., Ali, R., Belles, J. M., and Serrano, R. (1997). Comparative physiology of salt tolerance in *Candida tropicalis* and *Saccharomyces cerevisiae*. *Microbiology* **143,** 1125–1131.

Garciadeblas, B., Benito, B., and Rodriguez-Navarro, A. (2001). Plant cells express several calcium stress ATPases but apparently no sodium ATPase. *Plant Soil* **235,** 181–192.

Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **11,** 4241–4257.

Geiser, D. M., Taylor, J. W., Ritchie, K. B., and Smith, G. W. (1998). Cause of sea fan death in the West Indies. *Nature* **394,** 137–138.

Gibb, F. M., Wethered, J. M., and Jennings, D. H. (1986). The effect of monovalent ions on enzyme activity in *Dendryphiella salina*. *In* ''The Biology of Marine Fungi'' (S. T. Moss, ed.), pp. 27–43CUP, Cambridge, UK.

Goldman, G. H., McGuire, S. L., and Harris, S. D. (2002). The DNA damage response in filamentous fungi. *Fung. Gen. Biol.* **35,** 183–195.

Gorner, W., Durchschlag, E., Martinez-Pastor, M. T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., and Schuller, C. (1998). Nuclear localization of the C_2H_2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev.* **12,** 586–597.

Griffin, D. H. (1994). ''Fungal Physiology.'' Wiley-Liss, New York.

Hahnenberger, K. M., Jia, Z., and Young, P. G. (1996). Functional expression of the *Schizosaccharomyces pombe* Na^+/H^+ antiporter gene, *sod2*, in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 5031–5036.

Han, K. H., and Prade, R. A. (2002). Osmotic stress-coupled maintenance of polar growth in *Aspergillus nidulans*. *Molec. Micro.* **43,** 1065–1078.

Hasegawa, P. M., Bressan, R. A., Zhu, J. K., and Bohnert, H. J. (2000). Plant cellular and molecular responses to high salinity. *Ann. Rev. Pla. Phys. and Pla. Mol. Biol.* **51,** 463–499.

Hawksworth, D. L. (2001). The magnitude of fungal diversity: The 1.5 million species estimate revisited. *Mycol. Res.* **105,** 1422–1432.

Hernandez-Saavedra, N. Y., and Romero-Geraldo, R. (2001). Cloning and sequencing the genomic encoding region of copper–zinc superoxide dismutase enzyme from several marine strains of the genus *Debaryomyces (Lodder and Kreger-van Rij). Yeast* **18,** 1227–1238.

Hocking, A. D. (1986). Effects of water activity and culture age on the glycerol accumulation patterns of five fungi. *J. Gen. Microbiol.* **132,** 269–275.

Hocking, A. D. (1993). Responses of xerophilic fungi to changes in water activity. *In* ''Stress Tolerance of Fungi'' (D. H. Jennings, ed.), pp. 233–256. Marcel Dekker, New York, Basel, Hong Kong.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* **66,** 300–372.

Hondmann, D. H. A., and Visser, J. (1994). Carbon metabolism. *In* ''*Aspergillus*: 50 years'' (S. D. Martinelli, and J. R. Kinghorn, eds.), *Progress in Industrial Microbiology,* **29,** 61–139. Elsevier, Amsterdam.

Iwaki, T., Tamai, Y., and Watanabe, Y. (1999). Two putative MAP kinase genes, *ZrHOG1* and *ZrHOG2*, cloned from the salt-tolerant yeast *Zygosaccharomyces rouxii* are functionally homologous to the *Saccharomyces cerevisiae HOG1* gene. *Microbiology* **145,** 241–248.

Jelinsky, S. A., and Samson, L. D. (1999). Global response of *Saccharomyces cerevisiae* to an alkylating agent. *PNAS* **96,** 1486–1491.

Jennings, D. H. (1995). ''The Physiology of Fungal Nutrition.'' CUP, Cambridge, UK.

Jennings, D. H., and Burke, R. M. (1990). Compatible solutes — The mycological dimension and their role as physiological buffering agents. *New Phytol.* **116,** 277–283.

Jones, E. B. G., and Mitchell, J. I. (1996). Biodiversity of marine fungi. *In* ''Biodiversity. International Biodiversity Seminar.'' (A. Climerman, and N. Gunde-Climerman, eds.), pp. 31–42. UNESCO.

Katz, D., and Rosenberger, R. F. (1970). A mutation in *Aspergillus nidulans* producing hyphal walls which lack chitin. *Biochim. Biophys. Acta* **208,** 452–460.

Knight, H., and Knight, M. R. (2001). Abiotic stress signaling pathways: Specificity and cross-talk. *TIPS* **6,** 262–267.

Kohlmeyer, J., and Kohlmeyer, E. (1979). ''Marine Mycology, The Higher Fungi.'' Academic Press, New York.

Kruckeberg, A. L. (1996). The hexose transporter family of *Saccharomyces cerevisiae*. *Arch. Microbiol.* **166,** 283–292.

Kupfer, D. M., Reece, C. A., Clifton, S. W., Roe, B. A., and Prade, R. A. (1997). Multicellular ascomycetous fungal genomes contain more than 8000 genes. *Fung. Genet. Biol.* **21,** 364–372.

Lee, D. W., Kim, S., Kim, S. J., Han, D. M., Jahng, K. Y., and Chae, K. S. (2001). The *lsdA* gene is necessary for sexual development inhibition by salt in *Aspergillus nidulans*. *Curr. Gen.* **39,** 237–243.

Lepingle, A., Casaregola, S., Neuveglise, C., Bon, E., Nguyen, H., Artiguenave, F., Wincker, P., and Gaillardin, C. (2000). Genomic exploration of the hemiascomycetous yeasts: 14. *Debaryomyces hansenii var. hansenii. FEBS Lett.* **487,** 82–86.

Liang, H., Ko, C. H., Herman, T., and Gaber, R. F. (1998). Trinucleotide insertions, deletions, and point mutations in glucose transporters confer K^+ uptake in *Saccharomyces cerevisiae*. *Molec. Cell Biol.* **18,** 926–935.

Liu, X., Osherov, N., Yamashita, R., Brzeska, H., Korn, E. D., and May, G. S. (2001). Myosin I mutants with only 1% of wild type actin-activated MgATPase activity retain essential *in vivo* function(s). *P.N.A.S.* **98,** 9122–9127.

Luard, E. J. (1982). Accumulation of intracellular solutes by two filamentous fungi in response to growth at low steady state osmotic potential. *J. Gen. Microbiol.* **128,** 2563–2590.

Maeda, T., Wurgler-Smith, S. M., and Saito, H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369,** 242–245.

Maeda, T., Takekawa, M., and Saito, H. (1995). Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3 containing osmosensor. *Science* **269,** 554–558.

Magan, N., Challen, M. P., and Elliott, T. J. (1995). Osmotic, matric, and temperature effects on *in vitro* growth of *Agaricus bisporus* and *A. bitorquis* strains *In* ''Mushroom Science XIV, Volume 2. Proceedings of the 14th International Congress on the Science and Cultivation of Edible Fungi, Oxford, UK, 17–22 Sep. 1995'' (T. J. Elliott, Ed.), pp. 773–780, A. A. Balkema, Rotterdam, Netherlands..

Maida, S., Fujii, M., Skrzynia, C., Pukkila, P. J., and Kamada, T. (1997). A temperature sensitive mutation of *Coprinus cinereus, hyt-1*, that causes swelling of hyphal tips. *Curr. Gen.* **32,** 231–236.

Martinelli, S. D., and Clutterbuck, A. J. (1971). A quantitative survey of conidiation mutants in *Aspergillus nidulans*. *J. Gen. Micro.* **69,** 261–268.

McGoldrick, C. A., Gruver, C., and May, G. S. (1995). *myoA* of *Aspergillus nidulans* encodes an essential myosin 1 required for secretion and polarized growth. *J. Cell Biol.* **128,** 577–587.

Money, N. P. (1994). Osmotic adjustment and the role of turgor in mycelial fungi. *In* ''The Mycota. Vol. 1. Growth, differentiation and sexuality'' (J. G. H. Wessels, and F. Meinhardt, eds.), pp. 67–88 Springer-Verlag, Berlin, Germany.

Money, N. P. (1997). Wishful thinking of turgor revisited: The mechanics of fungal growth. *Fung. Gen. Biol.* **22,** 173–187.

Moss, S. T. (1986). ''The Biology of Marine Fungi.'' CUP, Cambridge, UK.

Nilsson, A., and Adler, L. (1990). Purification and characterization of glycerol-3-phosphate dehydrogenase (NAD^+) in the salt tolerant yeast *Debaryomyces hansenii*. *Biochim. Biophys. Acta* **1034,** 180–185.

Northrop, F. D., Ljubojevic, S., and Davies, J. M. (1997). Influence of Na^+ and anions on the dimorphic transition of *Candida albicans*. *Microbiology* **143,** 3757–3765.

Ochiai, N., Fujimura, M., Motoyama, T., Ichiishi, A., Usami, R., Horikoshi, K., and Yamaguchi, I. (2001). Characterization of mutations in two-component histidine kinase gene that confer fludioxonil resistance and osmotic sensitivity in the *os-1* mutants of *Neurospora crassa*. *Pest. Manag. Sci.* **57,** 437–442.

O'Mahony, R. J., Burns, A. T. H., Millam, S., Hooley, P., and Fincham, D. A. (2002). Isotropic growth of spores and salt tolerance in *Aspergillus nidulans*. *Mycol. Res.* **106,** 1480–1486.

O'Neil, J., Bugno, M., Stanley, M. S., Barham-Morris, J. B., Woodcock, N. A., Clement, D. J., Clipson, N. J. W., Whitehead, M. P., Fincham, D. A., and Hooley, P. (2002). Cloning of a novel gene encoding a C_2H_2 zinc finger protein that alleviates sensitivity to abiotic stresses in *Aspergillus nidulans*. *Mycol. Res.* **106,** 491–498.

Onishi, H. (1963). Osmophilic yeasts. *Adv. Food Res.* **12,** 53.

Pascual-Ahuir, A., Posas, F., Serrano, R., and Proft, M. (2001). Multiple levels of control regulate the yeast cAMP-response element-binding protein repressor Sko1p in response to stress. *J. Biol. Chem.* **276,** 37373–37378.

Paton, F. M., and Jennings, D. H. (1988). Effect of sodium and potassium chloride and polyols on malate and glucose 6-phosphate dehydrogenase from the marine fungus *Dendryphiella salina*. *Trans. Br. Mycol. Soc.* **91,** 205–215.

Pfyffer, G. E., Boraschi-Gaia, C., Weber, B., Hoesch, L., Orpin, G. C., and Rast, D. M. (1990). A further report on the occurrence of acyclic sugar alcohols in fungi. *Mycol. Res.* **94,** 219–222.

Posas, F., Chambers, J. R., Heyman, J. A., Hoeffler, J. P., de Nadal, E., and Arino, J. (2000). The transcriptional response of yeast to saline stress. *J. Biol. Chem.* **275,** 17249–17255.

Pott, G. B., Miller, T., Bartlett, J. A., Palas, J. S., and Selitrennikoff, C. P. (2000). The isolation of *FOS-1*, a gene encoding a putative two-component histidine kinase from *Aspergillus fumigatus*. *Fung. Gen. Biol.* **31,** 55–67.

Prade, R. A., Ayoubi, P., Krishnan, S., Macwana, S., and Russell, H. (2001). Accumulation of stress and inducer-dependent plant-cell-wall-degrading enzymes during asexual development in *Aspergillus nidulans*. *Genetics* **157,** 957–967.

Prior, C., Potier, S., Souciet, J. L., and Sychrova, H. (1996). Characterization of the NHA1 gene encoding a Na^+/H^+ antiporter of the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **387,** 89–93.

Prista, C., Almagro, A., Loureiro-Dias, M. C., and Ramos, J. (1997). Physiological basis for the high salt tolerance of *Debaryomyces hansenii*. *Appl. Environ. Microbiol.* **63,** 4005–4009.

Ramesh, M. A., Laidlaw, R. D., Durrenberger, F., Orth, A. B., and Kronstad, J. W. (2001). The cAMP signal transduction pathway mediates resistance to dicarboximide and aromatic hydrocarbon fungicides in *Ustilago maydis*. *Fung. Gen. Biol.* **32,** 183–193.

Ramos, A. J., Magan, N., and Sanchis, V. (1999). Osmotic and matric potential effects on growth, sclerotia, and partitioning of polyols and sugars in colonies and spores of *Aspergillus ochraceus*. *Mycol. Res.* **103,** 141–147.

Redkar, R. J., Locy, R. D., and Singh, N. K. (1995). Biosynthetic pathways of glycerol accumulation under salt stress in *Aspergillus nidulans*. *Exp. Mycol.* **19,** 241–246.

Redkar, R. J., Lemke, P. A., and Singh, N. K. (1996a). Altered gene expression in *Aspergillus nidulans* in response to salt stress. *Mycologia* **88,** 256–263.

Redkar, R. J., Lemke, P. A., and Singh, N. K. (1996b). Isolation of differentially expressed cDNA clones from salt-adapted *Aspergillus nidulans*. *Curr. Gen.* **29,** 130–135.

Redkar, R. J., Herzog, R. W., and Singh, N. K. (1998). Transcriptional activation of the *Aspergillus nidulans gpdA* promoter by osmotic signals. *Appl. Environ. Microbiol.* **64,** 2229–2231.

Reiser, V., Ruis, H., and Ammerer, G. (1999). Kinase activity-dependent nuclear export opposes stress-induced nuclear accumulation and retention of Hog1 mitogen-activated protein kinase in the budding yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10,** 1147–1161.

Reoyo, E., Espeso, E. A., Penalva, M. A., and Suarez, T. (1998). The essential *Aspergillus nidulans* gene *pmaA* encodes an homologue of fungal plasma membrane H^+-ATPases. *Fung. Gen. Biol.* **23,** 288–299.

Rep, M., Krantz, M., Thevelein, J. M., and Hohmann, S. (2000). The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. *J. Biol. Chem.* **275,** 8290–8300.

Saloheimo, A., Aro, N., Ilmen, M., and Pentilla, M. (2000). Isolation of the *ace1* gene encoding a Cys2–His2 transcription factor involved in regulation of activity of the cellulase promoter *cbh1* of *Trichoderma reesei*. *J. Biol. Chem.* **275,** 5817–5825.

Shawcross, S. G., Baker, S. M., Strike, P., and Faulkner, B. M. (1994). A sodium fluoride-sensitive mutant of *Aspergillus nidulans*. *Fung. Gen. News.* **41,** 81.

Smith, G. W., Ives, L. D., Nagelkerken, I. A., and Ritchie, K. B. (1996). Caribbean sea fan mortalities. *Nature* **383,** 487.

Spathas, D. H. (1978). A salt sensitive mutant on chromosome VI of *Aspergillus nidulans*. *Aspergillus Newsletter* **14,** 28.

Specht, C. A., Liu, Y., Robbins, P. W., Bulawa, C. E., Iartchouk, N., Winter, K. R., Riggle, P. J., Rhodes, J. C., Dodge, C. L., Culp, D. W., and Borgia, P. T. (1996). The *chsD* and *chsE* genes of *Aspergillus nidulans* and their roles in chitin synthesis. *Fung. Gen. Biol.* **20,** 153–167.

Stanley, M. S., Hooley, P., and Clipson, N. J. W. (1995). A rapid method of heterologous gene cloning using cotransformation of lambda genomic DNA banks in *Aspergillus nidulans*. *Fungal Genetics Newsletter* **42,** 76–77.

Tarutani, Y., Ohsumi, K., Arioka, M., Nakajima, H., and Kitamato, K. (2001). Cloning and characterization of *Aspergillus nidulans vpsA* gene which is involved in vacuolar biogenesis. *Gene* **268,** 23–30.

Thome, P. E., and Trench, R. K. (1999). Osmoregulation and the genetic induction of glycerol-3-phosphate dehydrogenase by NaCl in the euryhaline yeast *Debaryomyces hansenii*. *Mar. Biotech* **1,** 230–238.

Thome-Ortiz, P. E., Pena, A., and Ramirez, J. (1998). Monovalent cation fluxes and physiological changes of *Debaryomyces hansenii* grown at high concentrations of KCl and NaCl. *Yeast* **14,** 1355–1371.

Timberlake, W. E., and Clutterbuck, A. J. (1994). Genetic regulation of conidiation. *In* ''*Aspergillus*: 50 years on'' (S. D. Martinelli, and J. R. Kinghorn, eds.), pp. 383–427. Elsevier.

Tresner, H. D., and Hayes, J. A. (1971). Sodium chloride tolerance of terrestrial fungi. *Appl. Microbiol.* **22,** 210–213.

van der Green, P., Ruijter, G. J. G., and Visser, J. (1995). An extreme *creA* mutation in *Aspergillus nidulans* has severe effects on D-glucose utilization. *Microbiology* **141,** 2301–2306.

Van Eck, J. H., Prior, B. A., and Brandt, V. E. (1989). Accumulation of poly-hydroxy alcohols by *Hansenula anomola* in response to water stress. *J. Gen. Microbiol.* **135,** 3505–3513.

Virginia, M., Appleyard, C. L., McPheat, W. L., and Stark, M. J. R. (2000). A novel ''two-component'' protein containing histidine kinase and response regulator domains required for sporulation in *Aspergillus nidulans*. *Curr. Genet.* **37,** 364–372.

Watanabe, Y., Miwa, S., and Tamai, Y (1995). Characterization of a Na^+/H^+ antiporter gene closely related to the salt tolerance of yeast *Zygosaccharomyces rouxii*. *Yeast* **11,** 829–838.

Webster, J., and Davey, R. A. (1984). Sigmoid conidial shape in aquatic fungi. *Trans. Brit. Mycol. Soc.* **83,** 43–52.

Wernars, K., Goosen, T., Wennekes, B. M., Swart, K., van den Hondel, C. A., and van den Broek, H. W. (1987). Cotransformation of *Aspergillus nidulans*: A tool for replacing fungal genes. *Mol. Gen. Genet.* **209,** 71–77.

Wooton, J. C., Fraser, M. J., and Baron, A. J. (1980). Efficient transformation of germinating *Neurospora* conidia using total nuclear fragments. *Neurospora Newsletter* **27,** 33.

Yale, J., and Bohnert, H. J. (2001). Transcript expression in *Saccharomyces cerevisiae* at high salinity. *J. Biol. Chem.* **276,** 15996–16007.

Zaragoza, O., and Gancedo, J. M. (2000). Pseudohyphal growth is induced in *Saccharomyces cerevisiae* by a combination of stress and cAMP signalling. *Ant. Van Leeuw.* **78,** 187–194.

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Mycotoxin Research in South Africa

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I. South Africa—A Short Guide

As the name suggests, South Africa is a country that sits at the bottom of the continent of Africa. It is a place of extreme contrasts and contradictions. It has the most beautiful scenery and modern cities but also has informal settlements and ''squatter camps,'' poverty, and a high rate of diseases such as AIDS and tuberculosis with intensive agriculture and mining as sources of income. Many of its current difficulties stem from 40 years of an oppressive apartheid regime, which now thankfully has given way to a democratic constitution which has been in place for 8 years. It is part first world and part third world, which tends to reflect the composition of its population, the third world being represented by poor Black African (75%) and people of mixed blood (Colored 10%) and the first world by whites of European origin (12%) and Asians (3%) originally from India, although this demarcation is by no means exclusive and is changing in response to the new dispensation. The number of ethnic groups encompassed by these major racial divisions are several and varied, ranging from the Khoisan speakers or

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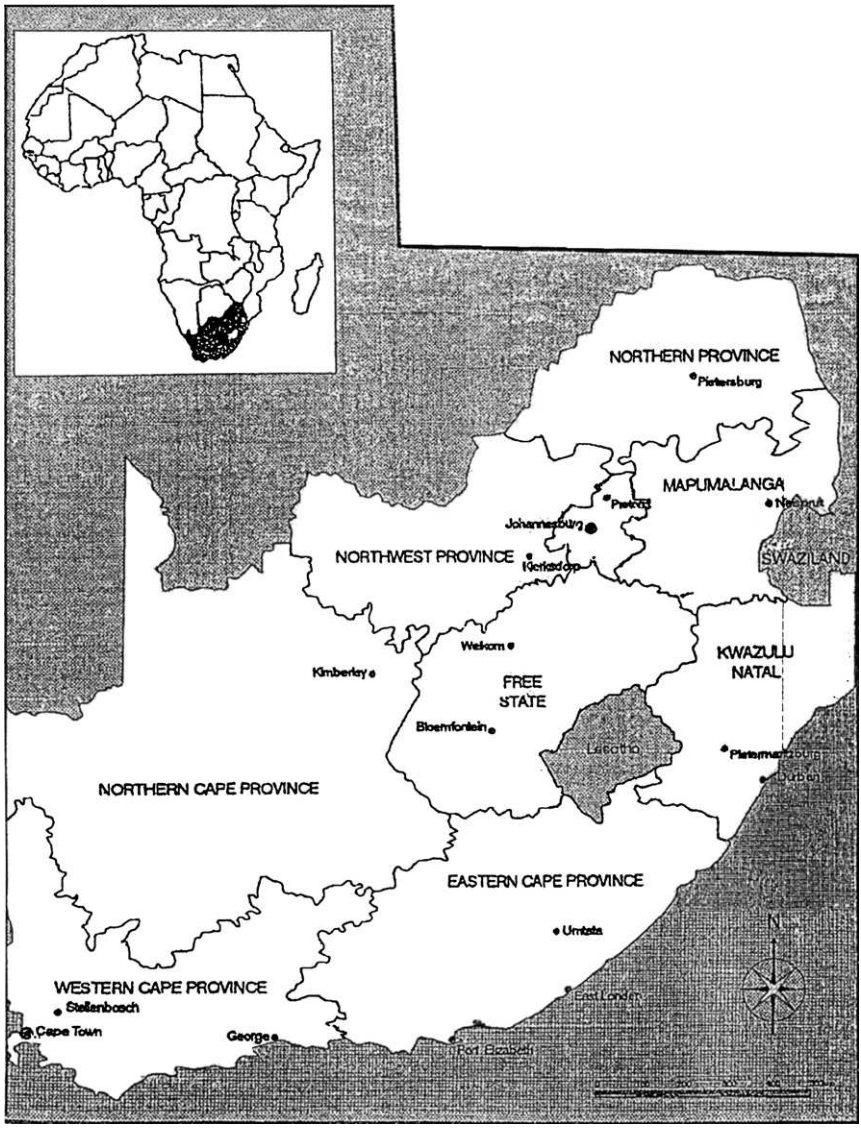

FIG. 1. Map of South Africa showing the provinces.

original Bushmen to Zulus, Xhosas, Afrikaaners, and various European language–speaking peoples. There are 11 official languages in South Africa. English is most used as a second language.

The country is divided into nine provinces (Fig. 1), which range in climate from the more temperate, cooler Western Cape to the subtropical maritime KwaZulu Natal and the higher-altitude Gauteng and Mpumalanga. Because of this range of conditions, nearly all types of agricultural commodities can be grown in South Africa. The Cape is the source of fruit, including a thriving grape and wine industry. Although the main staple of the African people is maize, which has replaced the African crop of sorghum, other cereals are grown, including wheat and barley in several provinces where higher altitude allows these temperate crops to flourish. There is a large meat and poultry industry, which makes great demands on feed mills and fodder crops and pasture. The result is a range of feed and food components which are susceptible to fungal infections and contamination by mycotoxins.

II. Introduction to South African Mycotoxin Research

South African science has long and successful history in the evaluation of mycotoxins with respect to identification, occurrence, and effects in both animal and human health. As with most research on mycotoxins and mycotoxicoses, the point of departure is taken as the identification of the aflatoxins and cyclopiazonic acid as causal agents of so-called turkey X disease (Blount, 1961). The early research on mycotoxins in South Africa included work not only on the aflatoxins but also on ochratoxin and sterigmatocystin (Purchase and Theron, 1967). Very soon after this, groups of scientists under the direction of Pieter (Piet) Steyn and Walter (Wally) Marasas commenced work on identifying mycotoxins, the fungi that produced them, and the associated diseases in animals and humans. In short order, over 100 mycotoxins were identified, characterized, or metabolically investigated. South Africa became a world leader in mycotoxin research. International collaborations were set up between South African mycotoxicologists and those in many countries of the world.

Once the euphoria of the ''time of plenty'' of mycotoxin discovery was passed, work commenced on more practical aspects, such as the importance of mycotoxins to South African agriculture and human health. The focus of this research is esophageal cancer, the contamination of agricultural commodities with mycotoxins, and chronic animal and human diseases. (Gqaleni *et al.*, 1997). This work became centered in two groups, located in Cape Town and Natal. The Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) (previously NRIND, National Research Institute for Nutritional

Diseases) under the directorship of Professor Wally Marasas, studied primarily the etiology of esophageal cancer in the Transkei (now part of the Eastern Cape Province) while the group under Professor Mike Dutton in Natal (now the Province of KwaZulu Natal) concentrated on agricultural problems. Other groups were also active in the area of mycotoxin research, such as the previous government's agricultural boards (e.g., Maize Board; see Vijoen *et al.*, 1993) and the Council for Scientific and Industrial Research (CSIR) (see Trinder, 1988, 1989). Perhaps the most important discovery of this period was that of the fumonisins as potential carcinogens by the PROMEC group with implications for the esophageal cancer research area. During the 1990s, both the PROMEC and Natal groups formed strong collaborative links to investigate esophageal cancer and the potential role of environmental agents in the cause of this terrible disease which is prevalent in the African rural population of South Africa (Figs. 2 and 3). This coordination has proved highly successful and has been extensively funded by the Cancer Association of South Africa.

A. Pre-Aflatoxin Research

Although modern mycotoxicology is generally thought to commence with the discovery of the aflatoxins (Goldblatt, 1969; Mclean and Dutton, 1995), this is a rather narrow view. There are many reported cases of earlier disease conditions from around the world which were related

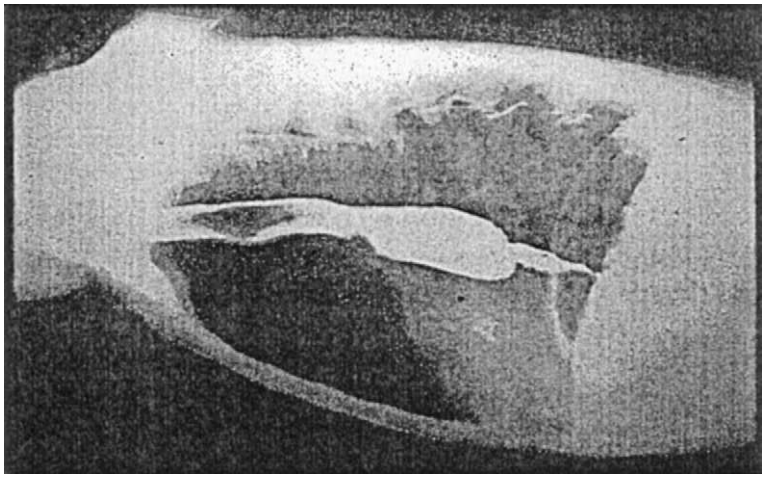

Fig. 2. Barium swallow showing cancerous obstruction in mid-esophagus. Permission of Professor A. A. Haffejee.

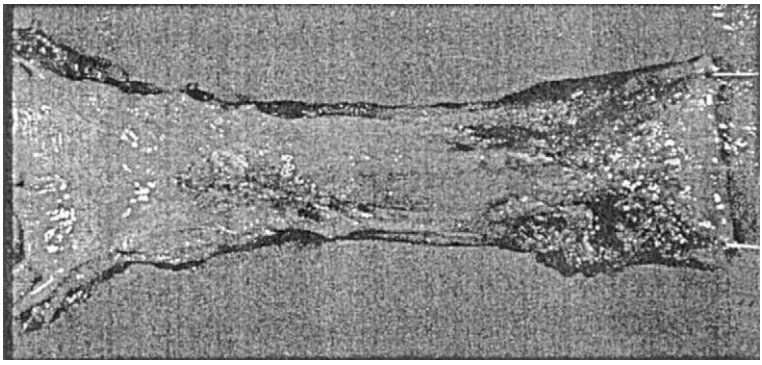

FIG. 3. Carcinoma of lower end of esophagus. Permission of Professor A. A. Haffejee

in some way to moldy foods and feeds. The classic case is that of ergotism, which was well documented in the Middle Ages in Europe and in the nineteenth century was shown to be a mycotoxicosis (Bennett and Bentley, 1999). Other examples include yellow rice poisoning in Japan (Saito *et al.*, 1971) and alimentary toxic aleukia in Russia (Joffe, 1978). In South Africa, it was noticed early on that esophageal cancer was prevalent in certain areas of the Transkei (Rose, 1965), as well as in other countries such as China and Iran. Because of the regional character of this disease, it was suspected that some unknown environmental factor was involved. Other veterinary diseases in South Africa were also described and related to fungal toxins (e.g., Mitchell, 1919).

B. THE GOLDEN AGE OF MYCOTOXIN DISCOVERY: MYCOTOXIN ISOLATION AND CHARACTERIZATION

The Golden Age commenced after the identification of the aflatoxins and the discovery of their powerful carcinogenic properties. The first toxin to be identified by South African scientists was ochratoxin A (Van der Merwe *et al.*, 1965). It was isolated from a strain of *Aspergillus ochraceus*, hence the name. Ironically, ochratoxin A is a major problem in the temperate climates of Northern Europe but for some reason is rarely found in South Africa. Nevertheless, the discovery of ochratoxin led to an intensive investigation of toxic fungal products and their modes of biosynthesis. Such studies were made possible by the development of techniques such as ^{13}C nuclear magnetic resonance (NMR) spectroscopy, which allowed Professor Steyn's group to investigate toxins produced from fungal cultures fed labeled precursors.

A few examples of the many that this group worked on will serve to exemplify the work. Some of the earlier characterization studies were those of secalonic acid D (Steyn, 1970), diplosporin (Chalmers *et al.*, 1978), fusarin C (Gelderblom *et al.*, 1984), and that of the austocystins (Steyn and Vleggar, 1974). In the latter study, six metabolites related to sterigmatocystin derived from *Aspergillus ustus* were characterized. It was a classical study illustrating the power of NMR spectroscopy and chemical deduction. The compounds themselves were of metabolic interest as they contained the bisdihydrofurano structure found in aflatoxin and its biosynthetic congeners. In fact, austocystin F is the hydroxy derivative of sterigmatin, a metabolite produced by *A. versicolor*. A similar study on a group of metabolites, the austalides, gives another example of the use of advanced instrumentation to solve molecular structure. The methods used included NMR spectroscopy and x-ray diffraction (Horuk *et al.*, 1981). Later, more use was made of isotope feeding to mycotoxin-producing fungi, where the toxins were specifically labeled according to the labeled precursor used. Thus, a combination of skilled culturing technique by Amelia De Jesus was brilliantly complemented by the advanced NMR spectroscopy under the leadership of Piet Steyn. This partnership elucidated the ^{13}C assignments in *Fusarium verticilliodes* pigments (Steyn *et al.*, 1979); the biosynthesis of cyclopiazonic acid (De Jesus *et al.*, 1981a); the biosynthesis of citreoviridin (Steyn *et al.*, 1982) using ^{13}C labeling and ^{18}O labeling (Steyn and Vleggar, 1985); penitrems (De Jesus *et al.*, 1981b, 1983), and the structure of the janthitrems (De Jesus *et al.*, 1984).

The efforts of Professor Steyn and his group greatly expanded our knowledge and understanding of mycotoxins, reflected in the many publications generated by this group. For reviews, see, for example, Steyn, 1977a,b, 1979, 1984, and 1995, and Steyn *et al.*, 1980. It should also be acknowledged that these studies were supported by other biochemists such as Piet Thiel and a group of dedicated mycologists, including Christian Rabie and Wally Marasas, the latter being a world author on *Fusarium* taxonomy (Marasas *et al.*, 1984).

C. Metabolic Studies

The elucidation of secondary metabolism and the biosynthesis of secondary metabolites is an interesting aside to the mainstream of biochemistry, which in the mid-twentieth century was more focused on intermediary metabolism of essential or ‘‘primary’’ metabolites. As mycotoxins are a subclass of secondary metabolites, an understanding of this phase of metabolism is a prerequisite to understanding that of

mycotoxins. The scope of this chapter does not allow for an in-depth discussion of the subject and the reader is directed to suitable treatises on secondary metabolism, e.g., Firn and Jones (2000) and Walton (2000). The unraveling of the metabolic pathways and the attendant molecular biology of mycotoxins are not just academic exercises, because an understanding allows for the control of their production in agricultural and related situations. The maintenance of food quality and the health of both animals and humans depends upon the minimization of mycotoxin contamination. One of the first mycotoxins to be studied in depth from the metabolic point of view was aflatoxin B_1. A convenient approach, as has been mentioned, was the use of NMR spectroscopy utilizing ^{13}C and other amenable isotopes and elements. As in the case of mycotoxins in general, the team under Steyn added greatly to our understanding of aflatoxin B_1 synthesis using these techniques (De Jesus *et al.*, 1980; Steyn *et al.*, 1975; Gorst-Allman *et al.*, 1977; Steyn *et al.*, 1979). However, to a biochemist this was not entirely a satisfactory or rigorous solution, as pathways in intermediary metabolism are characterized by the enzymes that catalyze the individual steps in the pathway. For various reasons, the enzymology of secondary metabolism is technically difficult (Dutton, 1988; Bentley and Bennett, 1999). However, work toward this goal was attempted by several groups and Dutton's group in South Africa continued at the University of Natal work already begun in the United Kingdom on enzymology (Dutton and Anderson, 1982). The first enzyme identified was a methyl transferase responsible for converting sterigmatocystin, an intermediate in aflatoxin B_1 biosynthesis to O-methylsterigmatocystin (Jeenah and Dutton, 1984). The significance of this was not appreciated at the time and it was concluded that O-methylsterigmatocystin was a metabolic shunt product. Later work (e.g., Bhatnagar and Cleveland, 1988) indicated that O-methylsterigmatocystin was in fact a compulsory intermediate between the step sterigmatocystin $\rightarrow$ aflatoxin B_1. At the time, this created rational objections in that methylated derivatives are usually more metabolically inert, certainly toward oxygenases required to cleave one of the aromatic rings of sterigmatocystin, and it also introduced what appeared to be an unnecessary step. A further series of cell-free experiments were carried out by Gengan *et al.*, 1999, which confirmed that sterigmatocystin had to be alkylated prior to its conversion to aflatoxin B_1. Several synthesized alkyl derivatives, however, could undergo this conversion. The propyl derivative was more rapidly converted than the other alkyl derivatives, including methyl. The conclusions from these studies, however, were somewhat at odds with results from molecular biology studies (e.g., Keller *et al.*,

1993; Motomura *et al.*, 1999), where it seems quite clear that O-methylsterigmatocystin is an obligatory intermediate in aflatoxin B_1 biosynthesis and other alkyl derivatives are not involved. It certainly is true to say that secondary metabolism is always full of surprises and is very different from that of intermediary metabolism, e.g., hexanoate synthase in aflatoxin B_1 biosynthesis (Hitchman *et al.*, 2001). For aflatoxin B_1 biosynthesis, the full story may be difficult to finally resolve because of the types of enzymes involved in the latter stages of the pathway. Some of these are oxygenases presumably related to the cytochrome P450 type, which are membrane bound and therefore not amenable to easy isolation as functional proteins, although several of these oxygenases have been identified at the genetic level (Yu *et al.*, 1997; Yabe *et al.*, 1999; Keller *et al.*, 2000).

One of the reactions involved in the anthraquinonoid part of the pathway was isolated and partly characterized and named norsolorinic acid dehydrogenase (Chuturgoon and Dutton, 1991). It is responsible for the interconversion of norsolorinic acid and averantin. Another interesting observation was that propionate herbicides, whose mechanism depends on the inhibition of acetyl coenzyme carboxylase, were found to block aflatoxin biosynthesis (Chuturgoon *et al.*, 1998). It was conjectured that this was due to the inhibition of malonyl coenzyme A, an important intermediate in polyketide synthesis.

A different type of study by McLean and coworkers at the University of Natal was undertaken to find out what effects aflatoxin B_1 had on plant metabolism and development. Aflatoxin B_1 inhibited tobacco callus and regenerating plantlets, rootlet formation being more inhibited than shoot (McLean *et al.*, 1995). The effects were progressively pronounced with increase in aflatoxin B_1 concentration correlated with a decrease in chlorophyll concentration in comparison to control plantlets (Mclean *et al.*, 1994a,b). Similar results were found in aflatoxin B_1–treated germinating immature maize embryos (Mclean *et al.*, 1992, 1993).

III. Mycotoxins and Agriculture in South Africa

South Africa has a large agricultural industry so much research and investigation has been done on mycotoxins in commodities and animal disease and metabolism (e.g., Westlake *et al.*, 1987a, 1987b, 1989). As has been mentioned, much of this research mirrors work done elsewhere in the world. Screening programs conducted on food (Luck *et al.*, 1976a,b; Dutton and Westlake, 1985a; Lotter and Khrom, 1988) and other agricultural products (Rabie *et al.*, 1986, Thiel *et al.*, 1986;

Sydenham *et al.*, 1989; Dutton and Westlake, 1985b; Dutton and Kinsey, 1995, 1996; Viljoen *et al.*, 1993) have shown that South African commodities have mostly the same toxins found elsewhere with the odd exception of the ochratoxins, which are found only rarely (one positive found by Viljoen *et al.*, 1993). Currently, the major threat is the ubiquitous occurrence of fumonisin B_1. In most domesticated species, levels have to climb into the ppm levels before disease symptoms in animals occur, due to the toxin being poorly absorbed from the gastrointestinal tract (Shephard *et al.*, 1994a). However, there have been various outbreaks of mycotoxicoses in South Africa, e.g., in dogs (Bastianello *et al.*, 1987). In pigs, poor production is often reported with vulvovaginitis being the most obvious mycotoxicosis when the maize-growing season favors zearalenone production (Aucock *et al.*, 1980; Sydenham *et al.*, 1988). Poultry are highly susceptible to toxigenic strains of *Diplodia maydis* (Rabie *et al.*, 1987), and sheep have suffered from mycotoxin-related disease conditions, such as lupinosis (Van Warmelo *et al.*, 1970), stachybotryotoxicosis (Schneider *et al.*, 1979), and facial eczema (Marasas *et al.*, 1972). Horses developed equine leucoencephalomalcia when fed material contaminated with *Fusarium verticilliodes* (Marasas *et al.*, 1976; Pienaar *et al.*, 1981) whereas ruminants have been reported to suffer from paspalum staggers (Ehret *et al.*, 1968) and diplodiosis (Mitchell, 1919). The discovery of the fumonisins in 1988 resolved the cause of leukoencephalomalacia. It was shown that horses fed pure fumonisin B_1 developed the disease (Marasas *et al.*, 1988; Thiel *et al.*, 1991).

Other problems in South African cereals are related to sorghum production. Sorghum is a more drought-tolerant crop than maize and hence is not so susceptible to mycotoxin contamination. Problems do arise, however, where sorghum is malted for use in the indigenous rural sorghum beer production (Trinder, 1989). Malting practices tend to be of the traditional sort where a layer of moist sorghum is laid on a concrete floor, yielding a dry upper crust, a sprouted middle layer, and a waterlogged bottom layer that is full of fungi and bacteria (Fig. 4).

South African fruits, a major export, are also susceptible to mycotoxin contamination. The occurrence of patulin in apples and apple juice has been studied by both PROMEC (Sydenham *et al.*, 1997) and the Natal groups (Dutton and Westlake, 1985a). In both cases, patulin has been detected and efforts have been made to reduce levels of the toxin to below legal limits. Removal of infected apples and washing of apples in flumes has done a lot to control the situation to allow export to continue. This control of toxin levels is important in all exported commodities due to the introduction of limits by the Codex

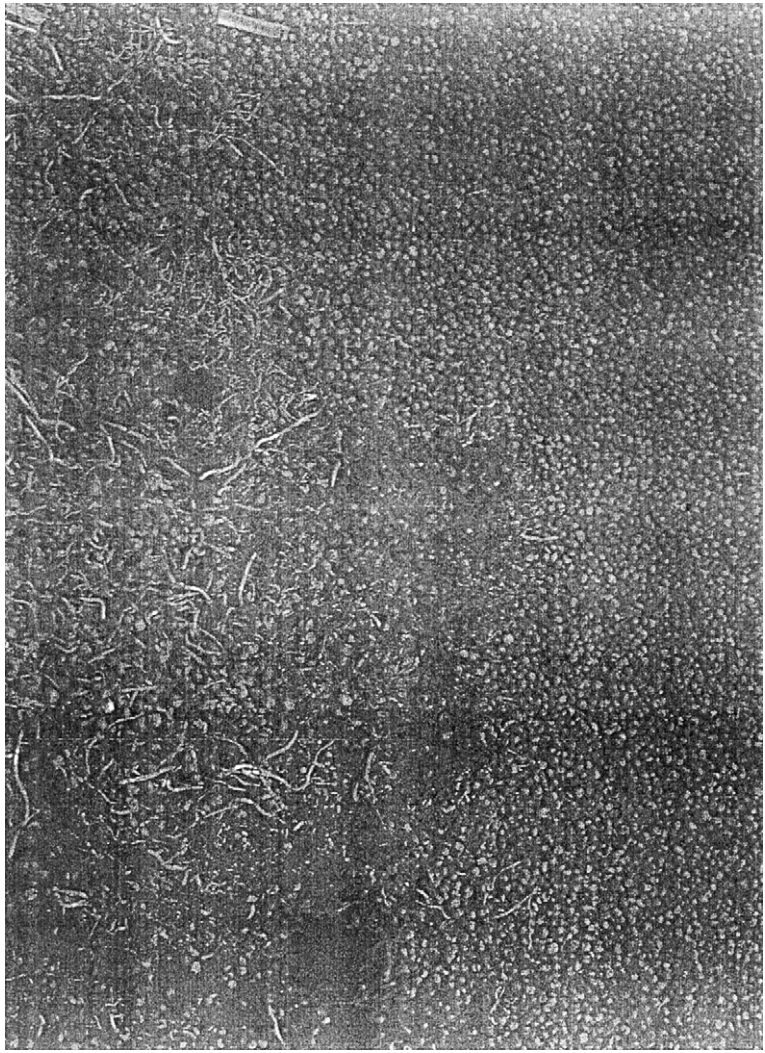

FIG. 4. Sorghum being malted on a concrete slab. Note areas of fungal growth. Permission of Professor J. Taylor.

Alimentarius committee. It has been estimated that the current control levels alone will cost Africa U.S. $0.5 billion per annum in lost exports (Otsuki *et al.*, 2001a,b). The quality control of agricultural produce in terms of mycotoxin level is of prime economic importance.

IV. Chronic Disease

A. General

There have been many chronic diseases of animals and humans reported in Southern Africa, some of which are putatively related to environmental toxic agents. Many arise due to the ingestion of indigenous plants that were not the normal forage of farm animals brought in by European settlers, particularly in arid areas such as the Karoo, where plants can accumulate novel toxins (Kellerman *et al.*, 1988). As has been mentioned, veterinary mycotoxicoses are similar to those found in other parts of the world, e.g., vulvovaginitis in pigs (Aucock, 1980). On the other hand, the identification of chronic mycotoxin-related disease in humans is much more problematic. There are rigorous criteria that need satisfying before such relationships can be made, e.g., a dose–response relationship between the mycotoxin and the disease (Richard and Thurston, 1986). Hsieh (1989) reviews this subject and lists five requirements, number one being the occurrence of the mycotoxin in foodstuffs. The minefield and confusion surrounding this subject is admirably reviewed in an article by Bennett and Klich (2003).

Esophageal cancer was reported as occurring at above average incidence in areas of the Transkei as early as the late 1950s (Burrell, 1957). A more concrete association of a disease with mycotoxin ingestion was that of hepatocellular carcinoma. On the discovery of aflatoxin B_1 as a powerful liver carcinogen, a correlation was made with consumption of contaminated groundnuts (Hag Elamein *et al.*, 1988). It is clear that aflatoxin B_1 contributes to the occurrence of liver cancer together with other agents, e.g., hepatitis B virus (Sylla *et al.*, 1999). This situation is mirrored in other chronic diseases of an environmental origin, where several factors converge to cause the condition. This is especially true of cancer induction in general, making it difficult to say with certainty what agent has what role. Cancers are the third most common killer in South Africa, and over the years the South African National Cancer Institute has compiled several reports on the prevalence and incidence of cancers (e.g., Sitas *et al.*, 1997). The cancer statistics show different rates of occurrence for different population groups and different types of cancer. For example, the five top cancers in African males (Black) were in order: esophagus, prostate, lung, liver, and mouth, whereas in White males it was prostate, bladder, colorectal, lung, and melanoma (Table I). Several of the more common conditions found in southern Africa will now be considered.

TABLE I

SUMMARY LIFETIME RISKS (LR) FOR THE TOP FIVE CANCERS IN THE REPUBLIC OF SOUTH AFRICA BY SEX AND POPULATION GROUP

Male			Female	
Population group	Cancer	LR	Cancer	LR
Asian	Stomach	1 in 64	Breast	1 in 20
	Prostate	1 in 67	Cervix	1 in 58
	Colorectal	1 in 71	Stomach	1 in 68
	Bladder	1 in 74	Uterus	1 in 91
	Lung	1 in 91	Colorectal	1 in 94
	All cancers	1 in 7	All cancers	1 in 6
Black	Esophagus	1 in 39	Cervix	1 in 26
	Prostate	1 in 58	Breast	1 in 68
	Lung	1 in 89	Esophagus	1 in 97
	Liver, bile duct	1 in 137	Uterus	1 in 204
	Mouth	1 in 185	Liver, bile duct	1 in 400
	All cancers	1 in 8	All cancer	1 in 9
Colored	Prostate	1 in 35	Cervix	1 in 30
	Stomach	1 in 52	Breast	1 in 33
	Lung	1 in 53	Colorectal	1 in 139
	Esophagus	1 in 56	Stomach	1 in 164
	Bladder	1 in 88	Lung	1 in 227
	All cancers	1 in 5	All cancers	1 in 8
White	Prostate	1 in 20	Breast	1 in 15
	Bladder	1 in 37	Colorectal	1 in 52
	Colorectal	1 in 40	Melanoma	1 in 80
	Lung	1 in 41	Cervix	1 in 83
	Melanoma	1 in 65	Ovary	1 in 108
	All cancers	1 in 4	All cancers	1 in 5

Permission Dr. F. Sitas.

B. LIVER CANCER

Hepatocellular carcinoma is relatively common among people living in sub-Saharan Africa, areas in west Africa (Onyemelukwe *et al.*, 1982), central Africa (Coulter *et al.*, 1986), and eastern Africa (Peers and Lindsell, 1973). In southern Africa, as in the rest of the continent, the correlation of liver cancer and estimated exposure to aflatoxin is a

graded dose response with extremely high daily doses (15 μg/adult/day aflatoxin) being recorded in some areas (Keen and Martin, 1971; van Rensburg *et al.*, 1974, 1975). Van Rensburg *et al.* (1985) reviewed the situation in the Transkei and Mozambique and demonstrated a direct correlation ($r = 0.95$) of the log liver cancer incidence versus log intake of aflatoxin from different parts of the world. The levels of liver cancer were found to be very high in areas of southern Africa with incidence levels of up to 13 cases per 100,000 population correlated to aflatoxin intake in Swaziland (Peers *et al.*, 1976). Consequently, it was hypothesized that hepatocarcinoma could be associated to groundnut consumption as a staple. The connection, however, was not straightforward, as hepatocarcinoma also correlated with occurrence of hepatitis, in particular, hepatitis B, infection of the liver (Peers *et al.*, 1987).

In several studies (e.g., Dutton *et al.*, 1998), aflatoxin B_1 and hepatitis B antibodies were identified in liver biopsy material from liver cancers. Both aflatoxin B_1 and fumonisin B_1 have been identified in liver cancer material by immunocytochemical means. Of 20 liver cancer samples examined, 14 were positive for aflatoxin B_1, 12 were positive for fumonisin B_1, with 8 being positive for both (Padayachee, B. Med. Sci. Honours Project, 2002, University of Natal). It would seem, however, that the major carcinogen is aflatoxin B_1, as transversion mutation in the p53 gene is found in human hepatocarcinoma. This mutation is a biomarker of aflatoxin exposure (Lasky and Magder, 1997).

C. Idiopathic Congestive Cardiopathy

This term was coined by a physician, Dr. George Campbell (Madadeni and Themba), who in the 1980s observed an unusual disease in rural hospitals where he practiced involving general major organ failure (Campbell, 1990). The symptoms of idiopathic congestive cardiopathy were damage to the heart (which became swollen), edema (mainly in the extremities of the leg, also indicating heart failure), massive dilation of the left deep neck vein, and failure of the kidneys and liver. Other associated observations were gynecomastasia and loss of skin pigmentation. The condition was mainly, but not exclusively, found in older men. Dr. Campbell concluded that that the syndrome was a result of a long toxic process that also involved malnutrition, avitaminosis, smoking, and alcohol abuse. An environmental toxin that triggered or exacerbated the condition was also a possible etiological agent. Much effort went into screening food that was consumed by the rural people who were affected (Dutton *et al.*, 1993) but nothing significant was found, although *Fusarium* spp. and some of their toxins were

sporadically present. However, fumonisins were not screened. Recent findings have shown that fumonisin B_1 can affect the heart (Haschek *et al.*, 2001), causing damage to the left heart chamber and lung edema in pigs as well as in horses (Smith *et al.*, 2002). It is still open to debate whether fumonisin B_1 is the missing etiological agent in idiopathic congestive cardiopathy. Further, zearalenone was often found in rural maize (Marasas *et al.*, 1977) and its presence could be connected with gynecomastasia (Campbell, 1990), although alternatively this may be due to liver damage. Kojic acid was also detected, which has been shown to interfere with pigment production (Curto *et al.*, 1999).

D. Respiratory Disease

Chronic respiratory diseases are common around the world. Many are connected to allergenic responses that can become life threatening in the form of asthma and related pathologies. In underdeveloped countries, allergic reactions may be exacerbated by malnutrition and underlying disease such as tuberculosis (TB). Many allergenic agents are of fungal origin in the form of spores, fragments of cell wall, and mycotoxins. Sometimes final disease in the lung progresses to frank mycosis in the form of, e.g., aspergillosis, where *Aspergillus fumigatus* and other species invade the lung tissue. HIV-positive individuals who are in advanced stages of AIDS are particularly susceptible to infectious agents such as *Cryptococcus* (Sugar, 1991).

The problem of respiratory distress due to poor air quality, often because fungi have grown in the system and are being dispersed in the air, is found in so-called ''sick building'' syndrome common in air-conditioned buildings. Similar illnesses can occur in any buildings if conditions are right. In South Africa, this ranges from informal settlements (squatter camps) to poorer housing to public buildings such as office blocks and hospitals. The group at the University of Natal began a screening program to investigate air quality and respiratory problems in informal settlements and poorer housing under the direction of Dr. N. Gqaleni (Gqaleni *et al.*, 1999; Danaviah *et al.*, 2000). Similar study was done on poorer urban housing (Sekota *et al.*, 2000a) and efforts were made to control the growth of fungi using commercial fungicides (Sekota *et al.*, 2000b).

E. Esophageal Cancer

Esophageal cancer is a research topic under active investigation by the mycotoxin research groups in South Africa. An esophageal cancer consortium has been formed between the PROMEC and Natal groups,

funded by the Cancer Association of South Africa. Studies have been concentrating on the fungal and mycotoxicosis aspects.

Early on, major efforts were made by the PROMEC group to find foodborne agents that could be related to the disease, including the isolation of fungi and known mycotoxins. The main food staple of the rural African people along the eastern seaboard of South Africa is maize, so it is not surprising to find that the predominant fungi isolated were members of the *Fusarium* genus (Marasas *et al.*, 1979, Thiel *et al.*, 1982). A search for a *Fusarium* toxin that could produce cancer in a model animal, the rat, resulted in the discovery of the fumonisin B_1 and B_2 (Gelderblom *et al.*, 1988; Bezuidenhout *et al.*, 1988). Fumonisin B_1 and fumonisin B_2 were found in maize classed as ''good'' in both high and low esophageal cancer areas of the Transkei. There was a significant difference in a survey done in 1985, i.e., fumonisin B_1 ranged from 0 to 550 μg/kg in the low areas and 50 to 7900 μg/kg for high (Sydenham *et al.*, 1990). In a 1989 survey, fumonisin B_1 levels went up to 5380 μg/kg in ''good'' maize high esophageal cancer areas but there was no significant difference between low and high esophageal cancer areas (Sydenham *et al.*, 1990; Rheeder *et al.*, 1992). The practice of using maize with high levels of fungal infection for home-brewing beer is problematic. Some evidence suggests a correlation between esophageal cancer incidence and the consumption of this beverage (Segal *et al.*, 1988).

Fumonisins are present in commercially grown maize, although at lower levels than for rural material. Maize meal from South Africa had levels of fumonisin B_1 ranging up to 475 μg/kg and that from Egypt had levels between 1780 and 2980 μg/kg (Sydenham *et al.*, 1991). A rather novel method was devised by the PROMEC group in South Africa in using hair of primates dosed with fumonisin B_1 for analysis (Sewram *et al.*, 2001). They showed that there is a correlation between exposure and toxin residue in the hair. This method may provide an excellent noninvasive way of monitoring the human population for fumonisin B_1 exposure.

The sustained thrust of research into mycotoxins and associated chronic diseases in South Africa has been and continues to be dependent upon PROMEC and the leadership of Professor Wally Marasas. His contribution can be gauged by the large number of publications on the subject and overviews and reviews written over the years (e.g., Marasas and van Rensburg, 1979; Marasas, 1982; Marasas *et al.*, 1984, 1993, 1995, Marasas, 1996).

The Mycotoxin Research Unit at the University of Natal, South Africa, commenced its work on esophageal cancer in 1995. Access to cancerous tissues through the services of surgeons at the Nelson R.

Mandela Medical School, most notably Professor A. A. Haffejee, has facilitated such research. Two approaches have been used. One method was to probe tissue using antibodies to fumonisin and visualizing them by either gold probes or dye reactions, i.e., by immunocytochemical means. The first study focused on esophageal cancer tissue and peri-cancer tissue surgically removed from African patients with the disease. Tissue was probed with a fumonisin B_1 antibody, which was then detected using gold probes attached to antibodies sensitive to the primary one and electron microscopy. Stringent controls were included and it was found that eight of the esophageal cancer samples were positive but not the peri-cancer tissues. The experiments were done blind. It was interesting to find that the two tissues not positive were from South African Indian patients, the other eight all being African patients originating or living in the Transkei. Although at first sight these results seem to support the notion that fumonisin B_1 is directly involved with human esophageal cancer, on reflection that conclusion created more questions than it answered (Myburg, M. Med. Sci., Thesis, University of Natal, 1998).

If fumonisin B_1 is involved in esophageal cancer, as suggested, then there are also other ways in which the disease is initiated or promoted. The two South African Indian patients showed no sign of fumonisin in their tissues. Did the fumonisin B_1 enter the cancerous tissue prior to, hence being causal, or after the cancer formation? Did the fumonisin enter from the gastrointestinal tract side or from the internal blood vessel side? Why did this tissue specifically accumulate fumonisin B_1? How did the fumonisin B_1 bind to the tissue? Of these questions, the last seemed the most amenable to answer. That fumonisin B_1 is strongly tissue-bound seems fairly certain as the tissues subjected to immunocytochemical labeling were washed well and dehydrated with alcohol in which fumonisin B_1 is soluble. Currently, the possibility that trans-glutaminase is responsible for the binding process, with fumonisin B_1 as an acceptor amine for cross-linking to protein, is being investigated.

Another important question was the level of exposure to fumonisin B_1 that the rural African population faced in their daily lives. One approach developed by the group was the analysis of feces for the toxin and its breakdown products, based on the method of Shephard *et al.* (1994b), who attempted to reconcile the radiolabeled fumonisin dosed to monkeys by recovering the dose from the sum total found in tissues, urine, and feces. The bulk of the toxin was found in feces. It was either passed straight through the gastrointestinal tract or was excreted via the bile into the gastrointestinal tract. When samples of faeces collected

from school children for parasite investigation were also analyzed for fumonisin (Chelule *et al.*, 2000), the results were disturbing. Twenty-three percent of samples were positive, ranging from 0.6 to 20 μg/g fumonisin B_1. In addition, 10% of the urban control samples were positive in the same range. In a rural population (Chelule *et al.*, 2001) 33% of samples were found to be positive with a range of 513 to 39000 ppb. As 6% of the urban controls were positive (with the highest level being 16200 ppb,) these results suggested that people living in the urban area of Durban consumed contaminated maize. South African esophageal cancer may mirror the situation in China, as fumonisin B_1 producing strains isolated in South Africa by the PROMEC group produce nitrosamines in conjunction with fumonisin B_1. Moreover, the cytochrome P450 genes found in the Chinese population are also present in the South African population (Chelule, PhD thesis, 2002). The relative roles of *Fusarium verticilliodes*, fumonisin B_1, and nitrosamines in the etiology of esophageal cancer remain under investigation.

F. Pre-Eclampsia and Eclampsia

Pre-eclampsia is a worldwide condition prevalent in pregnant Africans. It has been defined as hypertension in pregnant women where the blood pressure is 140/90 mm Hg and higher when taken on two occasions of 6 hours apart. The incidence of this condition is much higher in African populations, especially along the eastern seaboard of South Africa, than that found in the industrialized world, e.g., 18% of all admissions to the Obstetric Unit, King Edward Eighth Hospital, Durban, have some degree of hypertension (Moodley, 1999, personal communication). In South Africa, the condition often progresses into eclampsia, which is marked by convulsions, and in KwaZulu Natal is almost invariably fatal. The cause of pre-eclampsia/eclampsia is not known nor is the reason for its high prevalence in KwaZulu Natal. During the development of fumonisin B_1 analysis by HPLC in physiological fluids, samples of blood from pre-eclamptic patients were analyzed and found to contain the toxin (Moodley *et al.*, 2001). This observation came as a surprise because there is no known link between the disease and the toxin. Other factors are thought to be involved as prime causes in industrialized nations (Habeck, 2000). As already demonstrated in primates and rats, doses of fumonisin B_1 were poorly absorbed and eliminated, with very short half-lives in the blood. The results, however, were confirmed by ELISA and the identification of fumonisin B_1 by mass spectrometry (Moodley *et al.*, 2001).

Another trial was immediately commenced and fumonisin B_1 was identified in the blood, cord blood, and placental tissues of pre-eclamptic (Fig. 5) and eclamptic patients. It was also in the pregnant controls, with a statistically higher value for the eclamptic's blood over the other two groups (Coumi, 2000; Master's thesis, University of Natal). As with esophageal cancer patients, there is no direct evidence to connect the presence of fumonisin B_1 with pre-eclampsia; furthermore, its presence has to be explained in light of the results with animals dosed with fumonisin B_1. It seems that rural Africans are subjected to fumonisin B_1 in their diets from the womb (found in cord blood) to the tomb on a daily basis. It is likely, therefore, that their systems become saturated with the toxin over a very long period, possibly resulting in a basal level being present in the tissues and the total load at any time varying with current intake. Maize is the staple diet of these people and it can be consumed up to three times per day. Coincidentally, this fluctuation can be used to gauge the immediate exposure of these people to the toxin. As fumonisin B_1 causes chronic liver and kidney damage (Badria *et al.*, 1996), it is not unreasonable to suppose that malfunction of these organs contributes to the slower elimination of the toxin. The finding of the toxin in human cord blood is somewhat at variance with observations of other pregnant animals,

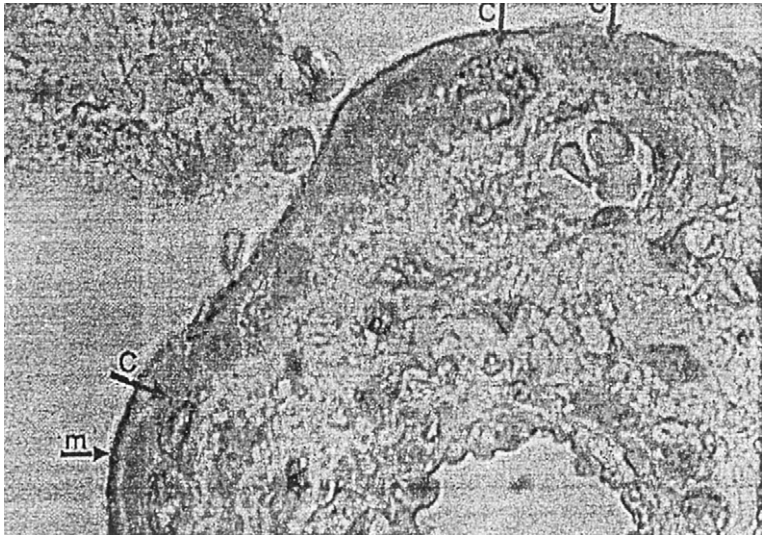

FIG. 5. Placental tissue from a pre-eclamptic patient, stained for fumonisin B_1 by immunocytochemical means (DAB stain, brown positive, C = cytotrophoblastic cells, m = microvillous brush border).

TABLE II

FUMONISIN B_1 LEVELS FOUND IN THE BLOOD OF 8 PATIENTS (5 AFRICAN, 2 WHITE, AND 1 INDIAN) WITH VARIOUS TYPES OF BRAIN LESION OR TUMORS TREATED AT WENTWORTH HOSPITAL, DURBAN

Tumor type	Age	Sex	Race	Concentration fumonisin B_1 (ng/ml)
Pineoblastoma	66	F	African	11.25
Metastatic cancer	53	F	White	459.40
Metastic cancer	59	M	African	164.64
Medulloblastoma	14	M	African	6.18
Choroid plexus papilloma	14	M	African	16.85
Low-grade glioma	5	F	African	6.32
Glioblastoma	2	F	Indian	41.97
Meningioma	53	M	White	9.10
Mean				89.5
Controls (N = 9) mean	25	M	African	6.8

where fumonisin B_1 administered at low levels did not pass the placental barrier (Voss *et al.*, 2001). In these experiments, however, fumonisin was embryotoxic at high maternal doses in pregnant pigs. (Zomborsky-Kovaks *et al.*, 2000) In conclusion, the presence of high levels of fumonisin B_1 may simply reflect the poverty of the susceptible population. All around the world, preeclampsia is correlated with poverty.

The presence of fumonisin B_1 in the blood and tissues of people dwelling in KwaZulu Natal was further confirmed by a study on tissue samples gathered from patients with various brain lesions, mostly different form of brain cancer. (Palanee, personal communication, PhD project, University of Natal). The purpose of the study was to see whether lesions similar to those found in equine leukoencephalomalacia could be discerned in humans and, if so, whether there was any evidence to support a role for the fumonisins in human brain disease. Again, fumonisin B_1 was commonly detected in the blood by HPLC and in tissue by immunocytochemical methods (Table II). The possibility that fumonisin B_1 can cause damage to the human central nervous system cannot be ignored.

G. OTHER CONDITIONS

Several rare disease conditions found in South Africa have raised questions as to whether they are mycotoxin-linked, mainly because of their restricted geographical distribution. Leiomyopathy (Rode *et al.*, 1992) is found in certain areas of the northern border region of the

Transkei (Hadley, personal communication, 1996). This disease is found mainly in male children of around 10 years in age and is characterized by a progressive fibrosis of smooth muscle of the colon. The only treatment is surgical, resulting in the child's having a colostomy. As the disease is progressive, it recurs until the child dies. In certain cases, fibrosis is found in the smooth muscle of the blood vessels. It is tempting to suggest that mycotoxins such as fumonisin B_1 are involved because of their accumulation in the feces. An equal possibility is the use of traditional medicinal plants as enemas in such children, which could also act in concert with the mycotoxin. Other diseases of limited distribution with possible connection to nutritional factors are onyalai (Rabie *et al.*, 1975) and meselini joint disease (Marasas and Van Rensburg, 1986). These conditions do seem to be connected to environmental agents but they do not meet appropriate epidemiological diagnostic criteria for mycotoxicoses.

V. Conclusion on Currently Investigated Chronic Disease

Rural Black populations in South Africa are exposed on a daily basis over most of their lives to fumonisin in combination with other mycotoxins, such as zearalenone (Pillay, 2002) and aflatoxin (Ramjee *et al.*, 1992). It seems highly probable that they also have an exposure to other *Fusarium* toxins such as trichothecenes, as well as other nonmycotoxin environmental toxins. Indeed, South Africa has recently been described as a ''toxicologist's goldmine'' (Stewart, 2002). What this toxin load does to a population which is already malnourished, and in which avitaminosis, alcohol abuse, and smoking are common, is difficult to gauge. A more worrying aspect is that many Africans are now HIV-positive (figures range from 20 to over 50% of the African population) and several of the mycotoxins that they are exposed to, or could be exposed to, are immunosuppressive agents of varying potencies. It is likely that mycotoxins contribute to the outcome and course of AIDS in rural African populations. The exact contribution of fumonisin B_1 to immunosuppression in humans is difficult to state due to its different potency in different species and the fact that it interferes with cellular signaling due to its effects on sphingolipids (Merrill *et al.*, 2001).

References

Aucock, H. W., Marasas, W. F. O., Meyer, C. J., and Chalmers, P. (1980). Field outbreak of hyperoestrogenism (vulvo-vaginitis) in pigs consuming maize infected by *Fusarium graminearum* and contaminated with zearalenone. *Journal of the South African Veterinary Association* **51,** 163–166.

Badria, F. A., Li, S., and Shier, W. T. (1996). Fumonisins as potential causes of kidney disease. *Journal of Toxicology-Toxin Reviews* **15,** 273–292.

Bastianello, S. S., Nesbit, J. W., Williams, M. C., and Lange, A. L. (1987). Pathological findings in a natural outbreak of aflatoxicosis in dogs. *Onderstepoort Journal of Veterinary Research* **54,** 635–640.

Bennett, J. W., and Bentley, R. (1999). Pride and prejudice: The story of ergot. *Perspectives in Biology and Medicine* **42,** 333–355.

Bennett, J. W., and Klich, M. A. (2003). Mycotoxins. *Clinical Reviews.* In Press.

Bentley, R., and Bennett, J. W. (1999). Constructing polyketides: From Collie to combinatorial biosynthesis. *Annual Reviews Microbiology* **53,** 411–446.

Bezuidenhout, S. C., Gelderblom, W. C. A., Gorst-Allman, R. M. M., Marasas, W. F. O., Spiteller, G., and Vleggaar, R. (1988). Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. *Journal of the Chemical Society, Chemical Communications,* 743–745.

Bhatnagar, D., and Cleveland, T. E. (1988). Fate of the methyl group during the conversion of sterigmatocystin into O-methylsterigmatocystin and aflatoxin B_1 by cell-free preparations of *Aspergillus parasiticus*. *Biochimie* **70,** 743–747.

Blount, W. P. (1961). Turkey ''X'' disease. *Turkeys* **9,** 52–67.

Burrell, R. J. W. (1957). Esophageal cancer in the Bantu. *South African Medical Journal* **39,** 401–409.

Campbell, G. D. (1990). Sporadic clinical human mycotoxicosis. *Nutrition and Health* **7,** 21–23.

Chelule, P. K., Gqaleni, N., Chuturgoon, A. A., and Dutton, M. F. (2000). The determination of fumonisin B_1 in feces: A short-term biomarker for assessment of exposure. *Biomarkers* **5,** 1–8.

Chelule, P., Gqaleni, N., Chuturgoon, A. A., and Dutton, M. F. (2001). A study of the occurrence of fumonisin B_1 in a rural community in Kwazulu Natal, South Africa. *Environmental Health Perspectives* **109,** 253–256.

Chalmers, A. A., Gorst-Allman, C. P., Kriek, N. P. J., Marasas, W. F. O., Steyn, P. S., and Vleggaar, R. (1978). Diplosporin, a new mycotoxin from *Diplodia macrospora*. *South African Journal of Chemistry* **31,** 111–114.

Chuturgoon, A. A., and Dutton, M. F. (1991). The appearance of an enzyme activity catalyzing the conversion of norsolorinic acid to averantin in *Aspergillus parasiticus*. *Mycopathologia* **1131,** 41–44.

Chuturgoon, A. A., Punchoo, R., Bux, S., and Dutton, M. F. (1998). The effect of a commercial herbicide on aflatoxin lipid biosynthesis in *Aspergillus parasiticus*, South Africa. *In* ''Mycotoxins and Phycotoxins, Developments in Chemistry, Toxicology, and Food Safety'' (M. Miraglia, H. van Egmond, C. Brera, and J. Gilbert, eds.), pp. 121–128. Alaken Inc., Fort Collins.

Coulter, J. B. S., Suliman, G. I., Lampugh, S. M., Mukhtar, B. I., and Hendrickse, R. G. (1986). Aflatoxins in liver biopsy from Sudanese children. *American Journal of Tropical Medical Hygiene* **35,** 360–365.

Curto, E. V., Kwong, C., Mermersdorfer, H., Glatt, H., Santis, C., Virador, V., Hearing, V. J., and Dooley, T. P. (1999). Inhibitors of mammalian melanocyte tyrosinase: *In vitro* comparison of alkyl esters of gentisic acid with other putative inhibitors. *Biochemical Pharmacology* **57,** 663–672.

Danaviah, S., Gqaleni, N., Chuturgoon, A. A., Dutton, M. F., Lalloo, U. G., and Jeenah, P. M. (2000). Inborne airborne molds and their relevance to pediatric respiratory health—A study of an informal settlement in Durban, South Africa. *In* ''Health Buildings 2000: Exposure, Human Response and Building Investigations''

Vol. 1 (O. Seppannen, and J. Sateri, eds.), pp. 293–298. SIY Indoor Information Oy, Helsinki, Finland.

De Jesus, A. E., Gorst-Allman, C. P., Steyn, P. S., Vleggaar, R., Wessels, P. L., Wan, C. C., and Hsieh, D. P. H. (1980). Conversion of averufin derived from 1,2-^{13}C acetate into aflatoxin B_1. *Journal of the Chemical Society Chemical Communications.* 389–390.

De Jesus, A. E. S., Steyn, P. S., Vleggaar, R., Kirby, G. W., Varley, M. J., and Ferreira, N. P. (1981a). Biosynthesis of cyclopiazonic acid. Steric course of proton removal during the cyclization of cyclopiazonic acid in *Penicillium griseofulvum*. *Journal of the Chemical Society, Perkin Transactions I,* 3292–3294.

De Jesus, A. E., Steyn, P. S., Van Heerden, F. R., Vleggaar, R., Wessels, P. L., and Hull, W. E. (1981b). Structure and biosynthesis of the penitrems A–F. Six novel tremorgenic mycotoxins from *Penicillium crustosum*. *Journal of the Chemical Society Chemical Communications,* 289–291.

De Jesus, A. E., Steyn, P. S., Van Heerden, F. R., Vleggaar, R., and Wessels, P. L. (1983). Tremorgenic mycotoxins from *Penicillium crustosum*: Isolation of penitrems A–F and the structure elucidation and absolute configuration of penitrem A. *Journal of the Chemical Society, Perkin Transactions I,* 1847–1856.

De Jesus, A. E., Steyn, P. S., Van Heerden, F. R., and Vleggaar, R. (1984). Structure elucidation of the janthitrems, novel tremorgenic mycotoxins from *Penicillium janthinellum*. *Journal of the Chemical Society, Perkin Transactions I,* 697–701.

Dutton, M. F. (1988). Enzymes and aflatoxin biosynthesis. *Microbiological Reviews* **52,** 274–295.

Dutton, M. F., and Anderson, M. S. (1982). Role of versicolorin A and its derivatives in aflatoxin biosynthesis. *Applied and Environmental Microbiology* **43,** 548–551.

Dutton, M. F., and Westlake, K. (1985a). Mycotoxins found in supermarket foods. *Journal of Dietetics and Home Economics* **13,** 96–99.

Dutton, M. F., and Westlake, K. (1985b). Occurrence of mycotoxins in cereals and animal feedstuffs in Natal, South Africa. *Journal of the Association of Official Analytical Chemists* **68,** 839–842.

Dutton, M. F., and Kinsey, A. (1995). Occurrence of mycotoxins in cereals and animal feedstuffs in Natal, South Africa 1994. *Mycopathologia* **131,** 31–36.

Dutton, M. F., and Kinsey, A. (1996). The occurrence of mycotoxins in cereals and animal feedstuffs in Kwazulu Natal, South Africa 1984–1993. *South African Journal of Animal Science* **26,** 53–57.

Dutton, M. F., Robertson, E. J., Mathews, C., and Beck, B. D. A. (1993). Occurrence of mycotoxins in maize in a rural area in South Africa and methods of prevention of contamination and elimination. Fungal infection and mycotoxin contamination of commercial maize. *In* ''Proceedings ICC International Symposium, Pretoria'' (J. R. N. Taylor, P. G. Randall, and J. H. Viljoen, eds.), pp. 823–834. CSIR, Pretoria, South Africa.

Dutton, M. F., Myeni, S., Early, D., Chuturgoon, A. A., and Bux, S. (1998). The detection and measurement of aflatoxin B_1 conjugates in humans in Natal, South Africa. *In* ''Mycotoxins, Phycotoxins, Developments in Chemistry, Toxicology, and Food Safety'' (M. Miraglia, H. van Egmond, C. Brera, and J. Gilbert, eds.), pp. 303–309. Alaken Inc., Fort Collins.

Ehret, W. J., Adelaar, T. F., and Kriek, N. P. J. (1968). An outbreak of *Claviceps paspaulum* poisoning in beef cattle. *Journal of the South African Veterinary Medical Association* **39,** 100–105.

Firn, R. D., and Jones, C. G. (2000). The evolution of secondary metabolism—A unifying model. *Molecular Microbiology* **37,** 989–994.

Gelderblom, W. C. A., Jaskiewicz, K., Marasas, W. F. O., Thiel, P. G., Horak, R. M., Vleggaar, R., and Kriek, N. P. J. (1988). Fumonisins—Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Applied and Environmental Microbiology* **54,** 1806–1811.

Gelderblom, W. C. A., Marasas, W. F. O., Steyn, P. S., Thiel, P. G., Van der Merwe, K. J., Van Rooyen, P. H., Vleggaar, R., and Wessels, P. L. (1984). Structure elucidation of fusarin C, a mutagen produced by *Fusarium moniliforme*. *Journal of the Chemical Society Chemical Communications,* 122–124.

Gengan, R., Dutton, M. F., Chuturgoon, A. A., and Mulholland, D. (1999). Synthesis of sterigmatocystin derivatives and their biotransformation to aflatoxin B_1 by a blocked mutant of *Aspergillus parasiticus*. *Mycopathologia* **144,** 115–122.

Goldblatt, L. (ed.) (1969). ''Aflatoxin, Scientific Background, Control, and Implications.'' Academic Press, New York.

Gorst-Allman, C. P., Pachler, K. G. R., Steyn, P. S., Wessels, P. L., and Scott, D. B. (1977). Carbon-13 nuclear magnetic resonance assignments of some fungal C20 anthraquinones, their biosynthesis in relationship to that of aflatoxin B_1. *Journal of the Chemical Society, Perkin Transactions I,* 2181–2188.

Gqaleni, N., Chuturgoon, A. A., and Dutton, M. F. (1997). Maize storage and health-related problems for the indigenous rural community: Challenges and advances for South Africa. *Science, Technology and Development* **15,** 192–198.

Gqaleni, N., Danaviah, S., Chuturgoon, A. A., and Dutton, M. F. (1999). An assessment of the occurrence of mold bioaerosols in the in–outdoor environment in an informal settlement in Durban. *In* ''Bioaerosols, Fungi, and Mycotoxins: Health Effects, Assessments, Prevention, and Control'' (E. Johanning, ed.). Eastern New York Occupational and Environmental Health Center, Albany, New York.

Habeck, M. (2000). Dissecting the risk factors for pre-eclampsia. *Molecular Medicine Today* **6,** 4.

Hag Elamin, N. H., Abdel-Rahim, A. M., and Khalid, A. E. (1988). Aflatoxin contamination of groundnuts in Sudan. *Mycopathologia* **104,** 25–31.

Haschek, W. M., Gumprecht, L. A., Smith, G., Tumbleson, M. E., and Constable, P. D. (2001). Fumonisin toxicosis in swine. *Environmental Health Perspectives* **109,** 252–257.

Hitchman, T. S., Schmidt, E. W., Trail, F., Rarick, M. D., Linz, J. E., and Townsend, G. A. (2001). Hexanoate synthase, a special type I fatty acid synthase in aflatoxin B_1 biosynthesis. *Bioorganic Chemistry* **29,** 293–307.

Horak, R. M., Steyn, P. S., Van Rooye, P. H., Vleggar, R., and Rabie, C. J. (1981). Structures of the austalides A–E, five novel toxic metabolites from *Aspergillus ustus*. *Journal of the Chemical Society Chemical Communications*. 1265–1267.

Hsieh, D. P. H. (1989). Potential human health hazards of mycotoxins. *In* ''Mycotoxins and Phycotoxins'' (S. Natori, K. Hashimoto, and Y. Ueno, eds.), pp. 69–79. Elsevier, Amsterdam, Netherlands.

Jeenah, M. S., and Dutton, M. F. (1984). The conversion of sterigmatocystin to O-methylsterigmatocystin and aflatoxin B_1 by a cell-free preparation. *Biochemistry Biophysics Research Communications* **116,** 1114–1118.

Joffe, A. Z. (1978). *Fusarium poae* and *F. sporotrichoides* as principal causal agents of alimentary toxic aleukia. *In* ''Fungi, Mycotoxins, Mycotoxicoses,'' Vol. 3, (T. D. Wyllie, and L. G. Morehouse, eds.), pp. 21–86. Marcel Dekker, New York.

Keen, P., and Martin, P. (1971). Is aflatoxin carcinogenic to man? The evidence in Swaziland. *Tropical and Geographic Medicine* **23,** 44–53.

Keller, N. P., Dischinger, H. C., Bhatnagar, D., Cleveland, T. E., and Ullah, A. H. J. (1993). Purification of a 40-kilodalton methyltransferase active in aflatoxin biosynthetic activity. *Applied and Environmental Microbiology* **59,** 479–484.

Keller, N. P., Watanabe, C. M. H., Kelkar, H. S., Adams, T. H., and Townsend, C. A. (2000). Requirement of monooxygenase mediated steps for sterigmatocystin biosynthesis by *Aspergillus nidulans*. *Applied and Environmental Microbiology* **66,** 359–362.

Kellerman, T. S., Coetzer, J. A. W., and Naude, T. W. (1988). *Plant poisonings and mycotoxicoses of livestock in Southern Africa*. Oxford University Press, Cape Town, South Africa.

Lasky, T., and Magder, L. (1997). Hepatocellular carcinoma p53 G>T transversions at codon 249: The fingerprint of aflatoxin exposure. *Environmental Health Perspectives* **105,** 392–397.

Lotter, L. H., and Krohm, H. J. (1988). Occurrence of aflatoxins in human foodstuffs in South Africa. *Bulletin of Environmental and Contamination Toxicology* **40,** 240–243.

Luck, H., Steyn, M., and Wehner, F. C. (1976a). A survey of milk powder for aflatoxin content. *South African Journal of Dairy Technology* **8,** 85–86.

Luck, H., Wehner, F. C., Plomp, A., and Steyn, M. (1976b). Mycotoxins in South African cheese. *South African Journal of Dairy Technology* **8,** 107–110.

Marasas, W. F. O. (1982). Mycotoxicological investigations on corn produced in esophageal cancer areas in Transkei. *In* ''Cancer of the Esophagus,'' Vol. 1, (C. J. Pfeiffer, ed.), pp. 29–38. CRC Press Inc., Boca Raton, FL.

Marasas, W. F. O. (1995). Fumonisins: Their implication for human and animal health. *Natural Toxins* **3,** 193–198.

Marasas, W. F. O. (1996). Fumonisins: History, worldwide occurrence, and impact. *In* ''Fumonisin in Food,'' (L. Jackson, ed.), pp. 1–17. Plenum Press, New York.

Marasas, W. F. O., and Van Rensburg, S. J. (1979). Mycotoxins and their medical and veterinary effects. *Plant Disease* **4,** 357–379.

Marasas, W. F. O., and Van Rensburg, S. J. (1986). Mycotoxicological investigations on maize and groundnuts from the endemic area of Mseleni joint disease in KwaZulu. *South African Medical Journal* **69,** 369–374.

Marasas, W. F. O., Adelaar, T. F., Kellerman, T. S., Minne, J. A., Van Rensburg, I. B. J., and Burroughs, G. W. (1972). First report of facial eczema in sheep in South Africa. *Onderstepoort Journal of Veterinary Research* **39,** 107–112.

Marasas, W. F. O., Van Rensburg, S. J., and Mirocha, C. J. (1976). Leukoencephalomalacia: A mycotoxicosis of equidae caused by *Fusarium moniliforme*. *Ondersterpoort Journal of Veterinary Research* **43,** 113–122.

Marasas, W. F. O., Kriek, N. P. J., Van Rensberg, S. J., Steyn, M., and Van Schalkwyk, G. C. (1977). Occurrence of zearalenone and deoxynivalenol mycotoxins produced by *Fusarium graminearum* in maize in South Africa. *South African Journal of Science* **73,** 346–349.

Marasas, W. F. O., Van Rensburg, S. J., and Mirocha, C. J. (1979). Incidence of *Fusarium* species and the mycotoxins deoxynivalenol and zearalenone in corn produced in esophageal cancer areas in Transkei. *Journal of Agricultural and Food Chemistry* **27,** 1108–1112.

Marasas, W. F. O., Nelson, P. E., and Toussoun, T. A. (1984). ''Toxigenic Fusarium Species, Identity, and Mycology.'' Pennsylvania State University Press, University Park, Pennsylvania.

Marasas, W. F. O., Kellerman, T. S., Gelderblom, W. C. A., Coetzer, J. A. W., Thiel, P. G., and van der Lugt, J. J. (1988). Leukoencephalomalacia in a horse induced by fumonisin B_1 isolated from *Fusarium moniliforme*. *Ondersterpoort Journal of Veterinary Research* **55,** 197–203.

Marasas, W. F. O., Thiel, P. G., Gelderblom, W. C. A., Shephard, G. S., Sydenham, E. W., and Rheeder, J. P. (1993). Fumonisins produced by *Fusarium moniliforme* in maize: Foodborne carcinogens of pan-African importance. *African Newsletter of Occupational Health and Safety Supplement* **2,** 11–18.

Mclean, M., and Dutton, M. F. (1995). Cellular interactions and metabolism of aflatoxin. *Pharmaceuticals and Therapeutics* **65,** 163–192.

Mclean, M., Berjak, P., Watt, M. P., and Dutton, M. F. (1992). The effects of aflatoxin B_1 on germinating maize *Zea mays*. embryos. *Mycopathologia* **119,** 181–190.

Mclean, M., Berjak, P., Watt, M. P., and Dutton, M. F. (1993). The influence of aflatoxin B_1 on the *in vitro* germination and growth of excised, mature *Zea mays* embryos. *Journal of Natural Toxins* **21,** 13–26.

Mclean, M., Watt, M. P., Berjak, P., and Dutton, M. F. (1994a). Effects of aflatoxin B_1 on *in vitro* cultures of *Nicotiana tabacum* var Samsun. I. Callus growth and differentiation. *Mycopathologia* **125,** 93–105.

Mclean, M., Watt, M. P., Berjak, P., Dutton, M. F., and Snyman, C. (1994b). Effects of aflatoxin B_1 on *in vitro* cultures of *Nicotiana tabacum* var Samsun. II. Root and shoot development in tobacco plantlets. *Mycopathologia* **125,** 107–117.

Mclean, M., Watt, M. P., Berjak, P., and Dutton, M. F. (1995). Aflatoxin B_1—Its effects on an *in vitro* system. *Journal of Food Additives and Contaminants* **123,** 435–444.

Merrill, A. H., Sullards, M. C., Wang, E., Voss, K. A. V., and Riley, R. T. (2001). Sphingolipid metabolism: Roles in signal transduction and disruption by fumonisins. *Environmental Health Perspectives* **109,** 283–289.

Mitchell, D. T. (1919). Poisoning of cattle by *Diplodia*-infected maize. *South African Journal of Science* **16,** 446–452.

Moodley, J., Moodley, D., Reddy, L., Dutton, M. F., and Chuturgoon, A. A. (2001). Fumonisin B1, an etiological role in pre-eclamsia. *Journal of Obstetrics and Gynecology* **21,** 599–600.

Motomura, M., Chihaya, N., Shinozawa, T., Hamasaki, T., and Yabe, K. (1999). Cloning and characterization of the O-methyltransferase I gene from *Aspergillus parasiticus* associated with the conversions of demethylsterigmatocystin to sterigmatocystin and dihydrodemethylsterigmatocystin to dihydrosterigmatocystin in aflatoxin biosynthesis. *Applied and Environmental Microbiology* **65,** 4987–4994.

Onyemelukwe, G. C., Ogbadu, G. N., and Salifu, A. (1982). Aflatoxin B1, B2, G1, and G2 in primary liver cell carcinoma. *Toxicology Letters* **10,** 309–312.

Otsuki, T., Wilson, J. S., and Sewadeh, M. (2001a). Saving two in a billion: Quantifying the trade effect of European food safety standards on African exports. *Food Policy* **26,** 495–514.

Otsuki, T., Wilson, J. S., and Sewadeh, M. (2001b). What price precaution? European harmonization of aflatoxin regulations and African groundnut export. *European Review of Agricultural Economics* **28,** 263–283.

Peers, F. G., and Linsell, C. A. (1973). Dietary aflatoxins and human liver cancer. A population-based study in Kenya. *British Journal of Cancer* **27,** 473–484.

Peers, F. G., Gilman, G. A., and Linsell, C. A. (1976). Dietary aflatoxins and human liver cancer. A study in Swaziland. *International Journal of Cancer* **17,** 167–176.

Peers, F. G., Bosch, X., Kaldor, J., Linsell, C. A., and Pluijmen, M. (1987). Aflatoxin exposure, hepatitis B infection, liver cancer in Swaziland. *International Journal of Cancer* **39,** 545–553.

Pienaar, J. G., Kellerman, T. S., and Marasas, W. F. O. (1981). Field outbreaks of leucoencephalomalacia in horses consuming maize infected by *Fusarium verticilliodes* in South Africa. *Journal of South African Veterinary Association* **52,** 21–24.

Pillay, D., Chuturgoon, A. A., Nevines, E., Manickum, T., Deppe, W., and Dutton, M. F. (2002). The quantitative analysis of zearalenone and derivatives in plasma of patients with breast and cervical cancer. *Clinical Chemistry and Laboratory Medicine* **40,** 946–951.

Purchase, I. F. H., and Theron, J. J. (1967). Research on mycotoxins in South Africa. *International Pathology* **8,** 3–7.

Rabie, C. J., van Rensburg, S. J., van der Watt, J. J., and Lubben, A. (1975). Onyalai—The possible involvement of a mycotoxin produced by *Phoma sorghina* in the etiology. *South African Medical Journal* **49,** 1647–1650.

Rabie, C. J., Sydenham, E. W., Thiel, P. G., Lubben, A., and Marasas, W. F. O. (1986). T-2 toxin production by *Fusarium acuminatum* isolated from oats and barley. *Applied and Environmental Microbiology* **52,** 594–596.

Rabie, C. J., Du Preez, J. J., and Hayes, J. P. (1987). Toxicity of *Diploidia maydis* to broilers, ducklings, and laying chicken hens. *Poultry Science* **66,** 1123–1128.

Ramjee, G., Berjak, P., Adhikari, M., and Dutton, M. F. (1992). Aflatoxins and kwashiorkor in Durban, South Africa. *Annals of Tropical Pediatrics* **12,** 241–247.

Rheeder, J. P., Marasas, W. F. O., Thiel, P. G., Sydenham, E. W., Shephard, G. S., and van Schalkwyk, D. J. (1992). *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. *Phytopathologia* **82,** 353–357.

Richard, J. L., and Thurston, J. R., eds. (1986). ''Diagnosis of Mycotoxicoses.'' Martinus and Nijhoff Publishers, Dordrecht, Netherlands.

Rode, H., Moore, S. W., Kaschula, R. O. C., Brown, R. A., and Cywes, S. (1992). Degenerate leiomyopathy in children, a clinico-pathological study. *Pediatric Surgery* **7,** 23–29.

Rose, E. F. (1965). Interim report on the survey of cancer of the esophagus in the Transkei. *South African Medical Journal* **39,** 1098–1101.

Saito, M., Enomoto, M., and Tatsuno, T. (1971). Yellow rice toxins: Luteoskyrin and related compounds, chlorine-containing compounds, and citrinin. *In* ''Microbial Toxins, Vol. VI: Fungal Toxins'' (A. Ciegler, S. Kadis, and S. J. Ajl, eds.), pp. 299–380. Academic Press, New York.

Schneider, D. J., Marasas, W. F. O., Kuys, J. C. D., Kriek, N. P. J., and Schalkwyk, G. C. (1979). A field outbreak of suspected stachybotryotoxicosis in sheep. *Journal of the South African Veterinary Association* **50,** 73–81.

Segal, I., Reinach, S. G., and De Beer, M. (1988). Factors associated with oesophageal cancer in Soweto, South Africa. *British Journal of Cancer* **58,** 681–686.

Sekota, M. M., Gqaleni, N., Chuturgoon, A. A., and Dutton, M. F. (2000a). The occurrence of indoor mold growth in residential homes of Durban Metro, South Africa. *In* ''Exposure, Human Response, and Building Investigations,'' (O. Seppannen, and J Sateri, eds.), **Vol. 3,** 347–352. SIY Indoor Information Oy, Helsinki, Finland.

Sekotha, M. M., Gqaleni, N., Swemmer, J., Chuturgoon, A. A., and Dutton, M. F. (2000b) Efficacy of a new biocide on mold growth. *In* ''Exposure, Human Response, and Building Investigations,'' Vol. 3, (O. Seppannen, and J. Sateri, eds.), pp. 439–444. SIY Indoor Information Oy, Helsinki, Finland.

Sewram, V., Nair, J. J., Nieuwoudt, T. W., Gelderblom, W. C. A., Marasas, W. F. O., and Shephard, G. S. (2001). Assessing chronic exposure to fumonisin mycotoxins: The

use of hair as a suitable noninvasive matrix. *Journal of Analytical Toxicology* **25,** 450–455.

Shephard, G. S., Thiel, P. G., Sydenham, G. S., Alberts, J. F., and Cawood, M. E. (1994a). Distribution and excretion of a single dose of the mycotoxin fumonisin B_1 in the non-human primate. *Toxicon* **32,** 735–741.

Shephard, G. S., Thiel, P. G., Sydenham, G. S., Vleggaar, R., and Alberts, J. F. (1994b). Determination of the mycotoxin fumonisin B_1 and identification of its partially hydrolyzed metabolites in the faeces of non-human primates. *Food Chemistry and Toxicology* **32,** 23–29.

Sitas, F., Blaauw, D., Terblanche, M., Madhoo, J., and Carrara, H. (1997). Incidence of histologically diagnosed cancer in South Africa, 1992: Johannesburg: ''National Cancer Registry,'' South African Institute for Medical Research.

Smith, G. W., Constable, P. D., Foreman, J. H., Eppley, R. M., Waggoner, A. L., Tumbleson, M. E., and Haschek, W. M. (2002). Cardiovascular changes associated with intravenous administration of fumonisin B_1 in horses. *American Journal of Veterinary Research* **63,** 538–545.

Stewart, M. J. (2002). South Africa: A toxicologist's goldmine. *Aatherapeutic Drug Monitoring* **24,** 172–177.

Steyn, P. S. (1970). The isolation structure and absolute configuration of secalonic acid D, the toxic metabolite of *Penicillium oxalicum*. *Tetrahedron* **26,** 51–57.

Steyn, P. S. (1977a). Mycotoxins, excluding aflatoxin, zearalenone, and the trichothecenes. *In* ''Mycotoxins in Human and Animal Health'' (J. V. Rodricks, C. W. Hesseltine, and M. A. Mehlman, eds.), pp. 419–431. Pathotox Publishers, Park Forest South, Illinois.

Steyn, P. S. (1977b). Some new mycotoxins. *Pure and Applied Chemistry* **49,** 1771–1778.

Steyn, P. S. (1979). The biosynthesis of polyketide-derived mycotoxins. *Pure and Applied Chemistry* **52,** 189–204.

Steyn, P. S. (1984). Ochratoxins and related dihydroisocoumarins. *In* ''Mycotoxins—Production, Isolation, Separation, and Purification. Developments in Food Science,'' Vol. 8, Chapter 9. Elsevier, Amsterdam, Netherlands.

Steyn, P. S. (1995). Mycotoxins, general view, chemistry, and structure. *Toxicology Letters* **82/83,** 843–851.

Steyn, P. S., and Vleggaar, R. (1974). Austocystins. Six novel dihydrofuro (3′,2′,4,5) furo (3,2,-B) xanthones from *Aspergillus ustus*. *Journal of the Chemical Society, Perkin Transactions I,* 2250–2256.

Steyn, P. S., and Vleggaar, R. (1985). Oxygen-18 labeling studies on the biosynthesis of citereoviridin. *Journal of the Chemical Society, Chemical Communications,* 1531–1532.

Steyn, P. S., Vleggaar, R., and Wessels, P. L. (1975). Biosynthesis of aflatoxin B_1 from (2-^{13}C) and (1,2-^{13}C) acetate. *Journal of the Chemical Society, Chemical Communications,* 193–195.

Steyn, P. S., Wessels, P. L., and Marasas, W. F. O. (1979). Pigments from *Fusarium moniliforme*. Structure and ^{13}C nuclear magnetic resonance assignments of an azaanthraquinone and three naphthoquinones. *Tetrahedron* **35,** 1551–1555.

Steyn, P. S., Vleggaar, R., Wessels, P. L., and Scott, D. B. (1979). Biosynthesis of versiconal acetate, versiconol acetate and versiconol, metabolites from culture of *Aspergillus parasiticus* treated with dichlorvos. The role of versiconal acetate in aflatoxin biosynthesis. *Journal of the Chemical Society: Perkin Transactions I,* 460–463.

Steyn, P. S., Vleggaar, R., and Wessels, P. L. (1980). The biosynthesis of aflatoxin and its congeners. pp. 103–155. Academic Press, New York.

Steyn, P. S., Vleggar, R., Wessels, P. L., and Woudenberg, M. (1982). Biosynthesis of citreoviridin. A carbon-13 study. *Journal of the Chemical Society, Perkin Transactions I,* 2175–2178.

Sugar, A. M. (1991). Overview: *Cryptococcus* in the patient with AIDS. *Mycopathologia* **114,** 153–157.

Sydenham, E. W., Thiel, P. G., and Marasas, W. F. O. (1988). Occurrence and chemical determination of zearalenone and alternariol monomethyl ether in sorghum-based mixed feeds associated with an outbreak of suspected hyperestrogenism in swine. *Journal of Agricultural and Food Chemistry* **36,** 621–625.

Sydenham, E. W., Thiel, P. G., Marasas, W. F. O., and Nieuwenhuis, J. J. (1989). Occurrence of deoxynivalenol and nivalenol in *Fusarium graminearum*-infected undergrade wheat in South Africa. *Journal of Agricultural and Food Chemistry* **37,** 921–926.

Sydenham, E. W., Thiel, P. G., Marasas, W. F. O., Shephard, G. S., Van Schalkwyk, D. J., and Koch, K. R. (1990). Natural occurrence of some *Fusarium* mycotoxins in corn from low and high esophageal cancer prevalence areas of the Transkei, southern Africa. *Journal of Agricultural and Food Chemistry* **38,** 1900–1903.

Sydenham, E. W., Shephard, G. S., Thiel, P. G., Marasas, W. F. O., and Stockenstrom, S. (1991). Fumonisin contamination of commercial corn-based human foodstuffs. *Journal of Agricultural and Food Chemistry* **39,** 2014–2018.

Sydenham, E. W., Vismer, H. F., Marasas, W. F. O., Brown, N. L., Schlechter, M., and Rheeder, J. P. (1997). The influence of deck storage and initial processing on patulin levels in apple juice. *Food Additives and Contaminants* **14,** 429–434.

Sylla, A., Diallo, M. S., Castegnaro, J. J., and Wild, C. P. (1999). Interactions between hepatitis B virus infection and exposure to aflatoxin in the development of hepatocellular carcinoma: A molecular epidemiological approach. *Mutation Research* **428,** 187–196.

Thiel, P. G., Meyer, C. J., and Marasas, W. F. O. (1982). Natural occurrence of moniliformin together with dexoynivalenol and zearalenone in Transkeian corn. *Journal of Agricultural and Food Chemistry* **30,** 308–312.

Thiel, P. G., Gelderblom, W. C. A., Marasas, W. F. O., Nelson, P. E., and Wilson, T. M. (1986). Natural occurrence of moniliformin and fusarin C in corn screenings known to be hepatocarcinogenic in rats. *Journal of Agricultural and Food Chemistry* **34,** 773–775.

Thiel, P. G., Shepard, G. S., Sydenham, E. W., Marasas, W. F. O., Nelson, P. E., and Wilson, T. M. (1991). Levels of fumonisins B_1 and B_1 in feeds associated with confirmed cases of equine leukoencephalomalcia. *Journal of Agricultural and Food Chemistry* **39,** 109–111.

Trinder, D. W. (1988). A survey of aflatoxins in industrially brewed South African sorghum beer and beer strainings. *Journal of the Institute of Brewing* **95,** 307–309.

Trinder, D. W. (1989). Distribution of aflatoxins in industrially milled sorghum malt. *South African Journal of Food Science and Nutrition* **1,** 20–21.

Van Der Merwe, K. J., Steyn, P. S., Fourie, L., Scott de, B., and Theron, J. J. (1965). Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus*. *Nature* **205,** 1112–1113.

Van Rensburg, S. J., Van Watt, J. J., Purchase, I. F. H., Coutinho, I. P., and Markham, R. (1974). Primary liver cancer rate and aflatoxin intake in a high cancer area. *South African Medical Journal* **48,** 2508A–2508C.

Van Rensburg, S. J., Kirsipuu, A., Pereira Coutinho, L., and Van Watt, J. J. (1975). Circumstances associated with the contamination of food by aflatoxin in a high primary liver cancer area. *South African Medical Journal* **49,** 877–883.

Van Rensburg, S. J., Cook-Mozaffari, P., van Schalwyk, D. J., van Watt, J. J., Vincent, T. J., and Purchase, I. F. H. (1985). Hepatocellular carcinoma and dietary aflatoxin in Mozambique and Transkei. *British Journal of Cancer* **51,** 713–726.

Van Warmelo, K. T., Marasas, W. F. O., Adelaar, T. F., Kellerman, T. S., Van Rensburg, I. B. J., and Minn, J. A. (1970). Experimental evidence that lupinosis of sheep is a mycotoxicosis caused by the fungus *Phomopsis Ieptostromiformis*. *Journal of the South African Veterinary Medical Association* **41,** 235–247.

Viljoen, J. H., Marasas, W. F. O., and Thiel, P. G. (1993). Fungal infection and mycotoxin contamination of commercial maize. *In* ''Proceedings ICC International Symposium, Pretoria'' (J. R. N. Taylor, P. G. Randall, and J. H. Viljoen, eds.), pp. 837–851. CSIR, Pretoria, South Africa.

Voss, K. A., Riley, R. T., Norred, W. P., Bacon, C. W., Meredith, F. I., Howard, P. C., Plattner, R. D., Collins, T. F. X., Hansen, D. K., and Porter, J. K. (2001). An overview of rodent toxicities, liver and kidney effects of fumonisins and *Fusarium moniliforme*. *Environmental Health Perspectives* **109,** 259–266.

Walton, J. D. (2000). Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: A hypothesis. *Fungal Genetics and Biology* **30,** 167–171.

Westlake, K., Mackie, R. I., and Dutton, M. F. (1987a). T-2 toxin metabolism by rumen bacteria and its effect on their growth. *Applied and Environmental Microbiology* **533,** 587–592.

Westlake, K., Mackie, R. I., and Dutton, M. F. (1987b). Effect of several mycotoxins on the specific growth rate of *Butyricvibrio fibrinosolvens* and toxin degradation *in vitro*. *Applied and Environmental Microbiology* **533,** 613–614.

Westlake, K., Mackie, R. I., and Dutton, M. F. (1989). *In vitro* metabolism of mycotoxins by bacterial, protozoal, and ovine ruminal fluid preparations. *Animal Feed Science and Technology* **25,** 169–173.

Yabe, K., Nakamura, M., and Hamasaki, T. (1999). Enzymatic formation of G-group aflatoxins and biosynthetic relationship between G- and B-group aflatoxins. *Applied and Environmental Microbiology* **65,** 3867–3872.

Yu, J., Chang, P. K., Cary, P. W., Bhatnagar, D., and Cleveland, T. E. (1997). *avnA*, a gene encoding for cytochrome P-450 monooxygenase, is involved in the conversion of averantin to averufin in aflatoxin biosynthesis in *Aspergillus parasiticus*. *Applied and Environmental Microbiology* **63,** 1349–1356.

Zomborszky-Kovacs, M., Vetesi, F., Kovacs, F., Bata, A., Toth, A., and Tornyos, G. (2000). Preliminary communication, examination of the harmful effect to fetuses of fumonisin B_1 in pregnant sows. *Teratogenesis, Carcinogenesis, and Mutagenesis* **20,** 293–299.

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Electrophoretic Karyotype Analysis in Fungi

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I. Introduction

The small size of fungal chromosomes (mostly 0.5 to 10 Mb) makes them amenable to separation by pulsed field gel electrophoresis (PFGE). In this technique, a genome is resolved into a series of chromosomal bands on an electrophoretic gel. The resultant ''electrophoretic karyotype'' shows the number and size of chromosomes, and hence, the estimated size of the genome. Many filamentous species contain apparently dispensable ''mini''-chromosomes as well as exhibiting extensive intraspecific chromosomal length polymorphism. Karyotypes are useful for strain identification and in the preparation of DNA for genomic analysis. In addition, molecular linkage maps can be built by using Southern blot or fluorescent *in situ* hybridization. Successful preparation of a fungal electrophoretic karyotype from a previously unstudied species is an empiric process and is not always successful. The efficient removal of the cell wall and obtaining sufficient numbers of protoplasts can pose a bottleneck. Moreover, pulsed field gels are

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sensitive to all electrophoretic parameters, including the concentration of agarose, the quality of the agarose, buffer strength, pulse switching interval, the voltage gradient, and temperature. As the number of fungal genome sequencing projects grows, electrophoretic karyotypes will be increasingly important for producing chromosome-specific libraries, for scaffolding physical maps, and for anchoring sequence data.

II. Background

In classical genetics, chromosome number and morphology were determined by direct microscopic examination of mitotic or meiotic figures. A ''karyotype'' was a photographic or digital display in which the mitotic chromosomes of a cell were lined up and numbered. By convention, the largest chromosome usually was labeled with an Arabic or Roman ''one'' with the rest of the chromosome set numbered in decreasing size order. Karyotype analysis has been used extensively in the cytogenetic characterization of higher eukaryotes, especially *Homo sapiens*, where a number of cytogenetic abnormalities have been associated with important clinical syndromes. (e.g., trisomy 21 is the cause of Down syndrome [Nora and Fraser, 1992]).

Cytogenetic karyotype analysis was rarely applied in mycology because of the extremely small size of fungal chromosomes and the difficulty of observing mitotic figures. Barbara McClintock (1945) obtained the first fungal karyotype using the acetoorcein squash technique and light microscopic analysis of meiotic figures in *Neurospora crassa*. Others succeeded with similar meiotic preparations of selected ascomycetes or by using electron microscopy of the synaptonemal complex (see Lu, 1996, for a review of chromosome cytology for fungi). In a few genetic models such as *Saccharomyces cerevisiae, Neurospora crassa*, and *Aspergillus nidulans*, recombination data allowed the creation of genetic linkage maps (O'Brien, 1993). For the vast majority of fungal species, however, neither cytological material nor appropriate genetic systems (i.e., stable mutants, sexual, or parasexual cycles) were available.

The small size of fungal chromosomes, long a disadvantage for cytogenetic analysis, has become an advantage for doing electrophoretic karyotyping by means of PFGE. With this technique, a genome is resolved into a series of chromosomal bands on an electrophoretic gel, thereby allowing researchers to bypass conventional cytological and genetic maps. In this chapter, we give a brief description of PFGE and review its application to fungi.

III. Techniques

A. Pulsed Field Gel Electrophoresis

PFGE is a technique that separates DNA molecules in agarose matrices by subjecting them to alternating electrophoretic fields. Developed by Schwartz and Cantor (1984), the acronym PFGE originally referred to pulsed field *gradient* electrophoresis. When it became apparent that a field gradient was neither important nor desirable, the abbreviation was changed to pulsed field *gel* electrophoresis (Birren and Lai, 1993). In PFGE, electrode arrays interrupt and reverse applied electric fields, thereby forcing molecules periodically to stop migrating and reorient themselves. When applied to intact chromosomes, the size and three-dimensional structure of the chromosomes determine the rate at which they migrate through the gel matrix. Large chromosomes require more time to reorient than small chromosomes. The resolution of a particular size range of chromosomes depends on the duration of the switching interval. Pulse times are selected so that chromosomes of a targeted size spend most of the duration of the pulse reorienting rather than moving through the gel. The long reorientation interval accounts for the long periods of time (24 to 150 hours) needed to fractionate large chromosomes. After electrophoresis is completed, the gel is stained with ethidium bromide and the genome is visualized as series of bands. Since chromosomes of the same size migrate at the same rate, bands containing more than one chromosome stain more intensely with ethidium bromide (Mills and McCluskey, 1990).

The original PFGE apparatus developed by Schwartz and Cantor (1984) used two perpendicularly oriented alternately pulsing electrophoretic fields. Although chromosome separation was possible, the variation in electrical field strength across the gel compromised the ability to make lane-to-lane comparisons. Thus, accurate determination of chromosome sizes by comparison with known standards was not possible. Subsequently, many novel approaches to instrumentation and design have improved the original method. Different configurations developed for the application of electric fields are usually known by their acronyms: e.g., CHEF (contour clamped homogeneous electric field), FIGE (field-inversion gel electrophoresis), OFAGE (orthogonal field alternation gel electrophoresis), RFGE (rotating field gel electrophoresis), TAFE (transverse alternating field electrophoresis), and so forth. For overviews of the different apparatus designs, see Anand (1986), Birren *et al.* (1988), Lai *et al.* (1989), Chu (1990), Eby (1990), Birren and Lai (1993), and Bustamente *et al.* (1993). For theoretical considerations of

the dynamics of DNA molecules in agarose matrices, see Deutsch and Madden (1989), Schwartz and Koval (1989), and Smith *et al.* (1989).

Pulsed field gels are extremely sensitive to changes in experimental parameters. The single most important variable is the switch interval. In addition, agarose gels with large and uniform pore size are crucial. Special high-strength, high-quality agarose for PFGE has been developed by several companies, e.g., BioWhittaker SeaKem LE agarose, Bio-Rad Chromosomal Grade™ agarose, and BioProducts Fast Lane™ agarose.

In order to optimize the separation of chromosomes of different molecular weights, empiric modifications of the pulse frequency, the agarose gel concentration, the buffer concentration, and so forth must be tested. Optimization of these parameters can require considerable effort. *Pulsed Field Gel Electrophoresis, A Practical Guide* by Birren and Lai (1993) offers many useful tips for manipulating the relevant experimental variables in order to achieve success. Another useful source for troubleshooting is Smith *et al.* (1988), who present a number of photographs of failed gels ''that are not usually published'' that can help diagnose problems such as uneven pulsing, overloading, or improper loading.

B. Preparation of DNA and Instrumentation

To preserve the integrity of chromosome-size DNA molecules, careful preparation of the DNA is critical. Conventional methods that rely on phenol extraction and contain multiple pipetting steps shear the DNA too much. In their pioneering work, Schwartz and Cantor (1984) and Carle and Olson (1984) isolated chromosomes from intact yeast cells *after* they had been embedded in agarose. Agarose protects the DNA against breakage and allows the flow of the enzymatic solutions required for digestion of the cell wall and nuclear membrane. Variations on this agar-embed technique have been adopted for most subsequent applications of PFGE.

For filamentous fungi, which have a chitinous cell wall, and which have a lower ratio of cell mass to nuclear material than yeasts, it is often necessary to start by producing protoplasts (=spheroplasts). After the protoplasts are obtained in sufficient yield, they are embedded in agarose, and then subsequently treated with detergents and proteolyic enzymes to release chromosomal-sized DNA. Because the efficiency of the cell wall–degrading step can be monitored by direct microscopic observation, protocols that start with the protoplast step have also been adopted for a number of yeasts. In general, protoplast concentrations greater than 10^9 cells/ml are needed in order to obtain a successful

karyotype, and achieving sufficient quantities of protoplasts can be an obstacle. The most important variables are physiological status of the mycelium (actively growing cells are best) and the lytic enzymes used for removing the cell wall (a cocktail of chitinases and glucanases). There is an enormous literature on fungal protoplasting techniques; see Peberdy and Ferenczy (1985) and Peberdy (1995) for general reviews and Lodder *et al.* (1993) for emphasis on protoplast preparation from commercially important mushrooms.

It should be noted that many of the published protocols for protoplast preparation from filamentous species call for a commercial product called Novozyme, a mixture of crude enzymes from *Trichoderma hazarianum* that, unfortunately, is no longer marketed. Many molecular mycologists are still relying on supplies hoarded in their freezers. Good protoplast yields can also be obtained using a combination of chitinase and another commercial crude enzyme from *Trichoderma* sold by Sigma-Aldrich (St. Louis, Missouri) as Cellulase C9422 or C0898. See Hamlyn *et al.* (1991) for a comparison of the effectiveness of commercial enzymes for efficient protoplast isolation.

It is not absolutely necessary to remove the cell wall in order to obtain successful electrophoretic karyotypes. For example, excellent results were achieved with slightly ground mycelia of *Ustilago* and intact sporidia of *Tilletia* embedded into agarose and then simply treated with protease in the presence of ethylenediamine-tetraacetic acid (EDTA) and SDS. This method produced karyotypes of equal or better quality than those derived from protoplasts, although the mechanism whereby chromosome-size DNA was released from cells with intact cell walls was not determined (McCluskey *et al.*, 1990). The nonprotoplast approach has also been used successfully for *Leptosphaeria maculans* (Plummer and Howlett, 1993). Useful pointers for preparing fungal DNA for use in PFGE, using both protoplasting and nonprotoplasting methods, have been reviewed by Mills *et al.* (1995).

After the isolation of sufficient chromosomal-size DNA, the next important step is the choice of appropriate instrumentation. The original studies on *Saccharomyces* used OFAGE (Schwartz and Cantor, 1984; Carle and Olson, 1984) and a number of other yeast genera have also been analyzed successfully with this method (e.g., De Jong *et al.*, 1986; Boekhout *et al.*, 1991, 1992). In the 1990s, however, CHEF has become the method of choice for most fungal systems. A listing of published electrophoretic karyotypes of fungi, including instrumentation method, is presented in Table I.

With CHEF, 24 electrodes are arranged in a hexagon, with orientation angles of 120 or 60 (Chu *et al.*, 1986). The chromosomes are separated

TABLE I

FUNGAL ELECTROPHORETIC KARYOTYPES

Species	Method	Chromosomes		Genome size[a] (Mb)	Citation
		Band # (Mb)	Size range		
Oomycota[b]					
Bremia lactucae	CHEF	7–?	3.0–8.0		Francis and Michelmore, 1993
Phytophythora megasperma[c]	CHEF	9–?	1.4–?		Howlett, 1989
Phytophythora megasperma	CHEF	10–13			Judelson *et al.*, 1993
Pythium acanthicum[d]	CHEF	10–12	1.1–3.2	19.9–25.5	Martin, 1995
Pythium aphanidermatum[d]	CHEF	11–15	0.9–5.7	31.2–41.5	Martin, 1995
Pythium aristosporum	CHEF	15	1.1–4.2	35.6	Martin, 1995
Pythium arrhenomanes[d]	CHEF	9	1.7–4.0	23.9–24.8	Martin, 1995
Pythium deliense[d]	CHEF	12–15	1.5–5.7	34.7–40.7	Martin, 1995
Pythium graminicola[d]	CHEF	14–15	0.9–4.7	29.6–33.2	Martin, 1995
Pythium heterothallicum	CHEF	12	1.1–3.4	26.5	Martin, 1995
Pythium irregulare[d]	CHEF	7–10	1.5–5.5	21.6–30.8	Martin, 1995
Pythium myriotylum[d]	CHEF	11–12	1.4–5.5	33.2–38.5	Martin, 1995
Pythium nunn	CHEF	14	0.9–4.4	31.3	Martin, 1995
Pythium oligandrum[d]	CHEF	12–20	0.7–3.4	28.0–36.7	Martin, 1995
Pythium periplocum	CHEF	11	1.1–3.6	25.9	Martin, 1995
Pythium spinosum[d]	CHEF	8–10	2.0–4.8	25.0–31.4	Martin, 1995
Pythium splendens	CHEF	16	1.0–3.3	31.5	Martin, 1995
Pythium sylvaticum[d]	CHEF	9–13	1.5–5.2	28.7–37.7	Martin, 1995
Pythium torulosum	CHEF	10	1.5–4.2	23.9	Martin, 1995

Pythium ultimum[d]	CHEF	9–14	1.3–4.6	24.3–37.6	Martin, 1995
Pythium vanterpoolii	CHEF	9	1.2–3.9	18.8	Martin, 1995
Zygomycota					
Absidia glauca	RFGE	10	1.2–7.0	42.0	Kayser and Wostemeyer, 1991
Ascomycota					
Cochliobolus heterostrophus	CHEF & TAFE	15–16	1.3–3.7	35	Tzeng *et al.*, 1992
Dipodascus (Endomyces) magnusii	CHEF	13	1.2–5.7	38.0	Filipp *et al.*, 1995
Emericella (Aspergillus) nidulans	CHEF	6	2.9–5.0	31.0	Brody and Carbon, 1989
Emericella (Aspergillus) nidulans	CHEF	6	2.0–6.0	24.2	Xuei and Skatrud, 1994
Emericella (Aspergillus) nidulans	CHEF	6	2.9–5.0	31.0	Geiser *et al.*, 1996
Erysiphe graminis	FIGE	7	0.2–0.8[c]		Borbye *et al.*, 1992
Gibberella fujikuroi	CHEF	11	2.0–6.0	37.0	Tudzynski *et al.*, 1996
Hansenula polymorpha	OFAGE	3			De Jonge *et al.*, 1986
Kluyveromyces aestuarii	OFAGE	6–7		10.2	Sor and Fukuhara, 1989
Kluyveromyces africanus	OFAGE	12–14		17.0	Sor and Fukuhara, 1989
Kluyveromyces blattae	OFAGE	3–5		5.5–7.0	Sor and Fukuhara, 1989
Kluyveromyces cellobiovorus	OFAGE	6		10.6	Sor and Fukuhara, 1989
Kluyveromyces delphensis	OFAGE	8–9		13.2	Sor and Fukuhara, 1989
Kluyveromyces lodderae	OFAGE	13		15.5	Sor and Fukuhara, 1989
Kluyveromyces marxianus					De Jonge *et al.*, 1986
var. *lactis*	OFAGE	2			
var. *marxanus*	OFAGE	5			
Kluyveromyces marxianus					Steensma *et al.*, 1988
var. *lactis*	OFAGE	5			
var. *marxanus*	OFAGE	5–7			

(*continued*)

TABLE I (*Continued*)

Species	Method	Chromosomes: Band # (Mb)	Chromosomes: Size range	Genome size[a] (Mb)	Citation
Kluyveromyces marxianus	OFAGE				Sor and Fukuhara, 1989
var. *bulgaricus*[d]		7–10		10.0–13.2	
var. *dobzhanskii*		6		8.8	
var. *marxianus*[d]		6–12		8.2–18.0	
Kluyveromyces phaffii	OFAGE	8–11		10.6	Sor and Fukuhara, 1989
Kluyveromyces polysporus	OFAGE	8–9		11.2	Sor and Fukuhara, 1989
Kluyveromyces thermotolerans	OFAGE	7		10.2–10.6[d]	Sor and Fukuhara, 1989
Kluyveromyces waltii	OFAGE	4–5		7.2	Sor and Fukuhara, 1989
Kluyveromyces wickerhamii	OFAGE	7–8		13.6	Sor and Fukuhara, 1989
Kluyveromyces yarrowii	OFAGE	13–15		15.0	Sor and Fukuhara, 1989
Leptosphaeria maculans					Taylor *et al.*, 1991
Highly virulent	TAFE	6–8			
Weakly virulent	TAFE	12–14			
Leptosphaeria maculans[d]	CHEF	12–15	0.2–2.2	17.5–23.0	Plummer and Howlett, 1993
Lipomyces starkeyi	CHEF	11	0.7–2.8	15.0	Bignell *et al.*, 1996
Magnaporthe grisea	CHEF	6	3.0–10.0		Talbot *et al.*, 1993
Magnaporthe grisea	CHEF	7	0.2–10.3		Orbach *et al.*, 1996
Nectria haematococca	CHEF	10–15	0.4–7.0		Kistler and Benny, 1992
Neurospora crassa	CHEF	7	4.0–12.6	47.0	Orbach *et al.*, 1988
Ophiostoma ulmi	CHEF	6			Royer *et al.*, 1991
Podospora anserina	CHEF	7	3.9–6.3	33.4	Osiewacz *et al.*, 1990

Pyrenophora graminea[d]	CHEF	6–8	1.6–6.0[c]	42.8	Aragona *et al.*, 2000
Pyrenophora teres	CHEF	6	1.6–6.0[c]	35.0	Aragona *et al.*, 2000
Saccharomyces bayanus	CHEF	16			Fiscer *et al.*, 2000
Saccharomyces cariocanus	CHEF	16			Fiscer *et al.*, 2000
Saccharomyces carlsbergensis	OFAGE	17			De Jonge *et al.*, 1986
Saccharomyces cerevisiae	OFAGE, FIGE	16			Carle and Olson, 1985
Saccharomyces cerevisiae	CHEF	15	0.2–2.2		Chu *et al.*, 1986
Saccharomyces cerevisiae	OFAGE	17		14.0	De Jonge, 1986
Saccharomyces cerevisiae[d]	TAFE	11–20	0.3–2.0		Jager and Philippsen, 1989
Saccharomyces cerevisiae	CHEF	16			Fischer *et al.*, 2000
Saccharomyces dairensis	TAFE	8			Jager and Philippsen, 1989
Saccharomyces exiguus	TAFE	11			Jager and Philippsen, 1989
Saccharomyces kluyveri	TAFE	5			Jager and Philippsen, 1989
Saccharomyces kudriavzevii	CHEF	16			Fischer *et al.*, 2000
Saccharomyces mikatae	CHEF	16			Fischer *et al.*, 2000
Saccharomyces paradoxus	CHEF	16			Fischer *et al.*, 2000
Saccharomyces servazzii	TAFE	19			Jager and Philippsen, 1989
Saccharomyces unisporus	TAFE	10			Jager and Philippsen, 1989
Saccharomyces uvarum	TAFE	14–15	0.2–2.0		Jager and Philippsen, 1989
Schizosaccharomyces pombe	Multiple meth.	3	3.0–9.0	18.0	Smith, *et al.*, 1987
Sclerotinia sclerotiorum	CHEF	16	1.5–4.0	43.5	Fraissinet-Tachet *et al.*, 1996
Sordaria macrospora	TAFE and CHEF	7	3.7–7.5	39.5	Walz and Kuck, 1995
Basidiomycota					
Agaricus bisporus	CHEF	13	1.2–4.0	34	Kerrigan *et al.*, 1993
Agaricus bisporus	CHEF	13	1.4–3.7	31.0	Sonnenberg *et al.*, 1996

(*continued*)

TABLE I (*Continued*)

Species	Method	Chromosomes: Band # (Mb)	Chromosomes: Size range	Genome size[a] (Mb)	Citation
Cryptococcus(Filobasidiella) neoformans	OFAGE				Polacheck and Lebens, 1989
var. *gattii*		9	0.6–1.6		
var. *neoformans*		8	0.6–1.6[d]		
Entyloma arnoseridis	OFAGE	12–16			Boekhout *et al.*, 1992
Entyloma ficariae	OFAGE	15			Boekhout *et al.*, 1992
Filobasidiella neoformans	OFAGE	11			De Jonge *et al.*, 1986
Filobasidiella neoformans	CHEF	7–14	0.5–3.0		Boekhout *et al.*, 1993
Malassezia pachydermatis	CHEF	5		6.7–7.1	Boekhout and Bosboom, 1994
Melanotaenium endogenum	OFAGE	11			Boekhout *et al.*, 1992
Microbotryum violaceum	CHEF	12–15	1.0–5.7	11.0–15.4[d]	Perlin 1996
Phanerochaete chrysosporium					D'Souza *et al.*, 1993
BKMF-1767	TAFE	10	1.8–5.0	29.0	
ME-446	TAFE	11	1.8–5.0	32.0	
Pleurotus ostreatus	CHEF	11	1.4–4.7	35.1	Larraya *et al.*, 2000
Schizophyllum commune	TAFE	6	1.2–5.1	35–36	Horton and Raper, 1991
Schizophyllum commune	CHEF	11	1.6–4.7	35.6	Asgeirsdottir *et al.*, 1994
Tilletia caries (T. controversa)[e]	CHEF	14–20	0.3–4.5	32.3–39.9	Russell and Mills, 1993
Tilletiaria anomala	OFAGE	9–15[f]			Boekhout *et al.*, 1992
Tilletiopsis albescens	OFAGE	14–16			Boekhout *et al.*, 1992
Tilletiopsis fulvescens	OFAGE	17–19			Boekhout *et al.*, 1992
Tilletiopsis minor	OFAGE	10–15			Boekhout *et al.*, 1992

Tilletiopsis pallescens	OFAGE	12–14			Boekhout *et al.*, 1992
Tilletiopsis washingtonensis	OFAGE	12–20			Boekhout *et al.*, 1992
Ustilago hordei[d]	CHEF	15–19	0.2–3.2	18.4–26.0	McCluskey and Mills, 1990
Ustilago maydis	OFAGE	20	0.3–2.0		Kinscherf and Leong, 1988
Ustilago tritici	CHEF	13	0.3–3.0		Mills and McCluskey, 1990
Deuteromycota					
Acremonium chrysogenum	TAFE & CHEF	8	2.6–6.0	32.0	Walz and Kuck, 1991
Acremonium flavum	TAFE & CHEF	7	2.9–6.0	26.5	Walz and Kuck, 1991
Acremonium strictum	TAFE & CHEF	7	2.9–5.9	27.7	Walz and Kuck, 1991
Alternaria alternata	CHEF	9–11	0.6–6.5		Akamatsu *et al.*, 1997
''Other'' *Alternaria* spp.	CHEF	8–10	0.9–6.4		Akamatsu *et al.*, 1997
Aspergillus flavus	CHEF	5–8	3.0–7.0		Keller *et al.*, 1992
Aspergillus flavus	CHEF	7	2.3–7.0	31.2	Foutz *et al.*, 1995
Aspergillus fumigatus	CHEF	5	1.7–4.8	15.8	Tobin *et al.*, 1997
Aspergillus niger	CHEF	4	3.5–6.6	35.5–38.5	Debets *et al.*, 1990
Aspergillus niger	CHEF	8			Verdoes *et al.*, 1994
Aspergillus oryzae	TAFE	8	2.8–7.0	35	Kitamoto *et al.*, 1994
Aspergillus parasiticus	CHEF	5–7	3.0–7.0		Keller *et al.*, 1992
Aspergillus versicolor	CHEF	6	3.0–7.0		Keller *et al.*, 1992
Beauveria bassiana	CHEF	5–8		34.3–44.1	Viaud *et al.*, 1996
Cephalosporium acremonium	TAFE	8	1.7–4.0	22.5	Skatrud and Queener, 1989
Candida albicans	OFAGE	4–7		14.0	De Jonge *et al.*, 1986
Candida albicans	CHEF	6–13	1.1–2.9		Asakura *et al.*, 1991
Candida albicans	CHEF	8			Wickes *et al.*, 1991
Candida albicans	CHEF			16.0–17.0	Chu *et al.*, 1993

(*continued*)

TABLE I (*Continued*)

Species	Method	Chromosomes		Genome size[a] (Mb)	Citation
		Band # (Mb)	Size range		
Candida glabrata	CHEF	6–12	0.4–2.4		Asakura *et al.*, 1991
Candida molischiana	CHEF	7–8		12.0	Janbon *et al.*, 1995
Candida rugosa	CHEF	8	0.1–2.1	20.0	Brocca *et al.*, 1995
Candida stellatoidea	CHEF	12			Rikkerink *et al.*, 1990
Cladosporium fulvum	CHEF	9	1.9–5.4	44.0	Talbot *et al.*, 1991
Colletotrichum gloeosporoides					Masel *et al.*, 1990
type A	CHEF	13–15[d]	0.3–6.0[c]	23.5–24.3[d]	
type B	CHEF	6–8[d]	0.3–6.0[c]	17.5–19.2[d]	
Colletotrichum lindemuthianum	CHEF	9–12	0.7–7.0[c]		O'Sullivan *et al.*, 1998
Curvularia lunata	CHEF	12	1.4–4.0	29.7	Osiewacz and Ridder, 1991
Fusarium acuminatum	CHEF				Nagy and Hornok, 1994
subsp. *acuminatum*[d]		9	0.6–7.2	41.4–45.1	
subsp. *armeniacum*[d]		6	1.0–7.1	33.5–33.7	
Fusarium avenaceum	CHEF	7	0.4–6.5	27.0	Fekete *et al.*, 1993
Fusarium camptoceras	CHEF	7	1.0–6.5	28.1	Fekete *et al.*, 1993
Fusarium chlamydosporum	CHEF	8	1.0–6.5	29.5	Fekete *et al.*, 1993
Fusarium fusarioides	CHEF	6	0.9–6.5	29.9	Fekete *et al.*, 1993
Fusarium oxysporum	CHEF	11–14	0.8–6.7	41.0–51.5	Migheli *et al.*, 1993
Fusarium pallidoroseum	CHEF	8	1.3–6.5	29.5	Fekete *et al.*, 1993
Fusarium poae	CHEF	6	1.0–6.5	27.5	Fekete *et al.*, 1993
Fusarium solani	CHEF	13	0.4–6.1	39.9	Nazareth and Bruschi, 1994

Fusarium sporotrichioides	CHEF	6	1.2–6.5	27.7	Fekete *et al.*, 1993
Fusarium sporotrichioides	CHEF	6	1.2–7.0	20.4	Nagy *et al.*, 1995
Fusarium tricinctum	CHEF	9	1.2–6.5	29.2	Fekete *et al.*, 1993
Histoplasma capsulatum	CHEF & FIGE	7	2.0–5.7		Steele *et al.*, 1989
Hortaea werneckii	CHEF	12–18	0.5–2.3		Boekhout *et al.*, 1993
Itersonilia perplexans	OFAGE	10–12			Boekhout *et al.*, 1991
Itersonilia pyriformans	OFAGE	10–12			Boekhout *et al.*, 1991
Paecilomyces fumosoroseus	CHEF	6	3.1–7.8	30.1	Shimizu *et al.*, 1991
Penicillium janthinellum	RFGE	10	2.0–8.0	39–46	Kayser and Schultz, 1991
Phoma tracheiphila	OFAGE	12			Rollo *et al.*, 1989
Rhodosporidium toruloides	OFAGE	10	0.4–4.0		De Jonge *et al.*, 1986
Rhodotorula mucilaginosa	OFAGE	9	0.2–1.0		De Jonge *et al.*, 1986
Septoria nodorum[d]	CHEF	14–19	0.5–3.5	28–32	Cooley and Caten, 1991
Septoria tritici[d]	TAFE	14–16	0.3–3.5	29.5–32.6	McDonald and Martinez, 1991
Tolypocladium inflatum	CHEF	7	1.1–6.6	30.5	Stimberg *et al.*, 1992
Trichoderma reesei	CHEF	6	3.3–11.9	38.0	Gilly and Sands, 1991
Trichoderma longibrachiatum(reesei)	CHEF	7	2.8–6.9	33.0	Mantyla *et al.*, 1992

[a]Where given, estimates are based on comparison of electrophoretic mobilities.
[b]Oomycota are included here out of tradition although they are no longer considered ''true'' fungi.
[c]Maximum size resolved; may contain chromosomes of larger size.
[d]Ranges due to differences between isolates.
[e]Considered one species.
[f]Showed weak resolution of bands; difficult to estimate.

in straight bands, facilitating direct comparisons with standards. A CHEF system using programmable, autonomously controlled electrodes has been commercialized by Bio-Rad (Richmond, CA). Sensing circuitry adjusts the voltage across electrode pairs to compensate for variations in temperature, power supply output, buffer conductivity, and gel thickness. The CHEF system reduces total run times for DNAs of sizes greater than 2 Mb and, because it can separate a wide range of DNA fragments, is well suited for the analysis of genomes in which the actual size of the chromosomes to be separated is not known before-hand (Birren and Lai, 1993).

Whatever instrumentation is used, appropriate size markers are extremely important. Chromosome size of newly investigated species is estimated by comparing the migration distances of their electrophoretic bands with standards of known electrophoretic mobility. Yeast chromosomes have been sized accurately in conjunction with restriction mapping so they are commonly used as sizing standards for filamentous species. For small chromosomes, synthetic ladders of *S. cerevisiae* chromosomes (0.2 to 2 Mb) are employed, while for larger chromosomes, the three chromosomes (3.5, 4.6, and 5.7 Mb) of *Schizosaccharomyces pombe* are commonly used. Bio-Rad sells preprepared agarose plugs of these yeast chromosome standards.

For many fungi, chromosome size extends over a fairly wide range and cannot be resolved by any one set of electrophoretic conditions. In such cases, a combination of two or more runs may be required. Large chromosomal DNA requires long switch intervals, low voltages, and longer times, while the run conditions for smaller chromosomes call for short switch intervals, high voltage, and shorter times. For example, successful separation of chromosomes of *Fusarium solani* was achieved with a combined switch interval of 45 min for 52 hr, followed by 30 min for 68 h at 60 V, to yield twelve bands ranging from 2.08 to 6.08 Mb. When a switch interval of 60 seconds for 15 h followed by 90 seconds for 9 h at 150 V was applied, an additional small band of 0.42 Mb was resolved, indicating that *F. solani* has a total of 13 chromosomes (Nazareth and Bruschi, 1994).

IV. Chromosome Numbers and Size

Until the advent of electrophoretic karyotyping, chromosome number and estimated genome size were known for only a few well-studied fungal models, such as *Aspergillus nidulans* (Timberlake, 1978). Now, karyotypic analysis has been extended to almost all major groups of fungi except the chytrids, with published reports of over 100

species (see Table I). Most fungi have a vegetative chromosome number between 5 and 20. Estimated genome size ranges from approximately 10 to 40 Mb. In addition to giving mycologists this hitherto inaccessible view of basic chromosome number and genome size, PFGE has also provided a number of interesting new insights about fungal genome architecture. Perhaps the most striking finding is that most species studied to date exhibit chromosome length polymorphisms, that is, homologous chromosomes in different strains of the same species can have quite different chromosome sizes. Chromosomal polymorphism is common in the yeasts *S. cerevisiae* (Bidenne *et al.*, 1992; Carle and Olson, 1985) and *Candida albicans* (Magee and Magee, 1987; Chu *et al.*, 1993). Filamentous fungi also show extensive polymorphism; these include phytopathogenic species (Cooley, 1992), basidiomycetes such as *Microbotryum violaceum* (Perlin, 1996), and many asexual species (Zolan, 1995). In one case, 15 spores from a single pseudoperithecium of *Leptosphaeria maculans* displayed 10 different karyotypes (Plummer and Howlett, 1993). Polymorphisms have been correlated with genetic translocations, gains and losses of apparently nonessential sequences, and differences in the number of long tandem repeats of rDNA. Kistler and Miao (1992) have hypothesized that the extent of chromosomal polymorphism is inversely correlated with the frequency of meiosis, i.e., asexually reproducing fungi should have more polymorphism. Even so, chromosome length polymorphism occurs commonly in species with meiotic cycles and they segregate regularly in the sexual progeny of *Ustilago hordei* (McCluskey and Mills, 1990). See Zolan (1995) for an excellent review of fungal chromosome polymorphism.

PFGE analysis of fungi has also revealed another form of karyotype variation. Many species possess a variable number of small, ''dispensable'' chromosomes. These chromosomes are usually within the range of 0.5 to 2.0 Mb. Their small size, instability during meiosis, and dispensable nature makes them similar to the ''B'' chromosomes described from other eukaryotes (Jones, 1982). However, unlike classical B chromosomes, they sometimes carry functional genes (Miao *et al.*, 1991b; Xu, 1995; Van Etten *et al.*, 1998). The first report was from *Nectria haematococca*, where the loss of a 1.6 Mb chromosome was associated with loss of pathogenicity (Miao *et al.*, 1991a). The pisatin demethylase gene that specifies the enzyme involved in the detoxification of pisatin, a pea phytoalexin, is located on the dispensable 1.6 Mb chromosome (Miao *et al.*, 1991b). Where reported, these fungal supernumerary chromosomes (sometimes called minichromosomes) appear to be more polymorphic than standard fungal chromosomes (Masel *et al.*, 1990). The variable karyotype of species such as *Magnaporthe grisea* is due

largely to differences in the number of these minichromosomes (Orbach, 1996). In *Colletotrichum lindemuthianum*, supernumerary chromosome number ranged from 2 to 6, an attribute of the genome that was described as ''hypervariable'' (O'Sullivan *et al.*, 1998). Not all small chromosomes are supernumerary. In *C. albicans*, the minichromosome turned out to be a translocation product (Chu *et al.*, 1993).

V. Applications of PFGE

A. Genetic Mapping

One of the most important applications of PFGE has been for genetic mapping. With some modifications, conventional blotting and hybridization protocols can be used to assign cloned genes and other probes to linkage groups by Southern blot and/or fluorescent *in situ* hybridization. This approach is much faster than parasexual analysis and is a boon to linkage studies in fungi lacking sexual cycles. For successful Southern transfer of DNA larger than 2 Mb from standard agarose gels onto membranes, DNA must be cleaved by acid depurination or ultraviolet (UV) irradiation. The UV method for nicking DNA is more reliable. However, it should be noted unless DNA is stained with ethidium bromide, it will not be nicked and thus will not be transferred. When several chromosomes are co-migrating or when large chromosomes are not properly resolved, the application of rare-cutting endonucleases may aid in localizing genes on chromosomal fragments (Kempken and Waltz, 1996).

In one application, Keller *et al.* (1992) were able to compare the location of conserved genes (*argB, benA, trpC, niaD, pyrG*) in *Aspergillus flavus, Aspergillus parasiticus*, and *Aspergillus nidulans*. The map location of gene probes in *A. flavus* and *A. parasiticus* was similar, supporting the placement of these aflatoxigenic species into a different taxonomic section from *A. nidulans*. Foutz *et al.* (1995) extended this kind of analysis by using PFGE to assign parasexually defined linkage groups to seven *A. flavus* chromosomes. Hybridization analysis was also useful for genetic studies in the cultivated mushroom *Agaricus bisporus*, where Sonnenberg *et al.* (1996) exploited known gene probes, restriction fragment length polymorphism (RFLP) markers, and cDNA as expressed sequence tags (ESTs) to assign at least two markers to each of the 13 chromosomes of this economically important species. In another application, McDonald and Martinez (1991) used the hybridization approach to confirm the disomy of one RFLP locus in *Septoria tritici*.

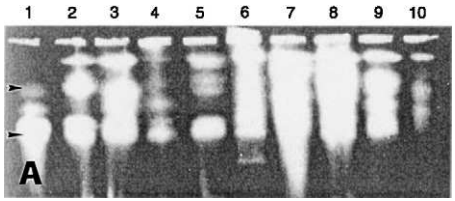

FIG. 1. Electrophoretic karyotypes of selected Aspergilli. 1. *Aspergillus nidulans* (SRRC 1079); 2. *A. tamari* (SRRC 1244); 3. *A. flavus* (SRRC 2112); 4. *A. flavus* (Papa 1300); 5. *A. flavus* (SRRC 2045); 6. *A. flavus* (Papa 650–33); 7. *A. oryzae* (SRRC 2104); 8. *A. sojae* (SRRC 1126); 9. *A. parasiticus* (SRRC 1039); 10. *A. parasiticus* (SRRC 162). In lane 1, arrows point to the largest (5.0 Mb) and smallest (2.9 Mb) chromosomes of *A. nidulans*.

B. Strain Identification

Chromosome length polymorphism can be harnessed to provide an adjunct to taxonomic classification of individual species or strains within species. For example, Vaughan-Martini *et al.* (1993) used CHEF analysis to examine the type strains of 10 species of the genus *Saccharomyces*. Six species showed uniform karyotypes, while *S. dairensis, S. exiguus, S. sarvazzii*, and *S. unisporus* were heterogeneous. In *Ustilago hordei*, different karyotypes were found for each of the 14 races studied, even though the individual strains from different races were sexually compatible (McCluskey and Mills, 1990). The four serotypes A, B, C, and D of the human pathogen *Cryptococcus neoformans* could be separated into three groups based on chromosomal polymorphism; serotype D showed extensive polymorphism even among individual isolates (Polacheck and Lebens, 1989). Different clinical isolates of *C. albicans* have different karyotypes (Magee and Magee, 1987; Mertz *et al.*, 1988) as do unstable morphological mutants of this species (Rustchenko-Bulgac, 1991).

In some cases, differences in karyotype variation can be used to compare species with a genus. *A. nidulans* isolated from Great Britain showed little apparent size variation (Geiser *et al.*, 1996), while *Aspergillus flavus* and its allies (Keller *et al.*, 1992) and *Aspergillus niger* and its allies (Debets *et al.*, 1990) exhibit considerable polymorphism (see Fig. 1).

C. Analysis of Transformed Strains

PFGE can be used to locate recombinant DNA directly on individual chromosomes. In *Sordaria macrospora*, for example, Southern hybridization showed that all vector molecules were integrated into only one

chromosome in each transformant, i.e., vector copies were integrated at a single locus. Integration of the transforming DNA was associated with structural changes of the transformed chromosomes (Walz and Kuck, 1995). Increase in chromosome size after transformation was reported for *Aspergillus niger* (Debets *et al.*, 1990), *Ophiostoma ulmi* (Royer *et al.*, 1991), and *Acremonium chrysogenum* (Walz and Kuck, 1991). In the echinocandin B–producing strain of *A. nidulans*, the introduction of a plasmid carrying a hygromycin resistance gene resulted in transformants with altered karyotypes. Several types of chromosomal changes were observed, including apparent chromosome loss, size alterations, and the appearance of large chromosomes. These karyotypes changes were mitotically stable (Xuei and Skatrud, 1994).

D. Preparative PFGE

In addition to being an analytic tool, PFGE can also be used as a preparative method to provide a source of purified chromosomal DNA for gene cloning and genomics research. After electrophoresis, DNA can be extracted from individual chromosome bands in the agarose gel. In many eukaryotic genomics projects, genomic DNA is sorted into chromosome-size pieces before library construction. Tiling paths of ordered clones can be assembled to produce a system of minimal clone overlap. Individual clones are then sequenced. Chromosome-specific libraries can also be used to help order genes from pre-existing libraries. Ordered libraries are valuable in chromosome walking and complementation strategies.

An early German *Neurospora* genome project utilized chromosomes that were first separated by PFGE. Physical maps were created by hybridization using a cosmid library. Chromosome sublibraries increased the efficiency of the mapping procedure (Aign *et al.*, 2001; Bhandarkar *et al.*, 2001; Kelkar *et al.*, 2001). PFGE also has been used successfully in physical mapping of *A. nidulans* (Brody *et al.*, 1991; Prade *et al.*, 1997), *C. albicans* (Magee and Scherer, 1998), *Pneumocystis carinii* (Arnold and Cushion, 1997), and *S. pombe* (Mizukami *et al.*, 1993). For an overview of the role of physical mapping in fungal genomics, see Bennett and Arnold (2001).

VI. Limitations of PFGE for Fungal Karyotype Analysis

Without a doubt, PFGE has increased our understanding of the chromosome constitution of the fungal kingdom. Appropriately, the

focus of previous reviews has been on the advantages of the method and the insights that have been derived from its application (Mills and McCluskey, 1990; Skinner *et al.*, 1991; Mills *et al.*, 1995; Walz and Kuck, 1995; Zolan, 1995). For this reason, the inherent limitations of the PFGE approach are often overlooked. In well-studied species, it is usually possible to circumvent these limitations, but for many species lacking genetic systems, the results of PFGE can be disappointing. Problems and pitfalls of PFGE karyotype analysis are listed here.

1. When chromosomes are of similar or equal size, they migrate at the same rate. Although increased intensity of ethidium bromide staining of bands is used to infer doublets and triplets, when a species has several chromosomes of similar or identical size, it can be extremely difficult to get an accurate chromosome count.

   For species with well-developed genetic systems, the altered migration rate of well-characterized translocation strains has been used to achieve separation for all chromosomes. This strategy was used successfully to develop karyotypes for *A. nidulans* (Brody and Carbon, 1989) and *S. cerevisiae* (Schwartz and Cantor, 1984). Another strategy was developed by Verdoes *et al.* (1994), who succeeded in generating a number of *A. niger* strains with altered electrophoretic karyotypes by introducing multiple copies of the *glaA* gene. Finally, chromosome-specific probes for individual linkage groups and heterologous telomeric probes can also be used to resolve chromosomes. However, for species that do not have well-developed genetic systems and that contain several chromosomes in the same size range, these options are difficult to implement.
2. When the chromosomes of a species have a large size range, it is necessary to use several electrophoretic conditions to obtain separation. For example, in their study of six strains of *Sclerotinia sclerotiorum*, Fraissinet-Tachet *et al.* (1996) used three sets of conditions to separate large chromosomes (A), small chromosomes (B), and possible mini-chromosomes (C). Conditions A and B yielded nine or ten bands, in which two or three bands were doublets and two bands were at least triplets. They concluded that *S. sclerotiorum* contains at least 16 chromosomes. For *Fusarium sporotrichoides*, one set of conditions was used that separated *S. pombe* chromosome-sized DNAs, while a second set of conditions was used to separate chromosomes less than 2 Mb in size (Fekete *et al.*, 1993). Uncovering the right algorithm for these chromosome separations can be extremely time consuming.

3. Artifacts such as chromosome fragments are not easily distinguished from true chromosomal length polymorphism or supernumerary chromosomes. Sometimes telomeric probes can be used to help estimate chromosome numbers but even this approach can give ambiguous results. In *Beauvaria bassiana*, for example, when genomic DNA was cut with three different restriction enzymes and probed with a heterologous telomeric probe, 6 to 16 bands were detected, indicating ''a minimum of 7 to 8 chromosomes'' (Viaud *et al.*, 1996).

   Further, most PFGE protocols do not resolve chromosomes larger than 8 Mb. Chromosomes of this size or larger migrate as a single band. When workers study a new species and fail to achieve adequate chromosome separation by PFGE because of large chromosome size or inability to obtain appropriate separation, these results usually do not get published.
4. Estimates of genome size have to be accepted as ''ballpark'' figures. The mobility of bands is sensitive to the amount of DNA loaded; increasing concentrations lead to lower mobility. Sizing errors can be as great as 30% (Birren and Lai, 1993). For example, when based on size comparisons of separated chromosomes, the estimated genome size of *Fusarium sporotrichoides* was 27.7 Mb (Fekete *et al.*, 1993) When genome size was calculated by summing the total size of *Not1* fragments, the projected genome size was approximately 20 Mb (Nagy *et al.*, 1995), a substantially lower figure. Similarly, the estimated genome size of *Neurospora crassa* was 43 Mb when based on PFGE but only 40 Mb when based on direct sequencing. In the case of *Aspergillus oryzae*, the reverse was true. The size estimate from PFGE was 35 Mb while the estimate from shotgun sequencing was 37 Mb (Machida, 2002).
5. Chromosomal polymorphism is extensive within and between fungal populations. Different strains of a single species often give distinctly different karyotypes. In addition, the number and size of mini-chromosomes is also highly variable (Mills and McCluskey, 1990; Scherer and Magee, 1990; Zolan, 1995). This enormous natural variation is in contrast to the prevailing paradigm of eukaryotic cytogenetics in which there is a standard ''reference'' karyotype for each species (Brown, 1972; Garber, 1972). For a large number of fungi, intraspecific karyotypic variability is the rule rather than the exception.

In summary, when working in the dark with previously unstudied fungi, it is not easy to know when and if adequate separation has been achieved. An examination of the species listed in Table I shows that

exact chromosome number could not be determined for over a third; it is likely that some of the numbers given for the other two-thirds are not accurate. In addition, it is important to remember that fungal karyotypes are less constant than karyotypes of other eukaryotes. Among fungi, karyotypic plasticity is the rule, not the exception.

VII. Summary

The resolution of chromosomal-sized DNAs by PFGE has many applications that include karyotyping, strain identification of similar species, characterization of transformed strains, building of linkage maps, and preparation of DNA for genomic analysis. Successful electrophoretic separation of chromosomes is an empiric process in which the initial concentration of intact chromosome-sized DNA and the optimization of electrophoretic parameters are the most important experimental variables. Nonetheless, inherent attributes of the genome architecture of certain species may thwart success. When a karyotype contains numerous chromosomes of the same size and/or many large (greater than 8 Mb) chromosomes, no amount of manipulation of the electrophoretic parameters will resolve individual chromosome bands using present technology. Further, fungi display a surprising amount of intraspecific variation in both chromosome number and size, making it difficult to establish a standard ''reference'' karyotype for many species.

Although PFGE is not a panacea for bringing genetics to species that lack classical genetic systems, it often does provide a way for developing a molecular linkage map in the absence of a formal genetic system. It is far faster than parasexual analysis in the discovery of linkage relationships. For genomics projects, DNA can be recovered from pulsed field gels and used to prepare chromosome-specific libraries. Where whole genome sequencing strategies are used, chromosomes separated by PFGE provide an anchor for sequencing data. Electrophoretic karyotypes can be probed with anonymous pieces of DNA from bacterial artificial chromosome (BAC) contigs, thereby facilitating the building of physical maps. In conclusion, despite its shortcomings, the PFGE technique underlies much of our current understanding of the physical nature of the fungal genome.

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References

Aign, V., Schulte, U., and Hoheisel, J. D. (2001). Hybridization-based mapping of *Neurospora crassa* linkage groups II and V. *Genetics* **157,** 1015–1020.

Akamatsu, H., Itoh, Y., Kodama, M., Otani, H., Kohmoto, K., and Taga, M. (1997). Molecular karyotypes for *Alternaria* plant pathogens known to produce host-specific toxins. *The 19th Fungal Genetics Conference at Asilomar* (Abstract #15).

Anand, R. (1986). Pulsed field gel electrophoresis: A technique for fractionating large DNA molecules. *Trends in Genetics* **2,** 278–283.

Aragona, M., Montigiani, M., and Porta-Puglia, A. (2000). Electrophoretic karyotypes of the phytopathenogenic *Pyrenophora graminea* and *P. teres*. *Mycological Research* **104,** 853–857.

Arnold, J., and Cushion, M. T. (1997). Constructing a physical map of the *Pneumocystis* genome. *Journal of Eukaryotic Microbiology* **44,** 8S.

Asakura, K., Iwaguchi, S., Homma, M., Sukai, T., Higashide, K., and Tanaka, K. (1991). Electrophoretic karyotypes of clinically isolated yeasts of *Candida albicans* and *C. glabrata*. *Journal of General Microbiology* **137,** 2531–2538.

Asgeirsdottir, S. A., Schuren, F. H. J., and Wessels, J. G. H. (1994). Assignment of genes to pulse-field separated chromosomes of *Schizophyllum commune*. *Mycological Research* **98,** 689–692.

Bennett, J. W., and Arnold, J. (2001). Genomics for fungi. *In* ''Biology of the Fungal Cell. The Mycota VIII.'' (R. Howard, and N. Gow, eds.), pp. 267–297. Springer-Verlag, Heidelberg, Germany.

Bhandarkar, S. M., Machaka, S. A., Shete, S. S., and Kota, R. N. (2001). Parallel computation of a maximum-likelihood estimator of a physical map. *Genetics* **157,** 1021–1043.

Bidenne, C., Blondin, B., Dequin, S., and Vezinhet, F. (1992). Analysis of the chromosomal DNA polymorphism of wine strains of *Saccharomyces cerevisiae*. *Current Genetics* **22,** 1–7.

Bignell, G. R., Bruce, I. J., and Evans, I. H. (1996). Electrophoretic karyotype of the amylolytic yeast *Lipomyces starkeyi* and cloning, sequencing, and chromosomal localization of its TRP1 gene. *Current Genetics* **30,** 83–88.

Birren, B., and Lai, E. (1993). ''Pulsed Field Gel Electrophoresis: A Practical Guide.'' Academic Press, Inc., New York.

Birren, B. W., Lai, E., Clark, S. M., Hood, L., and Simon, M. I. (1988). Optimized conditions for pulsed field gel electrophoresis separations of DNA. *Nucleic Acids Res.* **16,** 7563–7582.

Boekhout, T., and Bosboom, R. W. (1994). Karyotyping of *Melassezi* yeasts: Taxonomic and epidemiological implications. *Systemic and Applied Microbiology* **17,** 146–153.

Boekhout, T., Poot, G., and Hackman, P. (1991). Genomic characteristics of strains of *Itersonila*: Taxonomic consequences and lifestyle. *Canadian Journal of Microbiology* **37,** 188–194.

Boekhout, T., Renting, M., Scheffers, W. A., and Bosboom, R. (1993). The use of karyotyping in the systematics of yeast. *Antonie von Leeuwenhoek* **63,** 157–163.

Boekhout, T., Van Gool, J., Van den Boogert, H., and Jille, T. (1992). Karyotyping and G+C composition as taxonomic criteria applied to the systematics of *Tilletiopsis* and related taxa. *Mycological Research* **95,** 331–342.

Borbye, L., Linde-Laursen, I., Christiansen, S. K., and Giese, H. (1992). The chromosome complement of *Erysiphe graminis* f. sp. *hordei* analyzed by light microscopy and field inversion gel electrophoresis. *Mycological Research* **96,** 97–102.

Brocca, S., Grandori, R., Breviario, D., and Lotti, M. (1995). Localization of lipase genes on *Candida rugosa* chromosomes. *Current Genetics* **28,** 454–457.

Brody, H., and Carbon, J. (1989). Electrophoretic karyotype of *Aspergillus nidulans. Proceedings of the National Academy of Sciences* (USA) **86,** 6260–6263.

Brody, H., Griffith, J., Cuticchia, A. J., Arnold, J., and Timberlake, W. (1991). Chromosome-specific recombinant libraries from the fungus *Aspergillus nidulans*. *Nucleic Acids Research* **19,** 3105–3109.

Brown, W. V. (1972). ''Textbook of Cytogenetics.'' C. V. Mosby, St. Louis, Missouri.

Bustamente, C., Guerrieri, S., and Smith, S. B. (1993). Towards a molecular description of pulsed-field gel electrophoresis. *Trends in Biotechnology* **11,** 23–30.

Carle, G. F., and Olson, M. V. (1984). Separation of chromosomal DNA molecules from yeast by orthogonal-field-alteration gel electrophoresis. *Nucleic Acids Research* **12,** 5647.

Carle, G. F., and Olson, M. V. (1985). An electrophoretic karyotype for yeast. *Proceedings of the National Academy of Sciences* (USA) **82,** 3756–3760.

Chu, G. (1990). Pulsed-field gel electrophoresis: Theory and practice. *Methods* **1,** 129–142.

Chu, G., Vollrath, D., and Davis, R. W. (1986). Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science* **234,** 1582.

Chu, W., Magee, B., and Magee, P. (1993). Construction of an *SfiI* macrorestriction map of the *Candida albicans* genome. *Journal of Bacteriology* **20,** 6637–6651.

Cooley, R. N. (1992). The use of RFLP analysis, electrophoretic karyotyping and PCR in studies of plant pathogenic fungi. *In* ''Molecular Biology of Filamentous Fungi,'' (U. Stahl, and P. Tadeynski, eds.), pp. 13–25. EMBO Workshop, Berlin, Germany.

Cooley, R. N., and Caten, C. E. (1991). Variation in electrophoretic karyotype between strains of *Septoria nodorum*. *Molecular General Genetics* **228,** 17–23.

De Jonge, P., De Jongh, F. C. M., Meijers, R., Steenma, H. Y., and Scheffers, W. A. (1986). Orthogonal-field-alternation gel electrophoresis banding patterns of DNA from yeasts. *Yeast* **2,** 193–204.

Debets, A., Holub, E., Swart, K., van den Broek, H., and Bos, C. (1990). An electrophoretic karyotype of *Aspergillus niger*. *Molecular General Genetics* **224,** 264–268.

Deutsch, J. M., and Madden, T. L. J. (1989). Theoretical studies of DNA during gel electrophoresis. *Chemical Physics* **90,** 2476.

D'Souza, T. M., Dass, S. B., Rasooly, A., and Reddy, A. (1993). Electrophoretic karyotyping of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Molecular Microbiology* **8,** 803–807.

Eby, M. J. (1990). Pulsed-field separations: Continued evolution. *Bio/Technology* **8,** 243–245.

Fekete, C., Nagy, R., Debets, A., and Hornok, L. (1993). Electrophoretic karyotype and gene mapping in eight species of the *Fusarium* sections *Arthrosporiella* and *Sporotrichiella*. *Current Genetics* **24,** 500–504.

Filipp, D., Filipp, P., Nosek, J., and Hladka, M. (1995). Electrophoretic karyotype of *Dipodascus (Endomyces) magnusii*: Two main intraspecific chromosomal polymorphisms associated with the difference in total genome size. *Current Genetics* **29,** 81–87.

Fischer, G., James, S. A., Roberts, I., Oliver, S., and Louis, E. (2000). Chromosomal evolution in *Saccharomyces*. *Nature* **405,** 451–454.

Foutz, K. R., Woloshuk, C., and Payne, G. (1995). Cloning and assignment of linkage group loci to a karyotypic map of the filamentous fungus *Aspergillus flavus*. *Mycologia* **87,** 787–794.

Fraissinet-Tachet, L., Reymond-Cotton, P. R., and Fevre, M. (1996). Molecular karyotype of the phytopathogenic fungus *Sclerotina sclerotiorum*. *Current Genetics* **29,** 496–501.

Francis, D. M., and Michelmore, R. W. (1993). Two classes of chromosome-sized molecules are present in *Bremia lactucae*. *Experimental Mycology* **17,** 284–300.

Garber, E. D. (1972). ''Cytogenetics, An Introduction.'' McGraw Hill, New York.

Geiser, D., Arnold, M., and Timberlake, W. (1996). Wild chromosomal variants in *Aspergillus nidulans*. *Current Genetics* **29,** 293–300.

Gilly, J. A., and Sands, J. A. (1991). Electrophoretic karyotype of *Trichoderma reesei*. *Biotechnology Letters* **13,** 447–482.

Hamlyn, P. F., Bradshaw, R. E., Mellon, F. M., Santiago, C. M., Wilson, J. M., and Peberdy, J. F. (1991). Efficient protoplast isolation from fungi using commercial enzymes. *Enzyme Microbiology and Technology* **3,** 321–325.

Horton, J. S., and Raper, C. A. (1991). Pulsed-field gel electrophoretic analysis of *Schizophyllum commune* chromosomal DNA. *Current Genetics* **19,** 77–80.

Howlett, B. (1989). An electrophoretic karyotype for *Phytophthora megasperma*. *Experimental Mycology* **13,** 199–202.

Jager, D., and Philippsen, P. (1989). Many yeast chromosomes lack the telomere-specific Y′ sequence. *Molecular and Cellular Biology* **9,** 5754–5757.

Janbon, G., Magnet, R., Bigey, F., Arnaud, A., and Galzy, P. (1995). Karyotype studies on different strains of *Candida molischiana* by pulsed field electrophoresis. *Current Genetics* **28,** 150–154.

Jones, R. N. (1982). ''B Chromosomes.'' Academic Press, New York.

Judelson, H. S., Coffey, M. D., and Arredondo, F. R. (1993). Transformation of the oomycete pathogen *Phytophthora megasperma* occurs by DNA integration into a single or multiple chromosomes. *Current Genetics* **23,** 211–218.

Kayser, T., and Schultz, G. (1991). Electrophoretic karyotype of cellulolytic *Penicillium janthinellum* strains. *Current Genetics* **20,** 289–291.

Kayser, T., and Wostemeyer, J. (1991). Electrophoretic karyotype of the zygomycete *Absidia glauca*: Evidence for differences between mating types. *Current Genetics* **19,** 279–284.

Kelkar, H. S., Griffith, J., Case, M. E., Covert, S. F., and Hall, R. D. (2001). The *Neurospora crassa* genome: Cosmid libraries sorted by chromosome. *Genetics* **157,** 979–990.

Keller, N. P., Cleveland, T. E., and Bhatnagar, D. (1992). Variable electrophoretic karyotypes of members of *Aspergillus* section *Flavi*. *Current Genetics* **21,** 371–375.

Kempken, F., and Walz, M. (1996). The use of rare-cutting endonucleases in electrophoretic karyotyping of fungal genomes. *Fungal Genetics and Biology* **20,** 80–92.

Kerrigan, R. W., Royer, J. C., Baller, L. M., Kohli, Y., Horgen, P. A., and Anderson, J. B. (1993). Meiotic behavior and linkage relationships in the secondarily homothallic fungus *Agaricus bisporus*. *Genetics* **133,** 225–236.

Kinscherf, T. G., and Leong, M. (1988). Molecular analysis of the karyotype of *Ustilago maydis*. *Chromosoma* **96,** 427–433.

Kistler, H. C., and Benny, U. (1992). Autonomously replicating plasmids and chromosome rearrangement during transformation of *Nectria haematococca*. *Gene* **117,** 81–89.

Kistler, H. C., and Miao, V. P. W. (1992). New modes of change in filamentous fungi. *Annual Review of Phytopathology* **30,** 131–152.

Kitamoto, K., Kimura, K., Gomi, K., and Kumagai, C. (1994). Electrophoretic karyotype and gene assignment to chromosomes of *Aspergillus oryzae*. *Bioscience, Biotechnology, and Biochemistry* **58,** 1467–1470.

Lai, E., Birren, B. W., Clark, S. M., Simon, M. I., and Hood, L. (1989). Pulsed field gel electrophoresis. *Bio Techniques* **7,** 34–42.

Larraya, L. M., Perez, G., Penas, M., Baars, J. J. P., Mikosch, T., Pisabarro, A., and Ramirez, L. (2000). Molecular karyotype of the white rot fungus *Pleurotus ostreatus*. *Applied and Environmental Microbiology* **65,** 3413–3417.

Lodder, S., Wood, D., and Gull, K. (1993). A protoplasting technique with general applicability for molecular karyotyping of hymenomycetes. *Journal of General Mycrobiology* **139,** 1063–1067.

Lu, B. C. K. (1996). Chromosomes, mitosis, and meiosis. *In* ''Fungal Genetics: Principles and Practices.'' (C. J. Bos, ed.), pp. 119–177. Marcel Dekker, Inc., New York.

Machida, M. (2002). Progress of *Aspergillus oryzae* genomics. *Advances in Applied Microbiology* **51,** 81–106.

Magee, B. B., and Magee, P. T. (1987). Electrophoretic karyotypes and chromosome numbers in *Candida* species. *Journal of General Microbiology* **133,** 425–230.

Magee, P. T., and Scherer, S. (1998). Genome mapping and gene discovery in *Candida albicans*. *ASM News* **64,** 505–511.

Mantyla, A. L., Rossi, K. H., Vanhanen, S. A., Penttila, M. E., Suominen, P. L., and Nevalainen, K. M. H. (1992). Electrophoretic karyotyping of wild-type and mutant *Trichoderma longibrachiatum* (*reesei*) strains. *Current Genetics* **21,** 471–477.

Martin, F. N. (1995). Electrophoretic karyotype polymorphisms in the genus *Pythium*. *Mycologia* **87,** 333–353.

Masel, A., Braithwaite, K., Irwin, J., and Manners, J. (1990). Highly variable molecular karyotypes in the plant pathogen *Colletotrichum gloeosporioides*. *Current Genetics* **18,** 81–86.

McClintock, B. (1945). Preliminary observations of the chromosomes of *Neurospora crassa*. *American Journal of Botany* **32,** 671–678.

McCluskey, K., and Mills, D. (1990). Identification and characterization of chromosome length polymorphisms among strains representing fourteen races of *Ustilago hordei*. *Molecular Plant–Microbe Interactions* **3,** 366–373.

McCluskey, K., Russell, B. W., and Mills, D. (1990). Electrophoretic karyotyping without the need for generating protoplasts. *Current Genetics* **18,** 385–386.

McDonald, B. A., and Martinez, J. P. (1991). Chromosome length polymorphisms in a *Septoria tritici* population. *Current Genetics* **19,** 265–271.

Mertz, W. G., Connelly, C., and Hieter, P. (1988). Variation of electrophoretic karyotypes among clinical isolates of *Candida albicans*. *Journal of Clinical Microbiology* **26,** 842–845.

Miao, V. P. W., Covert, S. F., and Van Etten, H. D. (1991a). A fungal gene for antibiotic resistance on a dispensable (B) chromosome. *Science* **254,** 1773–1776.

Miao, V. P., Matthews, D. E., and VanEtten, H. D. (1991b). Identification and chromosomal locations of a family of cytochrome P-450 genes for pisatin detoxification in the fungus *Nectria haematococca*. *Molecular General Genetics* **226,** 214–223.

Migheli, Q., Berio, T., and Gullino, M. L. (1993). Electrophoretic karyotypes of *Fusarium* spp. *Experimental Mycology* **17,** 329–337.

Mills, D., and McCluskey, K. (1990). Electrophoretic karyotypes of fungi: The new cytology. *Molecular Plant–Microbe Interactions* **3,** 351–357.

Mills, D., McCluskey, K., Russell, B. W., and Agnan, J. (1995). Electrophoretic karyotyping: Methods and applications. *In* ''Molecular Methods in Plant Pathology'' (R. Singh, P, and U. S. Singh, eds.), pp. 81–96. Lewis Publishers, Inc., Chelsea, MI.

Mizukami, T., Chang, W. I., Garkatseve, I., Kaplan, N., and Lombardi, D. (1993). A 13Kb resolution cosmid map of the 14Mb fission yeast genome by nonrandom sequence-tagged site mapping. *Cell* **73,** 121–132.

Nagy, R., and Hornok, L. (1994). Electrophoretic karyotype differences between two subspecies of *Fusarium acuminatum*. *Mycologia* **86,** 203–208.

Nagy, R., Taborhegyi, E., Wittner, A., and Hornok, L. (1995). Mini-chromosomes in *Fusarium sporotrichioides* are mosaics of dispersed repeats and unique sequences. *Microbiology* **141,** 713–719.

Nazareth, S. W., and Bruschi, C. V. (1994). Electrophoretic karyotype of *Fusarium solani*. *Microbiology* **140,** 1373–1375.

Nora, J. J., and Fraser, F. C. (1992). ''Medical Genetics: Principles and Practice.'' Lea & Febiger, Philadelphia, PA.

O'Brien, S. J., ed. (1993). ''Genetic Maps: Locus of Complex Genomes.'' Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Orbach, M., Chumley, F., and Valent, B. (1996). Electrophoretic karyotypes of *Magnaporthe grisea* pathogens of diverse grasses. *Molecular Plant–Microbe Interactions* **9,** 261–271.

Orbach, M. J., Vollrath, D., Davis, R. W., and Yanofsky, C. (1988). An electrophoretic karyotype of *Neurospora crassa*. *Molecular and Cellular Biology* **8,** 1469–1473.

Osiewacz, H. D., Clairmont, A., and Huth, M. (1990). Electrophoretic karyotype of the ascomycete *Podospora anserina*. *Current Genetics* **18,** 481–483.

Osiewacz, H. D., and Ridder, R. (1991). Genome analysis of imperfect fungi: Electrophoretic karyotyping and characterization and characterization of the nuclear gene coding for glyceraldehyde-3-phosphate dehydrogenase of *Curvularia lunata*. *Current Genetics* **20,** 151–155.

O'Sullivan, D., Tosi, P., Creusot, F., Cooke, M., Phan, T., Dron, M., and Langin, T. (1998). Variation in genome organization of the plant pathogenic fungus *Colletotrichum lindemuthianum*. *Current Genetics* **33,** 291–298.

Peberdy, J. F. (1995). Fungal protoplasts. *In* ''Genetics and Biotechnology. The Mycota'' Vol. II (U. Kuck, ed.), pp. 49–60. Springer-Verlag, Berlin, Germany.

Peberdy, J. F., and Ferenczy, L., eds. (1985). ''Fungal Protoplasts.'' Mycology Series. Marcel Dekker, Inc., New York.

Perlin, M. H. (1996). Pathovars or formae speciales of *Microbotryum violaceum* differ in electrophoretic karyotype. *International Journal of Plant Sciences* **157,** 447–452.

Plummer, K. M., and Howlett, B. J. (1993). Major chromosome length polymorphisms are evident after meiosis in the phytopathogenic fungus *Leptosphaeria maculans*. *Current Genetics* **24,** 107–113.

Polacheck, I., and Lebens, G. A. (1989). Electrophoretic karyotype of the pathenogenic yeast *Cryptococcus neoformans*. *Journal of General Microbiology* **135,** 65–71.

Prade, R. A., Griffith, J., Kochut, K., Arnold, J., and Timberlake, W. E. (1997). *In vitro* reconstruction of the *Aspergillus nidulans* genome. *Proceedings of the National Academy of Sciences USA* **94,** 14564–14569.

Rikkerink, E., Magee, B., and Magee, P. (1990). Genomic structure of *Candida stellatoidea*: Extra chromosomes and gene duplication. *Infection and Immunity* **4,** 949–954.

Rollo, F., Ferracuti, T., and Pacilli, A. (1989). Separation of chromosomal DNA molecules from *Phoma tracheiphila* by orthogonal-field-alternation gel electrophoresis. *Current Genetics* **16,** 477–479.

Royer, J. C., Dewar, K., Hubbes, M., and Horgen, P. A. (1991). Analysis of a high frequency transformation system for *Ophiostoma ulmi*, the causal agent of Dutch elm disease. *Molecular General Genetics* **225,** 168–176.

Russell, B. W., and Mills, D. (1993). Electrophoretic karyotypes of *Tilletia caries, T. controversa*, and their F_1 progeny: Further evidence for conspecific status. *Molecular Plant–Microbe Interactions* **6,** 66–74.

Rustchenko-Bulgac, E. P. (1991). Variations of *Candida albicans* electrophoretic karyotypes. *Journal of Bacteriology* **173,** 6586–6596.

Scherer, S., and Magee, P. T. (1990). Genetics of *Candida albicans*. *Microbial Reviews* **54,** 226–241.

Schwartz, D. C., and Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* **37,** 67–75.

Schwartz, D. C., and Koval, M. (1989). Conformational dynamics of individual DNA molecules during gel electrophoresis. *Nature* **338,** 520–522.

Shimizu, S., Nishida, Y., Yoshioka, H., and Matsumoto, T. (1991). Separation of chromosomal DNA molecules from *Paecilomyces fumosoroseus* by pulsed field electrophoresis. *Journal of Invertebrate Pathology* **58,** 461–463.

Skatrud, P. L., and Queener, S. W. (1989). An electrophoretic molecular karyotype for an industrial strain of *Cephalosporium acremonium*. *Gene* **78,** 331–338.

Skinner, D. Z., Budde, A. D., and Leong, S. A. (1991). Molecular karyotype analysis in fungi. *In* ''More Gene Manipulations in Fungi.'' (J. W. Bennett, and L. L. Lasure, eds.), pp. 86–102. Academic Press, San Diego, CA.

Smith, C. L., and Cantor, C. R. (1987). Purification, specific fragmentation, and separation of large DNA molecules. *Methods Enzymology* **155,** 449–467.

Smith, C. L., Matsumoto, T., Niwa, O., Klco, S., Fan, J., Yanagida, M., and Cantor, C. R. (1987). An electrophoretic karyotype for *Schizosaccharomyces pombe* by pulsed field gel electrophoresis. *Nucleic Acids Research* **15,** 4481–4488.

Smith, C. L., Klco, S. R., and Cantor, C. R. (1988). Pulsed-field gel electrophoresis and the technology of large DNA molecules. *In* ''Genome Analysis: A Practical Approach'' (K. W. Davies, ed.), pp. 41–72. IRL Press, Washington D.C.

Smith, S. B., Aldridge, P. K., and Callis, J. B. (1989). Observations of individual DNA molecules undergoing gel electrophoresis. *Science* **243,** 520.

Sonnenberg, A. S. M., De Groot, P. W. J., Schaap, P. J., Baars, J. J. P., Visser, J., and Van Griensven, L. J. L. D. (1996). Isolation of expressed sequence tags of *Agaricus bisporus* and their assignment to chromosomes. *Applied and Environmental Microbiology* **62,** 4542–4547.

Sor, F., and Fukuhara, H. (1989). Analysis of chromosomal DNA patterns of the genus *Kluyveromyces*. *Yeast* **5,** 1–10.

Steele, P. E., Carle, G. F., Kobayashi, G. S., and Medoff, G. (1989). Electrophoretic analysis of *Histoplasma capsulatum* chromosomal DNA. *Molecular and Cellular Biology* **9,** 983–987.

Steensma, H. Y., de Jongh, F. C. M., and Linnekamp, M. (1988). The use of electrophoretic karyotypes in the classification of yeast: *Kluyveromyces marxianus* and *K. lactis*. *Current Genetics* **14,** 311–317.

Stimberg, N., Walz, M., Schorgendorfer, K., and Kuck, U. (1992). Electrophoretic karyotyping from *Tolypocladium inflatum* and six related strains allows differentiation of morphologically similar species. *Applied Microbiology and Biotechnology* **37,** 485–489.

Talbot, N. J., Oliver, R. P., and Coddington, A. (1991). Pulsed field gel electrophoresis reveals chromosome length differences between strains of *Cladosporium fulvum* (syn. *Fulvia fulva*). *Molecular General Genetics* **229,** 267–272.

Talbot, N. J., Salch, Y. P., Ma, M., and Hamer, J. E. (1993). Karyotypic variation within clonal lineages of the rice blast fungus, *Magnaporthe grisea*. *Applied and Environmental Microbiology* **59,** 585–593.

Taylor, J. L., Borgmann, I., and Seguin-Swartz, G. (1991). Electrophoretic karyotyping of *Leptosphaeria maculans* differentiates highly virulent from weakly virulent isolates. *Current Genetics* **19,** 273–277.

Timberlake, W. E. (1978). Low repetitive DNA content in *Aspergillus nidulans*. *Science* **202,** 973–975.

Tobin, M. B., Peery, R. B., and Skatrud, P. L. (1997). An electrophoretic molecular karyotype of a clinical isolate of *Aspergillus fumigatus* and localization of the MDR-like genes *AfuMDR1* and *AfuMDR2I*. *Diagnostic Microbiology and Infectious Disease* **29,** 67–71.

Tudzynski, B., Mende, K., Weltring, K., Kinghorn, J. R., and Unkles, S. E. (1996). The *Gibberella fujikuroi niaD* gene encoding nitrate reductase: Isolation, sequence, homologous transformation, and electrophoretic karyotype location. *Microbiology* **142,** 533–539.

Tzeng, T., Lyngholm, L. K., Ford, C. F., and Bronson, C. R. (1992). A restriction fragment length polymorphism map and electrophoretic karyotype of the fungal maize pathogen *Cochliobolus heterostrophus*. *Genetics* **130,** 81–96.

VanEtten, H., Jorgensen, S., Enkerli, J., and Covert, S. F. (1998). Inducing the loss of conditionally dispensable chromosomes in *Nectria haematococcai* during vegetative growth. *Current Genetics* **33,** 299–303.

Vaughan-Martini, A., Martini, A., and Cardinali, G. (1993). Electrophoretic karyotyping as a taxonomic tool in the genus *Saccharomyces*. *Antonie van Leeuwenhoek* **63,** 145–156.

Verdoes, J. C., Calil, M. G., Punt, P. J., Debets, F., Swart, K., Stouthamer, A. H., and Van den Hondel, C. (1994). The complete karyotype of *Aspergillus niger*: The use of introduced electrophoretic mobility variation of chromosomes for gene assignment studies. *Molecular General Genetics* **244,** 75–80.

Viaud, M., Couteaudlier, Y., Levis, C., and Riba, G. (1996). Genome organization in *Beauveria bassiana*: Electrophoretic karyotype, gene mapping, and telomeric fingerprint. *Fungal Genetics and Biology* **20,** 175–183.

Walz, M. (1995). Electrophoretic karyotyping. *In* ''Genetics and Biotechnology. The Mycota,'' (U. Kuck, ed.), Vol. II, pp. 63–73. Springer-Verlag, Berlin, Germany.

Walz, M., and Kuck, U. (1991). Polymorphic karyotypes in related *Acremonium* strains. *Current Genetics* **19,** 73–76.

Walz, M., and Kuck, U. (1995). Transformation of *Sordaria macrospora* to hygromycin B resistance: Characterization of transformants by electrophoretic karyotyping and tetrad analysis. *Current Genetics* **29,** 88–95.

Wickes, B., Staudinger, J., Magee, B., Kwon-Chung, K., Magee, P., and Scherer, S. (1991). Physical and genetic mapping of *Candida albicans*: Several genes previously assigned to chromosome 1 map to chromosome R, rDNA-containing linkage groups. *Infection and Immunity* **7,** 2480–2484.

Xu, J-R., Yan, K., Dickman, M. B., and Leslie, J. F. (1995). Electrophoretic karyotypes distinguish the biological species of *Gibberella fujikuroi* (*Fusarium* Section *Liseola*). *Molecular Plant–Microbe Interactions* **8,** 74–84.

Xuei, X., and Skatrud, P. L. (1994). Molecular karyotype alterations induced by transformations in *Aspergillus nidulans* are mitotically stable. *Current Genetics* **26,** 225–227.

Zolan, M. E. (1995). Chromosome-length polymorphism in fungi. *Microbiological Reviews* **59,** 686–698.

Tissue Infection and Site-specific Gene Expression in *Candida albicans*

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I. Introduction

Although it is estimated that at least one million fungal species exist worldwide, only a minority of 100,000 to 200,000 of these species are known to science. Several thousand of these fungi are known to be pathogenic for plants; however, only 150 fungal species are believed to be human pathogens (Kwon-Chung *et al.*, 1992). Most human fungal pathogens cause infections only in individuals with damaged immune systems. These fungi are known as opportunistic pathogens, the most well-known of them being *Candida albicans. C. albicans* is a polymorphic yeast which causes almost 80% of all nosocomial (hospital-acquired) fungal infections (Beck-Sague and Jarvis, 1993). In extremely low birth weight infants, the mortality due to systemic *Candida* infections (candidosis) can be as high as 37% (Friedman *et al.*, 2000). In addition to these life-threatening infections, *C. albicans* very frequently causes surface infections of mucosa and skin. Three-quarters of all healthy women experience at least one vaginal yeast infection (Sobel, 1985), and oral *Candida* infections affect up to 90% of all HIV-infected patients (Ruhnke, 2002).

In the healthy and normal host, *C. albicans* is considered to be a harmless commensal; however, several factors, such as low numbers of granulocytes (neutrophils $< 500/\mu l$), immunosuppression, AIDS,

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antibacterial therapy, polytrauma or major surgery, drug abuse, and diabetes mellitus (Rüchel, 1993), are known to be risk factors for candidosis (Fig. 1). While such changes in the host environment are essential events for *C. albicans* to cause disease, there are also a number of fungal factors which are crucial for fungal survival on and within the host, therefore contributing to pathogenesis. These attributes include the ability to grow at body temperature (37 °C), highly flexible nutrient requirements, molecular mimicry (exposing host like molecules to the cell surface or binding host molecules), adhesion factors, the yeast-to-hyphal transition (dimorphism), phenotypic switching (the ability to rapidly change sets of morphological and biochemical attributes), thigmotropism (contact sensing), and hydrolytic enzymes, e.g., phospholipases, lipases, and secretory proteinases (Odds, 1994 (Table I). Molecular biologists have recently discovered that, in addition to this large set of possible virulence attributes, some of these factors are not only encoded by single genes but by large gene families. For example, agglutinin-like adhesion molecules (Als) (Hoyer, 2001), lipases (Lips)

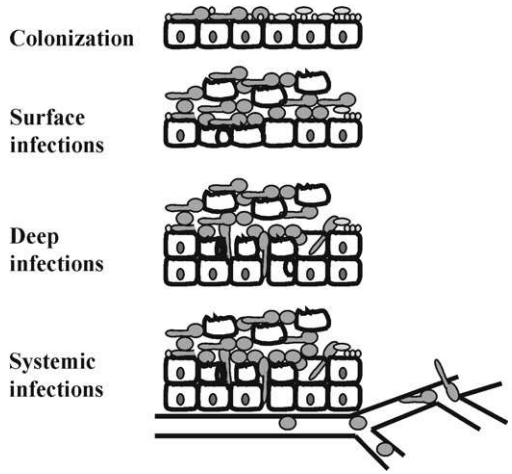

FIG. 1. Stages of *C. albicans* infection. Infections with *C. albicans* can be divided into four stages (Odds, 1994). Colonizing cells remain harmless as long as the normal microbiological flora and a healthy immune system control the fungus. Under certain circumstances, for example, after extensive antibacterial treatment, *C. albicans* may overgrow the normal microbial flora and may cause the very common infections of mucosal surfaces and the skin. Once the fungus penetrates into deeper tissue, it may cause deep infections. In severely immunocompromised patients, *C. albicans* cells can penetrate into the blood vessel system. From there, cells may disseminate throughout the body and may infect a wide range of organs and cause life-threatening disease. Adapted from Odds (1994) with permission from *ASM Press*.

TABLE I

VIRULENCE FACTORS AND FUNCTIONS

Virulence attribute	Description	Function
Adapted to body temperature	Growth at 37°C	Survival in and on human host
Highly flexible nutrient requirements	Broad sets of biochemical pathways to metabolize possible sources necessary for growth	Survival in and on human host
Molecular mimicry	Exposing hostlike molecules to the cell surface or binding host molecules	Evasion of the immune response
Adhesion factors	Proteins or glycoproteins or hydrophobicity	Attachment to host surfaces
Dimorphism	Ability to grow in a yeast or hyphal form	Evasion of the immune response, thigmotropism, physical forces for tissue penetration, adhesion
Phenotypic switching	Ability to rapidly change sets of morphological and biochemical attributes	Evasion of the immune response, adaptation to environmental changes
Thigmotropism (contact sensing)	Growth in contact to surfaces	Microscopically sensing wounds for penetration
Extracellular hydrolases	Phospholipases, lipases, proteinases	Damage of host cells and host components, hydrolysis of nutrients, adhesion, tissue invasion

(Hube *et al.*, 2000), and extracellular aspartic proteinases (Saps) (Hube and Naglik, 2001) are encoded by gene families with at least nine members (Fig. 2).

II. Secretory Lipases of *C. albicans*

Extracellular lipase (not phospholipase) activity is due to the expression of members of the *LIP* gene family. Ten members of this gene family have been cloned and characterized (Fu *et al.*, 1997; Hube *et al.*, 2000). The open reading frames of all 10 lipase genes encode highly similar proteins with up to 80% identical amino acid sequences

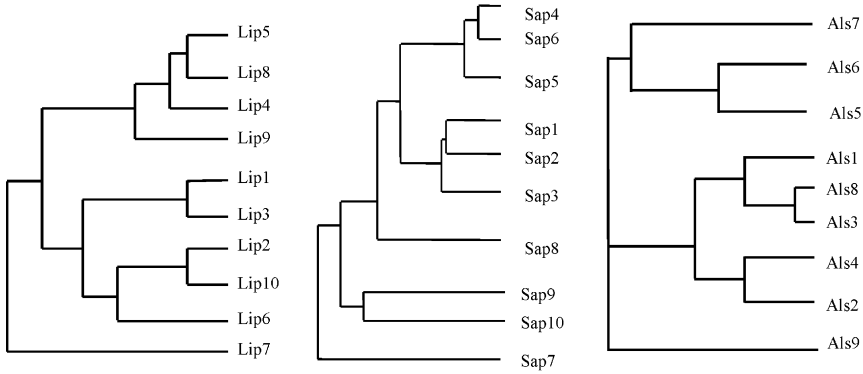

FIG. 2. Dendrogramms of the Lip (lipase), the Als (agglutinin-like sequences), and the Sap (secreted aspartic proteinase) protein families. The families contain subgroups with proteins up to 90% homologous. Three distinct groups are clustered within the Sap family. Sap 1–3 are up to 67% and Sap4–6 up to 89% identical, while Sap7 is only 20–27% identical to other Saps. Sap9 and Sap10 both have C-terminal consensus sequences typical for GPI proteins. Amino acid sequence identities of the *LIP* gene products ranged from 33% (between Lip2 and Lip7) to 80% (between Lip5 and Lip8). When clustered, the Lip isoenzyme family could be divided into two subgroups. Lip4, Lip5, Lip8, and Lip9, which were more than 73% identical to each other, and Lip1, Lip2, Lip3, Lip6, and Lip10, which were at least 54% identical to each other. Lip7 was the most divergent lipase in this isoenzyme family. Als proteins have a relatively conserved N-terminal domain but a very variable C-terminal domain in sequence and length, the amino acid identities of these products range from 19% (between Als8 and Als9) to 98% (between Als3 and Als8). Two distinct groups are clustered within this family. Als5–7 are up to 79% identical, while Als1, Als8, Als3, Als4, and Als2 are at least 54% identical. Als9 is the most distantly related member of this family, with a maximum of 29% identity (with Als7). Adapted from Stehr *et al.*, 2000; Hube and Naglik, 2002; and Hoyer, 2001 with permission from Blackwell Verlag and ASM Press.

(Hube and Naglik, 2002) (Fig. 3). This gene family may have evolved to adapt to the permanent association of *C. albicans* with the human and may have important functions in colonization and infection processes. One obvious role of these lipases may be the utilization of possible lipid substrates on human tissue, such as the skin or the intestinal tract. The high number of *LIP* genes may provide an adaptive advantage to persist on these surfaces, even in the absence of carbohydrates, and may assist *C. albicans* in competing with the normal microbial flora. Furthermore, the release of fatty acids due to lipolytic activity may modify the surrounding pH of fungal cells, thus optimizing the activity of other proteins, such as the secreted proteinases. As has been shown for other microbial lipases, *Candida* lipases may also directly affect the host immune system by inhibiting cell-mediated chemotaxis, by

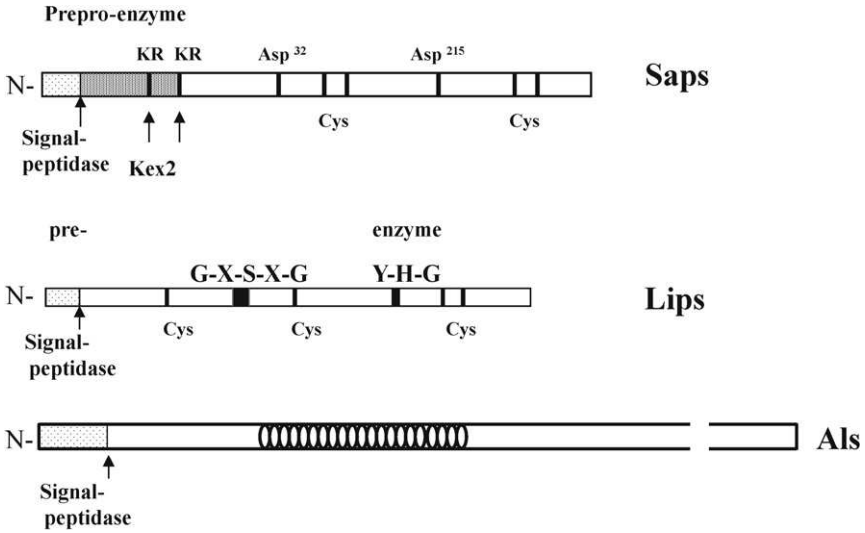

FIG. 3. Protein structure and motifs found in members of the Lip, Als, and Sap proteins. *SAP* genes are translated into preproenzymes. The first approximately 20 amino acids (signal peptides) are processed by the signal peptidase (arrow) in the endoplasmatic reticulum (ER). The propeptides of all Saps contain either Lys-Arg or Lys-Lys processing sites, which are targeted by the proteinase Kex2 (arrows) in the Golgi apparatus or by alternative activation processes (Hube and Naglik, 2002). Four conserved cysteine residues (Cys) are probably responsible for two conserved disulfide bridges and the two aspartate residues (Asp^{32} and Asp^{215} corresponding to the location in the mature Sap1 protein) are common to the active sites of all aspartic proteinases. *LIP* genes are translated into preenzymes with four conserved Cys residues and the consensus sequence G-X-S-X-G found in most lipolytic enzymes. A second region conserved in the lipases from *C. albicans* contains the sequence Y-H-G. The *ALS* gene products have a typical three-domain structure consisting of N- and C-terminal regions separated by a tandem repeat domain. The number of tandem repeat units, displayed as circles, vary across the family from 2 to 37. The N-terminal domain is conserved across the family, while the C-terminal is variable in sequence and length (symbolized by a cut in this domain) with approximately 290 up to 1468 amino acids difference in length. Adapted from Stehr *et al.*, 2000; Hube and Naglik, 2002; and Hoyer, 2001 with permission from Blackwell Verlag and ASM Press.

damaging phagocytotic cells or by triggering local inflammatory response.

III. Agglutinin-like Proteins of *C. albicans*

A family of agglutinin-like sequences (Als) has been characterized in *C. albicans* and shown to contain at least nine members, with some variations among strains (Hoyer *et al.*, 1995; Gaur and Klotz, 1997;

Hoyer *et al.*, 1998a,b; Hoyer and Hecht, 2000, 2001; Hoyer, 2001). *ALS* genes encode large N- and O-glycosylated GPI proteins linked to β-1,6-glucan in the cell wall (Kapteyn *et al.*, 2000). Their location at the surface of *C. albicans* cells was demonstrated by immunohistochemical detection (Hoyer *et al.*, 1999).

One prerequisite of colonization and infection of *C. albicans* is the attachment of the fungal cells to host surfaces. Adhesion is a complex phenomenon which involves interaction of protein and carbohydrate components, depending on the environmental conditions and strains used. Based on their surface location and their sequence similarities either to the *Saccharomyces cerevisiae* cell-surface adhesion glycoprotein α-agglutinin, AGα1 (*SAG1*), or to the *S. cerevisiae* cell wall protein Flo1, which is involved in flocculation, a putative role of Als proteins in *C. albicans* adhesion to host surfaces was postulated. In fact, Als1 has been demonstrated to mediate the adherence of *C. albicans* to epithelial and endothelial cells (Fu *et al.*, 1998) and Als5 was shown to bind to host extracellular matrix proteins (fibronectin, type IV collagen, and laminin) (Gaur and Klotz, 1997). Furthermore, immunohistological analysis has shown that Als antigens are produced in murine disseminated candidosis (Hoyer *et al.*, 1999).

Experimental analysis of Als1 and Als5 suggested that the amino terminal domains of these proteins are involved in adhesion (Gaur and Klotz, 1997; Fu *et al.*, 1998; Gaur *et al.*, 1999) and further sequence analysis suggested that the N-terminal domain of Als1 and Als5 may contain immunoglobulin (Ig)-like folding motifs, which would place Als proteins within the Ig superfamily of proteins to which many cell-adhesion molecules belong (Hoyer and Hecht, 2001). As their amino-terminal sequences are hydrophobic, Als proteins might also be involved in nonspecific binding mechanisms. In addition, Als proteins might have functions other than mediating adherence of *C. albicans* to host surfaces. For example, expression of Als1 is correlated with the filamentation process by acting downstream of the Efg1 pathway (Fu *et al.*, 2002). Nevertheless, in spite of a high sequence similarity between Als1 and the *S. cerevisiae* mating associated α-agglutinin (Sag1), Als1 is believed not to be involved in *C. albicans* mating (Magee *et al.*, 2002).

IV. Secretory Proteinases of *C. albicans*

Extracellular proteinase activity of *C. albicans* is due to the secretion of aspartic proteinases (Saps) encoded by 10 *SAP* genes (Monod *et al.*, 1994; Monod *et al.*, 1998; Felk *et al.*, 2002). It is not clear if all 10 genes

are present in all strains, but no other classes of extracellular proteinases have so far been discovered in *C. albicans. SAP* genes are translated into preproenzymes which are processed by the signal peptidase in the endoplasmatic reticulum and the Kex2 proteinase in the late Golgi (Hube and Naglik, 2002). The mature enzymes seem to be involved in a number of processes relevant for the pathogenesis of *C. albicans*. For example, Saps may contribute to cell damage and tissue invasion by the hydrolysis of host proteins such as keratin and collagen. Almost all investigated proteins of the human immune system, including the immunoglobulins IgG, IgA, sIgA, alpha-macroglobulin, proteins associated with leukocytes (cathepsin D and enzymes of the respiratory burst), and salivary proteins (lactoferrin and lactoperoxidase), are hydrolyzed by purified Sap2 proteinases (Rüchel, 1991). Since adhesion of *C. albicans* to epithelial and endothelial cells was shown to be inhibited by the classical aspartic proteinase inhibitor pepstatin A, Saps may also function in attachment processes, possibly by modifying surface proteins (Watts *et al.*, 1998). Finally, Sap proteinases may act directly on host proteins which, in turn, might activate cascade system such as the kallikrein-kinin system or the blood clotting cascade (Kaminishi *et al.*, 1990; Kaminishi *et al.*, 1994).

V. Why Gene Families (Part I)?

Although *C. albicans* is relatively similar to the comparatively harmless baker's yeast *Saccharomyces cerevisiae* (approximately 80% of all genes in *C. albicans* have homologous counterparts in *S. cerevisiae*), there are likely to be specific phenotypic differences (virulence factors) which allow the distinction between a pathogenic and a nonpathogenic fungus. For example, agglutinins exist in *S. cerevisiae*, but the differences between the agglutinins in these two species result in divergent functions. In *S. cerevisiae*, agglutinins interact between two cell types of facilitate mating; in *C. albicans*, agglutinins are implicated in adhesion to host cells but not in mating (Magee *et al.*, 2002). In contrast, sequences encoding the secreted Sap-like proteinases (except for the putative GPI-anchored proteinases Sap9 and 10) or lipases do not exist in *S. cerevisiae*. Furthermore, it is striking that the genome of *C. albicans* contains not a single gene for these factors but a whole arsenal of genes. Why does *C. albicans* (and perhaps other eukaryotic pathogenic microorganisms) possess such large gene families? There are a number of possible advantages for a pathogenic microorganism to possess such a variety of gene families. (1) These gene families may have evolved to allow the coordinated regulation of members of this family

together with other virulence attributes. (2) The different gene products may have adapted and coevolved to function in different tissues and environments during colonization and infection. (3) They may act by providing a second protein in line when another member of the family fails, is removed, or is otherwise lost. (4) They may encode proteins with similar activities but distinct and different functions; and finally, (5) the concomitant expression of a number of similar but functionally distinct genes of a gene family may result in a synergistic effect to promote colonization or infection. Thus, several gene products may act in unison to carry out a series of tasks to possibly provide the microorganism with a biological advantage.

A. Transcriptional Profile of Gene Families

One possibility which may explain why *C. albicans* possesses large gene families would be that the fungus provides specialized genes for different stages or types of infection in different tissue. If this were the case, one would expect the various members of the gene families to be differentially regulated and expressed in response to different environmental signals or associated with the different morphological forms of the fungus. Indeed, expression studies suggest that *LIP, ALS*, and *SAP* genes fulfill these requests.

B. *In vitro* Expression of the *LIP*-, ALS-, and *SAP*-Gene Families

Four of the 10 members of the *LIP* gene family were constitutively expressed under a wide range of conditions (Hube *et al.*, 2000). However, five additional lipase genes were shown to be regulated depending on the medium of growth. Six *LIP* genes were expressed in medium with lipids as the sole source of carbon. However, the same genes were expressed in media without lipids. Two more genes, *LIP2* and *LIP9*, were expressed exclusively in media which did not contain any lipids. The fact that most *LIP* genes were expressed in media without lipids suggests that *LIP* genes may have functions other than just providing nutrients for the cells. Furthermore, transcripts of seven lipase genes were detected during the yeast-to-hyphal transition, suggesting that expression of lipase genes occurs in the two dominant growth forms of *C. albicans*. These data indicate lipid-independent, highly flexible expression of a large number of *LIP* genes, possibly reflecting broad lipolytic activity, which may contribute to the persistence and virulence of *C. albicans* in human tissue.

The expression of the first described *ALS* member, *ALS1*, was regulated during medium changes (Hoyer *et al.*, 1995). *ALS3* and *ALS8*, which are highly similar genes, were expressed in association with the yeast-to-hyphal transition (Hoyer *et al.*, 1998a). *ALS4* was predominantly expressed in the late exponential and stationary phases of growth (Hoyer *et al.*, 1998b). No transcript of *ALS2* was detected under *in vitro* growth conditions. If *ALS2* is not a pseudogene, its expression might be regulated by a specific signal which might only occur *in vivo* (Hoyer *et al.*, 1998b). All these expression data show that genes in the *ALS* family are differentially regulated.

Under most proteinase-inducing conditions in the laboratory, the major proteinase gene expressed in *C. albicans* yeast forms was *SAP2* (Hube *et al.*, 1994). In contrast, *SAP1* and *SAP3* were discovered to be differentially expressed during phenotypic switching in certain strains (Morrow *et al.*, 1992; White *et al.*, 1993). Expression of *SAP8* was temperature-regulated (Monod *et al.*, 1998) and *SAP9* was constitutively expressed under most environmental conditions in both yeast and hyphal forms while expression of *SAP10* was medium dependent (Felk *et al.*, 2001). Since most aspartic proteinases are only active under acidic conditions, it was a surprising discovery that the *SAP4–6* genes were almost exclusively expressed during hyphal formation at neutral pH, even in defined proteinfree media (Hube *et al.*, 1994; White and Agabian, 1995). These studies demonstrated that the *SAP* gene family is differentially expressed *in vitro* and suggests that, in contrast to the induction of *SAP2*, expression of other *SAP* genes is not dependent on the presence of exogenous proteins or peptides.

The *in vitro* expression patterns of the *LIP*, *ALS*, and *SAP* gene families in *C. albicans* cells indicated that some of the individual genes are, in fact, differentially expressed according to the environmental conditions and the morphological form of growth. Therefore, it is possible that a specific expression pattern of these genes is dependent on the stage of infection or the type of tissue during *C. albicans* infections *in vivo*. A number of studies have been performed to investigate this hypothesis in relation to the expression of the *SAP* gene.

C. Oral Infections

Mucosal infections with *C. albicans* are extremely common diseases. In order to investigate the transcriptional pattern of *SAP* genes on oral epithelial tissue, Schaller *et al.* (Schaller *et al.*, 1998, 1999) used an *in vitro* model based on a proliferating epithelial cell line called RHE for ''Reconstituted Human Epithelium'' which mimics *C. albicans* oral

infections (Fig. 4). Within 12 h post infection of RHE, *C. albicans* caused severe tissue damage in this model. As soon as the first lesions became visible, transcripts of *SAP1* and *SAP3* were identified using RT-PCR. When the damage increased, other *SAP* transcripts were also detectable; however, expression of *SAP1* and *SAP3* was always dominant in the early stages. Interestingly, Naglik *et al.* (1999) demonstrated that expression of *SAP1* and *SAP3* was more frequently observed in human patients suffering from oral candidosis, as compared with asymptomatic *Candida* carriers, suggesting that these genes may play a role in tissue damage of the oral epithelia. This view was supported by the fact that mutants lacking *SAP1* and *SAP3* were significantly less virulent in the RHE model. In contrast, mutants lacking the hyphal associated genes *SAP4–6* caused tissue damage indistinguishable from

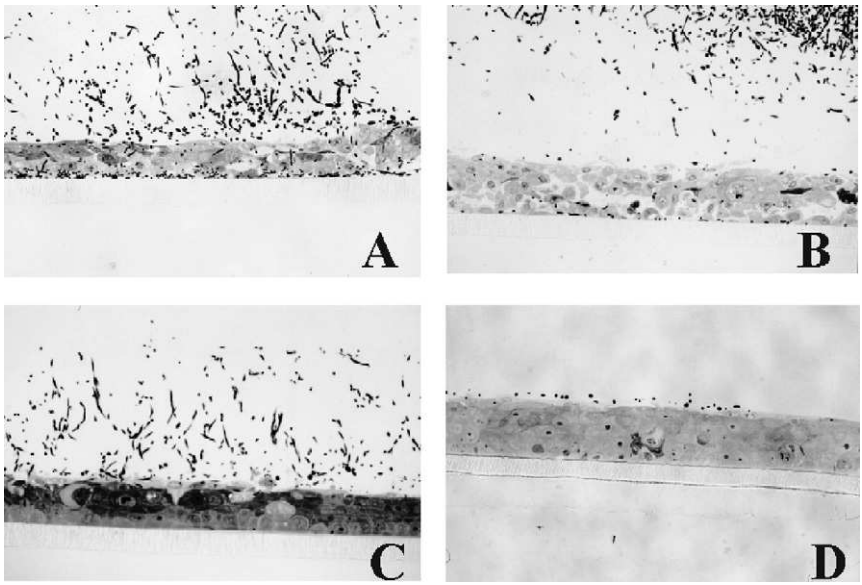

FIG. 4. Virulence of *C. albicans* wild-type and *SAP* mutant cells and effect of pepstatin A inhibition of Saps in a model of oral candidosis based on reconstituted human epithelium (RHE). Light micrograph of untreated RHE 12 h after inoculation with *C. albicans* wild type-(A), the *sap4–6* mutant (B), and *sap1/3* mutant (C) cells. *Candida* cells penetrated into the epithelium and caused severe mucosal erosion with vacuolation, edema, and enlarged intercellular spaces within all keratinocyte layers in wild-type and *sap4–6* mutant cells. In contrast, *sap1/3* mutant cells had a strongly reduced virulence phenotype causing only tissue damage on the surface of the epithelia layer. Addition of 15 μM pepstatin A to wild-type cells showed a clear protective effect of the epithelial layer (D). Adapted from Hube and Naglik, 2002 with permission from ASM Press. (Photographs by courtesy of M. Schaller).

wild-type cells (Schaller *et al.*, 1999). Therefore, *SAP1* and *SAP3*, but not *SAP4–6*, are likely to be important for oral infection. Although the *sap1/3* double mutant was reduced in its virulence potential, it still caused epithelial erosions, mostly in the latter phases of infection. When the *SAP* transcription pattern of the *sap1/3* mutant was analyzed, an upregulation of *SAP2*, *SAP5*, and *SAP8* was noticed, suggesting that *C. albicans* may partially compensate for the loss of important proteinase genes by the upregulation of alternative proteinase genes. However, when the RHE was treated with pepstatin A, an aspartic proteinase inhibitor, the epithelial tissue damage was clearly reduced, further supporting the view that these Sap proteinases contribute to the virulence properties of *C. albicans* during oral infections.

D. Systemic Infections

While mucosal infections are very common but often less serious, systemic infections are relatively rare and life-threatening diseases in severely immunocompromised individuals. Here, the fungus can penetrate the blood vessel system and disseminate throughout the body, eventually invading deeper tissues and organs. Extracellular proteinase activity has long been suggested to contribute to tissue invasion during this type of infection. In order to investigate whether *SAP* genes are expressed during systemic infections, the expression profile of the *SAP* gene family was analyzed by RT-PCR in organs from mice infected intraperitoneally (i. p.) with *C. albicans* (Felk *et al.*, 2002) (Fig. 5). Parenchymal organs, such as the liver and pancreas, are invaded by *C. albicans* wild-type hyphal cells between 4 and 24 hours after i. p. infection of mice. Within the first 72 h after infection, *SAP1*, *SAP2*, *SAP4–6*, and *SAP9* were the most commonly expressed proteinase genes. Immuno-labeling and electron microscopy studies suggest that Sap1–3 antigen are expressed on yeast and hyphal cells, while Sap4–6 antigen are predominantly expressed on hyphal cells in close contact to host cells, in particular, leukocytes. A triple mutant lacking *SAP1–3* had invasive properties similar to wild-type cells. In contrast, a triple mutant lacking *SAP4–6* showed strongly reduced invasiveness but still produced hyphal cells. When the tissue damage of liver and pancreas caused by *sap4*, *sap5*, and *sap6* single or *sap4/6*, *sap5/6*, and *sap4/5* double mutants was compared to the damage caused by wild-type cells, all mutants which lacked functional *SAP6* showed significantly reduced tissue damage. Furthermore, mutants lacking a key transcriptional factor for the production of hyphal cells, Efg1, which were avirulent in this model of systemic infection, not only failed to produce

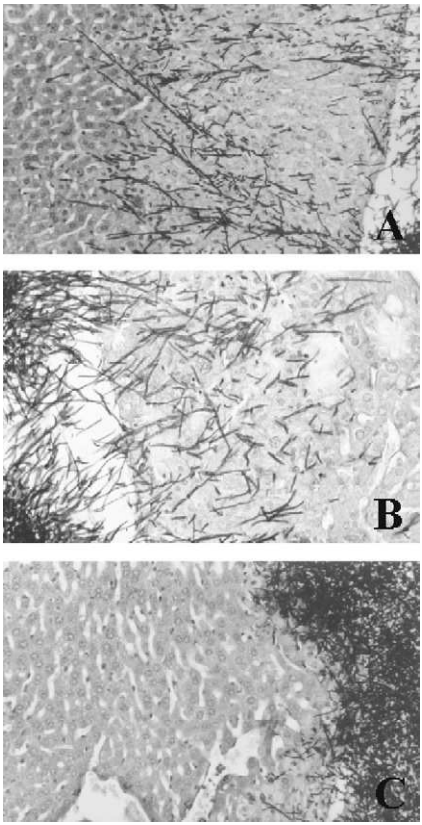

FIG. 5. Histology of liver tissue infected by *C. albicans* wild-type strains and *sap* mutants after i. p. infection of mice. Hyphal cells from the wild type (A) and a mutant strain lacking *SAP1-3* (B) deeply invaded liver tissue 24 h after i. p. infection. In contrast, hyphal cells of the *sap4-6* mutant poorly invaded the liver (C). Adapted from Felk *et al.*, 2002, with permission from the American Society for Microbiology. (Photographs by courtesy of M. Kretschmar).

hyphal cells but also had strongly reduced levels of *SAP4–6* transcripts. Therefore, the hyphal associated proteinase genes and particularly *SAP6*, but not *SAP1–3*, which were important for mucosal infections, are crucial for tissue invasion of parenchymal organs.

VI. Why Gene Families (Part II)?

These examples may show the function of gene families encoding virulence factors (Table II). The fact that certain *SAP* genes (*SAP4–6*) are associated with the formation of hyphal cells, an attribute which

TABLE II

THE POSSIBLE ROLE AND FUNCTIONS OF GENE FAMILIES ENCODING VIRULENCE FACTORS AND THE *SAP* GENE FAMILY AS AN EXAMPLE

Possible role, functions, and advantages of gene families encoding virulence factors	**Examples from the *SAP* gene family**
Coordinated regulation of members of the family together with other virulence attributes	Hyphal associated *SAP 4–6* expression
Adapted and evolved to function in different tissues and environments during colonization and infection	Differential expression of all *SAP* genes; different pH optima for SAP 1–6
Having a second protein in line when one fails, is removed, or is otherwise lost	Up-regulation of *SAP2, SAP5*, and *SAP8* in *SAP1/3* mutants
Similar activities, but distinct and different functions for the distinct members	*SAP 1–3* are important for mucosal, but not for systemic infections; *SAP 4–6* are important for systemic, but not for mucosal infections
Synergistic effects	Several *SAP* genes are expressed at the same time

is considered to be important for invasion and other virulence properties, shows that there is indeed a coordinated regulation of members of this gene family together with other virulence attributes. There is also evidence that Sap proteinases may have adapted and evolved to function in different tissues. First, the *SAP* genes were found to be differentially regulated *in vitro* and *in vivo* depending on the type of infection. Second, the purified Sap enzymes were found to have a range of different pH optima: while Sap3 was still active at pH 2.0 (optima pH 3.5), Sap4–6 still showed activity at pH 7.0 (optima pH 5.0) (Borg-von Zepelin *et al.*, 1998). Therefore, *C. albicans* provides enzymes specialized for the acid pH of the vaginal mucosa or the phagolysosome and for the neutral pH of the blood. There are also data which suggest that alternative genes may fill in for the loss of important genes. For example, when *SAP1* and *3*, those genes which are critical for artificial oral infections, were removed, *SAP2*, *5*, and *8* were upregulated. It is still not clear whether all Saps have similar activities but distinct and different functions; (4) however, it is now apparent that at least Sap9 and 10 have, in fact, distinct target proteins of fungal or host origin (Albrecht *et al.*, 2003). Finally, the observation that several *SAP* genes were expressed at the same time points during mucosal and systemic

infections suggests that the simultaneous activity of the gene products may also have a synergistic effect. Interestingly, several *SAP* and *ALS* genes were found to be co-localized on the same chromosomes and genes of these two families are regulated by similar mechanisms (Hoyer, 2001). Therefore, similar mechanisms may have triggered the genetic evolution of these gene families.

In summary, the data known about the expression and role of the *SAP* genes and their products provide evidence that *C. albicans* indeed takes advantage of the several possible merits of a large family of genes encoding virulence factors.

VII. Genome-wide Transcriptional Profiling

Expression profiling of the *SAP* gene family and the analysis of *sap* mutants showed that some members are important for mucosal tissue, while others are necessary for the invasion of parenchymal organs. However, it is generally accepted that to ensure the adaptation, survival, and proliferation of the fungal cells, a large number of different genes is required to fulfill the tissue-specific demands during the different types of infections by *C. albicans*. The preliminary assembly for the *C. albicans* genome sequence recently released by the Stanford Sequencing Center (http://www-sequence.stanford.edu/group/candida) has promoted the construction of genomic arrays for gene profiling studies (De Backer *et al.*, 2001; Lane *et al.*, 2001; Murad *et al.*, 2001a,b; Cowen *et al.*, 2002). With these arrays, it is now possible to simultaneously study the expression of a large number of fungal genes and to identify infection-associated genes. We have used arrays representing approximately one-third of the genome of *C. albicans* (2002 genes) (Fig. 6) and a cDNA subtraction protocol to investigate the expression profile when *C. albicans* cells enters the bloodstream as an essential step of systemic infections (Fradin *et al.*, 2003). By combining data obtained with these two methods, we were able to identify unique sets of different fungal genes specifically expressed in cells exposed to human blood (Fig. 7). By removing host cells and incubating in plasma, we were also able to identify several genes whose expression level was significantly influenced by the presence of these cells. For example, several genes encoding enzymes with antioxidant activities such as *SOD1* (Cu/Zn superoxide dismutase), IPF1222 (another *SOD*-like gene), *CAT1* (catalase), or *TRR1* (a thioredoxin reductase) were upregulated as a response to the challenge of host leukocytes, possibly reflecting a countermeasure to the oxidative burst of phagocytic cells. Furthermore, genes encoding proteins of the glyoxylate cycle

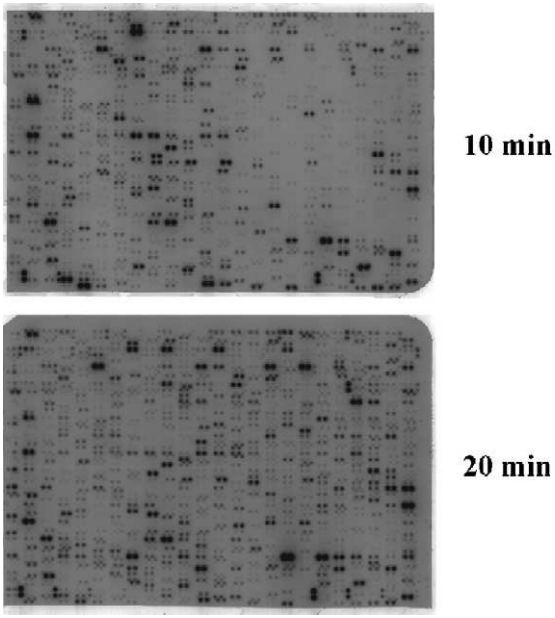

FIG. 6. Fluorescent images of microarray membranes hybridized with DIG-labeled cDNA from *C. albicans* SC5314 cells incubated in human blood for 10 and 60 min. PCR products were spotted as described previously (Murad *et al.*, 2001a). Differences in the expression profile are clearly visible. Adapted from Hube et al., 2002, with permission from Springer Verlag.

such as *MLS1* (Malate synthase 1) and *ICL1* (Isocitrate lyase 1) were strongly upregulated in blood as compared with plasma supporting previous data, which suggest that the glyoxylate cycle is required for intracellular survival of microorganisms after phagocytosis by host immune cells (McKinney *et al.*, 2000; Lorenz and Fink, 2001).

In general, *C. albicans* seems to be well able to adapt to the environmental conditions found in blood as the fungal cells proliferate and a large number of genes involved in protein synthesis are strongly expressed as soon as cells are removed from liquid culture medium and exposed directly to blood (Fradin *et al.*, 2003) (Fig. 8). In addition to survival in blood, *C. albicans* cells have to provide another set of gene products to allow the cells to attach to and penetrate through the endothelial surface layer of blood vessels. The yeast-to-hyphal transition, hyphal-associated factors, and hydrolytic enzymes have been described as factors involved in these processes (Odds, 1994; Hube *et al.*, 1997; Sanglard *et al.*, 1997; Ibrahim *et al.*, 1998; Clemons *et al.*,

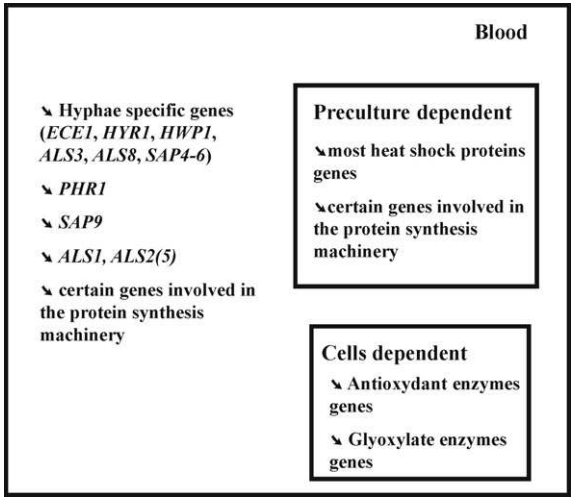

FIG. 7. Blood-, leucocyte-, and preculture-dependent gene expression of selected genes (Fradin *et al.*, 2003). Human blood or plasma (blood without cells) was infected with *C. albicans*, precultured in a distinct medium, and the expression profile was monitored using the arrays shown in Fig. 6. The expression of most heat shock protein genes and certain genes of the protein synthesis machinery was preculture-dependent. Hyphal-specific genes, such as *ECE1, HYR1, ALS1, ALS3*, and *SAP4–6*, the genes *PHR1* (pH-regulated gene), *SAP9*, and *ALS1* and certain genes involved in protein synthesis were shown to be blood-specific but independent from the blood cells. In contrast, the expression of genes encoding antioxidant enzymes and proteins of the glyoxylate cycle in blood was cell-dependent.

2000; Phan *et al.*, 2000; Felk *et al.*, 2002) and the majority of cells that enter the blood are transformed to hyphal cells (Fradin *et al.*, 2003). Consequently, a number of genes known to be hyphal-specific, such as the proteinase genes *SAP4–6*, the hypha-specific cell wall protein gene *HWP1*, and the hyphal-regulated surface protein *HYR1*, were increasingly expressed when cells were exposed to human blood. Other hyphal-associated genes such as *ALS3* and *ALS8* (Hoyer *et al.*, 1998a) may facilitate the attachment of the hyphal cells to endothelial cells *in vivo* (see preceding text). Hyphal-associated hydrolases linked to the cellular surface or secreted into the extracellular space, such as phospholipases and proteinases, may not only help the fungus to survive phagocytosis by granulocytes but may also support penetration through endothelial layers. The hyphal-associated *SAP* genes, *SAP4, SAP5*, and *SAP6*, which are known to be important virulence factors of systemic infections and tissue invasion (Sanglard et al., 1997; Felk

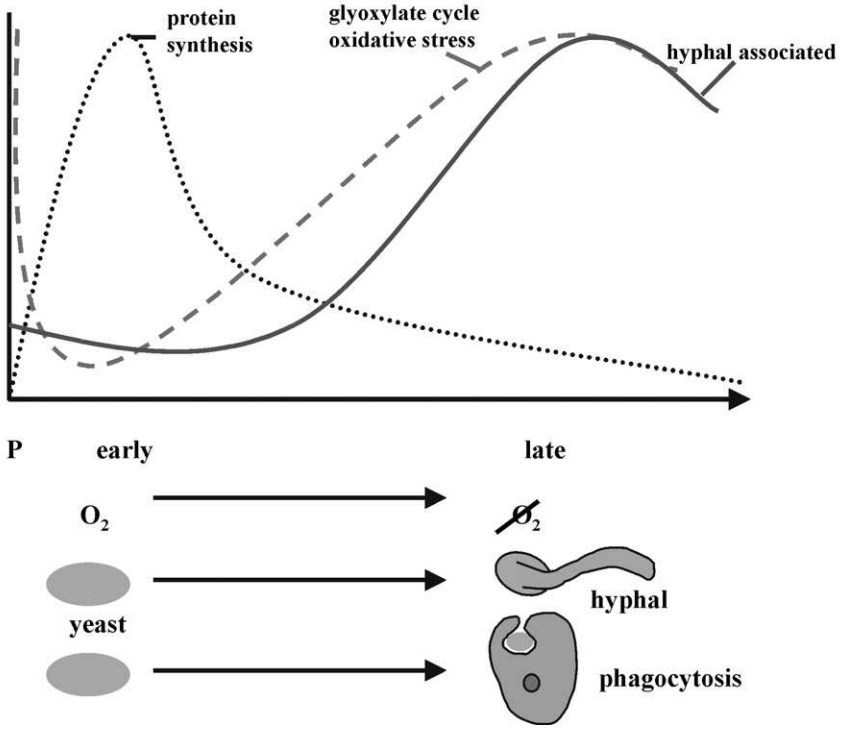

FIG. 8. Proposed schematic summary of the gene expression profile of *C. albicans* during experimental bloodstream infection based on the results by Fradin *et al.*, 2003. Genes coding for proteins involved in protein synthesis are upregulated in the early stages, but downregulated in the later stages. In contrast, genes involved in the glyoxylate cycle, oxidative stress, and hyphal-associated genes are poorly expressed in the early stages but upregulated in the later stages. During the time course experiment oxygen concentrations decreased, the majority of *C. albicans* cells formed hyphal cells, and numerous fungal cells were phagocytized by leukocytes. Adapted from Fradin *et al.*, 2003, with permission from Blackwell Oxford, UK.

et al., 2002), were upregulated in the mid- to late stages of incubation in blood. Furthermore, the phospholipase B gene *PLB5* was highly upregulated at the mid- to late stages during the artificial bloodstream infection. Since it is known that phospholipases contribute significantly to systemic infections (Leidich *et al.*, 1998; Mukherjee *et al.*, 2001), the expression of this gene may also help *C. albicans* to escape from the bloodstream.

VIII. Summary

C. albicans is able to survive and proliferate in and on a range of different tissues, either as a commensal or as a pathogen. During the different stages and types of infection, the fungal cells need a broad flexibility since each anatomic site has its own set of environmental pressures (Calderone and Fonzi, 2001). The fact that *C. albicans* possesses gene families encoding known virulence factors may reflect an adaptation to the wide range of environmental pressures that a *C. albicans* cell is likely to encounter during growth *in vivo*. In fact, specific members of each family are likely to be differentially expressed in different tissues and at different stages of infection, suggesting that these features have evolved as a consequence of these pressures. It remains to be investigated whether the members of the families have different functions or if they are just proteins with the same function but adapted to the specific demands of each anatomical site. Furthermore, with a few exceptions, the regulatory mechanisms responsible for the differential expression of individual members within gene families are not clear. However, the use of microarrays and other high-throughput technologies will certainly accelerate our knowledge of tissue-specific gene expression in microorganisms and will therefore help to understand why *C. albicans* is such a successful fungal commensal and pathogen.

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References

Albrecht, A., Felk, A., Pichova, I., Schaller, M., Kretschmar, M., Nichterlein, T., Schäfer, W., Monod, M., and Hube, B. (2003). Unpublished data.

Beck-Sague, C., and Jarvis, W. R. (1993). *J. Infect. Dis.* **167,** 1247–1251.

Borg-von Zepelin, M., Beggah, S., Boggian, K., Sanglard, D., and Monod, M. (1998). *Mol. Microbiol.* **28,** 543–554.

Calderone, R. A., and Fonzi, W. A. (2001). *Trends Microbiol.* **9,** 327–335.

Clemons, K. V., Calich, V. L., Burger, E., Filler, S. G., Grazziutti, M., Murphy, J., Roilides, E., Campa, A., Dias, M. R., Edwards, Jr., J. E., Fu, Y., Fernandes-Bordignon, G., Ibrahim, A., Katsifa, H., Lamaignere, C. G., Meloni-Bruneri, L. H., Rex, J., Savary, C. A., and Xidieh, C. (2000). *Med. Mycol.* **38**(Suppl. 1), 99–111.

Cowen, L. E., Nantel, A., Whiteway, M. S., Thomas, D. Y., Tessier, D. C., Kohn, L. M., and Anderson, J. B. (2002). *Proc. Natl. Acad. Sci. USA* **99,** 9284–9289.

De Backer, M. D., Ilyina, T., Ma, X. J., Vandoninck, S., Luyten, W. H., and Vanden Bossche, H. (2001). *Antimicrob. Agents Chemother.* **45,** 1660–1670.

Felk, A., Kretschmar, M., Nichterlein, T., Schäfer, W., and Hube, B. (2001). *Euroconference on Fungal Virulence Factors and Disease*. Seefeld, Austria.

Felk, A., Kretschmar, M., Albrecht, A., Schaller, M., Beinhauer, S., Nichterlein, T., Sanglard, D., Korting, H. C., Schafer, W., and Hube, B. (2002). *Infect. Immun.* **70,** 3689–3700.

Fradin, C., Kretschmar, M., Nichterlein, T., Gaillardin, C., d'Enfert, C., and Hube, B. (2003). *Mol. Microbiol.*

Friedman, S., Richardson, S. E., Jacobs, S. E., and O'Brien, K. (2000). *Pediatr. Infect. Dis. J.* **19,** 499–504.

Fu, Y., Ibrahim, A. S., Fonzi, W., Zhou, X., Ramos, C. F., and Ghannoum, M. A. (1997). *Microbiology* **143**(Pt.2), 331–340.

Fu, Y., Rieg, G., Fonzi, W. A., Belanger, P. H., Edwards, J. E., Jr., and Filler, S. G. (1998). *Infect. Immun.* **66,** 1783–1786.

Fu, Y., Ibrahim, A. S., Sheppard, D. C., Chen, Y. C., French, S. W., Cutler, J. E., Filler, S. G., and Edwards, Jr., J. E. (2002). *Mol. Microbiol.* **44,** 61–72.

Gaur, N. K., and Klotz, S. A. (1997). *Infect. Immun.* **65,** 5289–5294.

Gaur, N. K., Klotz, S. A., and Henderson, R. L. (1999). *Infect. Immun.* **67,** 6040–6047.

Hoyer, L. L., Scherer, S., Shatzman, A. R., and Livi, G. P. (1995). *Mol. Microbiol.* **15,** 39–54.

Hoyer, L. L., Payne, T. L., Bell, M., Myers, A. M., and Scherer, S. (1998a). *Curr. Genet.* **33,** 451–459.

Hoyer, L. L., Payne, T. L., and Hecht, J. E. (1998b). *J. Bacteriol.* **180,** 5334–5343.

Hoyer, L. L., Clevenger, J., Hecht, J. E., Ehrhart, E. J., and Poulet, F. M. (1999). *Infect. Immun.* **67,** 4251–4257.

Hoyer, L. L., and Hecht, J. E. (2000). *Yeast* **16,** 847–855.

Hoyer, L. L. (2001). *Trends Microbiol.* **9,** 176–180.

Hoyer, L. L., and Hecht, J. E. (2001). *Yeast* **18,** 49–60.

Hube, B., Monod, M., Schofield, D. A., Brown, A. J., and Gow, N. A. (1994). *Mol. Microbiol.* **14,** 87–99.

Hube, B., Sanglard, D., Odds, F. C., Hess, D., Monod, M., Schafer, W., Brown, A. J., and Gow, N. A. (1997). *Infect. Immun.* **65,** 3529–3538.

Hube, B., Stehr, F., Bossenz, M., Mazur, A., Kretschmar, M., and Schafer, W. (2000). *Arch. Microbiol.* **174,** 362–374.

Hube, B., and Naglik, J. (2001). *Microbiology* **147,** 1997–2005.

Hube, B., Albrecht, A., Bader, O., Beinhauer, S., Felk, A., Fradin, C., Kunze, D., and Niewerth, M. (2002). Pathogenitätsfaktoren bei Pilzinfektionen: Die Arbeiten der Nachwuchsgruppe 4 des Robert Koch-Instituts Bundesgesundheitsblatt. **45**, 159–165.

Hube, B., and Naglik, J. (2002). *In* ''Candida and Candidiasis,'' (R. A. Calderone, ed.), pp. 107–122. ASM Press, Washington, DC.

Ibrahim, A. S., Filler, S. G., Sanglard, D., Edwards, Jr., J. E., and Hube, B. (1998). *Infect. Immun.* **66,** 3003–3005.

Kaminishi, H., Tanaka, M., Cho, T., Maeda, H., and Hagihara, Y. (1990). *Infect. Immun.* **58,** 2139–2143.

Kaminishi, H., Hamatake, H., Cho, T., Tamaki, T., Suenaga, N., Fujii, T., Hagihara, Y., and Maeda, H. (1994). *FEMS Microbiol. Lett.* **121,** 327–332.

Kapteyn, J. C., Hoyer, L. L., Hecht, J. E., Muller, W. H., Andel, A., Verkleij, A. J., Makarow, M., Van Den Ende, H., and Klis, F. M. (2000). *Mol. Microbiol.* **35,** 601–611.

Kwon-Chung, K. J., Varma, A., Edman, J. C., and Bennett, J. E. (1992). *J. Med. Vet. Mycol.* **30,** 61–69.

Lane, S., Birse, C., Zhou, S., Matson, R., and Liu, H. (2001). *J. Biol. Chem.* **276,** 48988–48996.

Leidich, S. D., Ibrahim, A. S., Fu, Y., Koul, A., Jessup, C., Vitullo, J., Fonzi, W., Mirbod, F., Nakashima, S., Nozawa, Y., and Ghannoum, M. A. (1998). *J. Biol. Chem.* **273,** 26078–26086.

Lorenz, M. C., and Fink, G. R. (2001). *Nature* **412,** 83–86.

Magee, B. B., Legrand, M., Alarco, A. M., Raymond, M., and Magee, P. T. (2002). *Mol. Microbiol.* **46,** 1345–1351.

McKinney, J. D., Honer zu Bentrup, K., Munoz-Elias, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, Jr., W. R., and Russell, D. G. (2000). *Nature* **406,** 735–738.

Monod, M., Togni, G., Hube, B., and Sanglard, D. (1994). *Mol. Microbiol.* **13,** 357–368.

Monod, M., Hube, B., Hess, D., and Sanglard, D. (1998). *Microbiology* **144**(Pt.10), 2731–2737.

Morrow, B., Srikantha, T., and Soll, D. R. (1992). *Mol. Cell. Biol.* **12,** 2997–3005.

Mukherjee, P. K., Seshan, K. R., Leidich, S. D., Chandra, J., Cole, G. T., and Ghannoum, M. A. (2001). *Microbiology* **147,** 2585–2597.

Murad, A. M., d'Enfert, C., Gaillardin, C., Tournu, H., Tekaia, F., Talibi, D., Marechal, D., Marchais, V., Cottin, J., and Brown, A. J. (2001a). *Mol. Microbiol.* **42,** 981–993.

Murad, A. M., Leng, P., Straffon, M., Wishart, J., Macaskill, S., MacCallum, D., Schnell, N., Talibi, D., Marechal, D., Tekaia, F., d'Enfert, C., Gaillardin, C., Odds, F. C., and Brown, A. J. (2001b). *Embo. J.* **20,** 4742–4752.

Naglik, J. R., Newport, G., White, T. C., Fernandes-Naglik, L. L., Greenspan, J. S., Greenspan, D., Sweet, S. P., Challacombe, S. J., and Agabian, N. (1999). *Infect. Immun.* **67,** 2482–2490.

Odds, F. C. (1994). *ASM News* **60,** 313–318.

Phan, Q. T., Belanger, P. H., and Filler, S. G. (2000). *Infect. Immun.* **68,** 3485–3490.

Rüchel, R. (1991). *Immun. Infekt.* **19,** 108–111.

Rüchel, R. (1993). *Ann. Hematol.* **67,** 1–11.

Ruhnke, M. (2002). *In* ''Candida and Candidiasis'' (R. A. Calderone, ed.), pp. 307–325. ASM Press, Washington, DC.

Sanglard, D., Hube, B., Monod, M., Odds, F. C., and Gow, N. A. (1997). *Infect. Immun.* **65,** 3539–3546.

Schaller, M., Schafer, W., Korting, H. C., and Hube, B. (1998). *Mol. Microbiol.* **29,** 605–615.

Schaller, M., Korting, H. C., Schafer, W., Bastert, J., Chen, W., and Hube, B. (1999). *Mol. Microbiol.* **34,** 169–180.

Sobel, J. D. (1985). *Am. J. Obstet. Gynecol.* **152,** 924–935.

Stehr, F., Felk, A., Kretschmar, M., Schaller, M., Schafer, W., and Hube, B. (2000). *Mycoses* **43**(Suppl. 2), 17–21.

Watts, H. J., Cheah, F. S., Hube, B., Sanglard, D., and Gow, N. A. (1998). *FEMS Microbiol. Lett.* **159,** 129–135.

White, T. C., Miyasaki, S. H., and Agabian, N. (1993). *J. Bacteriol.* **175,** 6126–6133.

White, T. C., and Agabian, N. (1995). *J. Bacteriol.* **177,** 5215–5221.

LuxS and Autoinducer-2: Their Contribution to Quorum Sensing and Metabolism in Bacteria

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I. Introduction

It has been known for some time that the marine bacterium *Vibrio harveyi* regulates bioluminescence in concert with cell density via the production of two small diffusible signal molecules termed ''autoinducers.'' The structure of the first *V. harveyi* autoinducer was elucidated in 1989 as *N*-(3-hydroxybutanoyl)-L-homoserine lactone (Cao and Meighen, 1989; 1993), but the nature of the second (autoinducer-2: AI-2), remained elusive. In 1999, a gene termed *luxS*, required for the production of AI-2, was identified (Surette *et al.*, 1999), and putative homologues of this gene were found in diverse bacterial genera. Since then, there has been an exponential increase in the number of *luxS*-related publications. Clearly, the hope of discovering AI-2–based signaling in organisms lacking one of the well-established cell-to-cell communication systems has motivated groups with little previous experience to enter the quorum sensing field. Recent efforts culminated in the discovery of the biochemical conversion catalyzed by the LuxS protein, the crystallization of not fewer than four different LuxS homologues, and the remarkable finding that AI-2 appears to be a furanosyl borate diester. AI-2 production has now been demonstrated for a large number of bacterial species, leading to the hypothesis that AI-2 is employed for interspecies communication. However, LuxS plays an important role in bacterial metabolism, contributing to the recycling of SAM, which raises the question whether the LuxS/AI-2 system really constitutes a signaling system in all of the organisms in which it is found. Here, we examine critically the current knowledge base and discuss future directions for LuxS research.

II. Overview of Bacterial Cell-to-Cell Communication

Bacteria are known to release a large variety of small molecules, including siderophores, secondary metabolites, and metabolic end products. They also produce and respond to diffusible compounds best described as signal molecules (sometimes termed autoinducers or pheromones), which are known to play a crucial role in bacterial cell-to-cell communication. It is believed that in many instances these molecules are used to determine the size (or density) of bacterial populations: The signal molecule is released during growth and accumulates in the local environment of the population. Once a certain threshold concentration of the molecule (and consequently a critical population size: the quorum (Fuqua *et al.*, 1994)) has been reached, a concerted cellular response is initiated. It has been suggested that this

phenomenon, termed quorum sensing, enables bacterial populations to achieve results which are denied to the individual cell. The early production of virulence factors by pathogenic bacteria, for instance, may activate a host response, which could lead to their eradication. A well-timed concerted action, however, once the intruding cells have multiplied, may overcome the host defenses. In this context, the phytopathogen *Erwinia carotovora* represents an often cited example (Swift *et al.*, 1996; Whitehead *et al.*, 2002). The aggressive action of cell wall–degrading enzymes produced by this bacterium are known to elicit a defense reaction, which may only be overcome when the bacteria are present in large numbers.

Since 2002, however, the general validity of this concept has been questioned (Redfield, 2002). Instead, it was suggested that the production of diffusible molecules could also be advantageous for individual cells, enabling them to obtain information on the local environment by sensing the rate of diffusion rather than population size. Such a mechanism would enable individual cells to release diffusible exoproducts such as exoenzymes or siderophores only if diffusion rates are low enough to benefit from this production, for instance, through an increased local availability of nutrients. In its simplest form, quorum sensing may be regarded as a special category of diffusion sensing where—in a given environment—the critical concentration of the signal molecule required to trigger a response can only be achieved by more than one cell.

For some microbial phenomena, particularly those requiring complex coordination and differentiation of a large number of cells, cell-to-cell communication is clearly required and may even include direct signaling between cells in close contact, as exemplified by C-signaling during fruiting body development of *Myxococcus xanthus* (Jelsbak and Søgaard-Andersen, 2000). It is possible that the evolution of cell-to-cell communication had its origins within simple diffusion-sensing mechanisms employed by single cells, which were later extended to incorporate functions exhibiting beneficial effects only when carried out in a concerted manner by a larger bacterial population. A detailed discussion of these concepts is beyond the scope of the present chapter.

Whether used for quorum sensing or the determination of diffusion rates, the utilization of signal molecules is widespread in the eubacterial domain. Many different groups of molecules have been described which are known to regulate a diverse range of phenomena including bioluminescence, competence, and biofilm differentiation as well as the production of exoenzymes, siderophores, antibiotics, surfactants, and pigments. Well-studied examples include the family of

N-acyl-L-homoserine lactones (AHLs), which are exclusively produced by gram-negative bacteria, the γ-butyrolactones of *Streptomyces* species, and the post-translationally modified peptides commonly employed by gram-positive bacteria (Lazazzera and Grossman, 1998; Novick and Muir, 1999; Swift *et al.*, 2001; Whitehead *et al.*, 2001, Fuqua and Greenberg, 2002; Horinouchi, 2002).

AHL molecules are usually synthesized by enzymes belonging to the LuxI family, but can also be formed by other enzymes such as LuxM, VanM, and AinS (in *V. harveyi, Vibrio anguillarum*, and *Photobacterium fischeri*, respectively), or HtdS in *Pseudomonas fluorescens*. Members of the LuxI family and AinS have been shown to utilize the acyl chain of the appropriately charged acyl carrier protein (acyl-ACP) for AHL synthesis, while the homoserine lactone moiety is derived from *S*-adenosylmethionine (More *et al.*, 1996; Schaefer *et al.*, 1996; Jiang *et al.*, 1998; Hanzelka *et al.*, 1999). AHLs, which are thought to be membrane-diffusible, accumulate during growth and activate a transcriptional regulator of the LuxR family once a critical concentration has been reached. Some bacteria, such as *Pseudomonas aeruginosa, Yersinia* species, or *Rhizobium leguminosarum*, have been shown to possess two or more signaling circuits, which are organized in a hierarchical manner and consist of distinct LuxI and LuxR homologues and their cognate AHL molecules (Latifi *et al.*, 1996; Pesci *et al.*, 1997; Atkinson *et al.*, 1999; Wisniewski-Dye and Downie, 2002).

The peptide pheromones of gram-positive bacteria are created from larger precursors and often secreted by ATP-binding cassette (ABC) transporters. Their receptors are usually membrane-bound sensor kinases that transduce a signal across the membrane. In some cases, the peptide is transported into the cell by oligopeptide permeases, where it then interacts with intracellular receptors. Examples for peptide-regulated phenotypes include the regulation of virulence factors in *Staphylococcus aureus*, conjugal plasmid transfer in *Enterococcus faecalis*, and competence in *Bacillus subtilis* and *Streptococcus pneumoniae* (Lazazzera and Grossman, 1998; Novick and Muir, 1999).

The first example of a signal molecule generating enzyme present in both gram-negative and Gram-positive bacteria is LuxS (Surette and Bassler, 1998; Surette *et al.*, 1999), which catalyzes the formation of the autoinducer AI-2 (Schauder *et al.*, 2001). However, although AI-2 has all the characteristics of a signal molecule in some species, its function in others is less obvious. Moreover, LuxS appears to have a metabolic function integral to the activated methyl cycle. Here, we will discuss the features of AI-2 and LuxS, relating them first to potential roles within amino acid metabolism and subsequently to

their contribution to quorum sensing in diverse gram-negative and gram-positive bacteria.

III. Biochemistry of AI-2 Formation

A. The AI-2 Bioassay

At present, there is no biochemical assay for the direct detection and quantification of AI-2. Instead, AI-2 activity present in culture supernatants or formed during *in vitro* reactions is detected with a specific reporter strain first used by Bassler *et al.* (1994, 1997) to screen the supernatants of various bacterial cultures for signal molecule activity. In common with an earlier assay described by Greenberg *et al.* (1979), it assesses the ability of cell-free culture supernatants to induce the bioluminescent phenotype of *V. harveyi* prematurely. *V. harveyi* produces and responds to two different signal molecules, AI-1 and AI-2 (see Section IX.A). For the detection of AI-2, the *V. harveyi* strain BB170 is used, which has a mutation in the *luxN* gene (Bassler *et al.*, 1994) and is therefore incapable of responding to the AI-1 signal (in contrast to the *V. harveyi* wild-type strain used by Greenberg *et al.*, 1979). A *luxN* mutant, like the wild-type, displays cell density-dependent production of light, because it still produces and responds to AI-2. However, in the presence of exogenous AI-2, light production is induced early and can be compared with that of a control culture grown in the absence of autoinducer (Fig. 1). This assay has also been used for semiquantitative determination of AI-2 levels in culture supernatants and *in vitro* reaction mixtures (DeLisa *et al.*, 2001a; Schauder *et al.*, 2001; Winzer *et al.*, 2002a). By performing assays with serial dilutions of AI-2 containing samples, the relative concentration which generates half-maximal (early) induction of light production can be determined. It should be pointed out, however, that the induction of bioluminescence upon addition of culture fluids or *in vitro* reaction mixtures does not unequivocally prove the presence of AI-2. Theoretically, an inactive precursor molecule could be converted into an active molecule by the *V. harveyi* BB170 cells.

B. The AI-2 Synthesis Pathway

AI-2 is produced from *S*-adenosyl-L-methionine (SAM) in three enzymatic steps (Schauder *et al.*, 2001; Winzer *et al.*, 2002a). In the first step, SAM acts as a methyl donor in SAM-dependent methyltransferase reactions, which lead to the formation of *S*-adenosyl-L-homocysteine

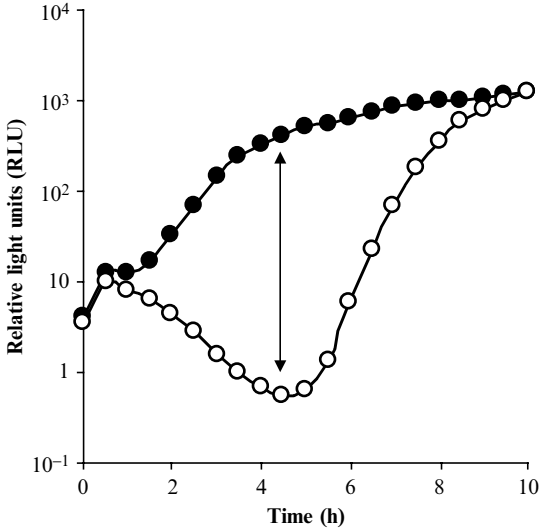


FIG. 1. Graphical representation of the typical light output over time of the *V. harveyi* light sensor strain BB170 in the presence and absence of exogenous AI-2. An overnight culture of *V. harveyi* reporter strain BB170 is inoculated into fresh medium in the presence or absence of AI-2 and incubated at 30 °C. The light output is displayed as relative light units (RLU), and time is measured in hours (h). In the presence of exogenous AI-2 (closed circles), early induction of bioluminescence is observed compared to in its absence (open circles). The point at which light output is compared to ascertain the fold increase resulting from the presence of AI-2 is indicated by the arrow.

(SAH). The second step is catalyzed by 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase. This enzyme (EC 3.2.2.9), here called Pfs because it is encoded by the *pfs* gene, is responsible for the hydrolytic cleavage of the glycosidic bond in both 5′-methylthioadenosine (MTA) and (SAH). When SAH serves as a substrate, adenine and *S*-ribosyl-L-homocysteine (SRH) are formed. LuxS finally converts SRH into homocysteine and AI-2 activity (see Figs. 2, 7A).

Schauder *et al.* (2001) identified the pathway for AI-2 synthesis using a genomic approach. The position of *luxS* in bacterial chromosomes was analyzed and it was noted that in the spirochete *Borrelia burgdorferi, luxS* is located downstream of *pfs* and *metK*. *metK* encodes the enzyme SAM synthetase, which catalyzes the formation of SAM from methionine and ATP. Therefore, it was assumed that SAM is a precursor in AI-2 formation, and that Pfs is part of a SAM-dependent pathway which provides a precursor of the LuxS-catalyzed reaction. This

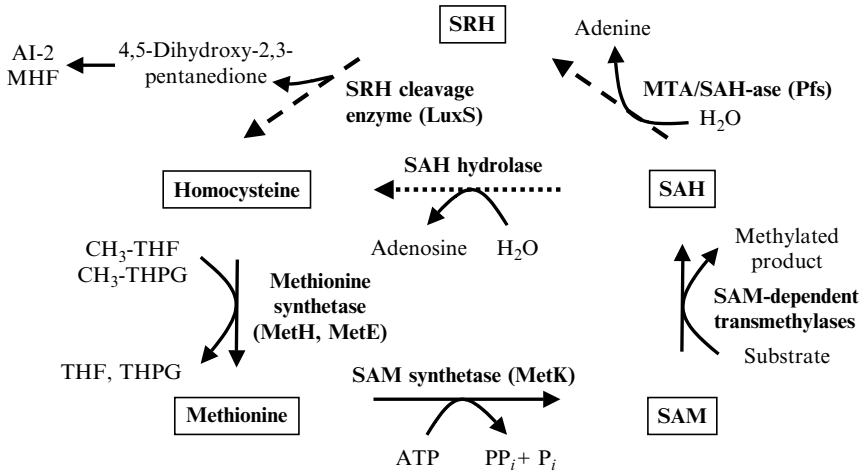


FIG. 2. The activated methyl cycle. Methionine is converted to *S*-adenosylmethionine (SAM) in a reaction catalyzed by the SAM synthetase (methionine adenosyltransferase). In SAM-dependent methyltransferase reactions, SAM is converted to *S*-adenosylhomocysteine (SAH). In some bacteria, SAH hydrolase catalyzes the hydrolysis of SAH to homocysteine and adenosine. Other bacteria, e.g., *E. coli*, produce homocysteine in two steps, catalyzed by methylthioadenosine/SAH nucleosidase (MTA/SAH-ase) and *S*-ribosylhomocysteine (SRH) cleavage enzyme. Methionine is then recycled from homocysteine. In *E. coli*, two enzymes catalyze the transfer of the methyl group of 5-methyltetrahydropteroryl glutamate (CH_3-THPG) to the sulfur of homocysteine to form methionine. One of these, the cobalamin-dependent methionine synthase (MetH) can also use N^5-methyltetrahydrofolate (CH_3-THF) as a methyl donor, whereas the cobalamin-independent methionine synthase (MetE) is restricted to CH_3-THPG. Adapted from Winzer *et al.* (2002b).

hypothesis was confirmed through a number of experiments, which involved the use of cell lysates, purified Pfs and LuxS proteins, as well as a number of different substrates. Cell lysates of *S. typhimurium*[1] *luxS* mutants, which contained a plasmid-borne copy of the *luxS* gene, produced AI-2 activity from SAM, SAH, SRH, but not MTA (Schauder *et al.*, 2001). In contrast, cell lysates of the corresponding *luxS* mutant containing the empty vector were not capable of generating AI-2 activity from any of these substrates. A defined *in vitro* assay containing SAH and the purified LuxS and Pfs proteins also produced AI-2 activity from SAH. It was subsequently established that SAH has to be converted to

[1] The correct name is *Salmonella enterica* serovar Typhimurium, but *Salmonella typhimurium* will be used in this chapter to improve readability.

SRH by the catalytic action of Pfs, before serving as a substrate in the LuxS reaction. These results demonstrated that the presence of SRH and LuxS is sufficient to generate AI-2 *in vitro*—although the possibility remains that AI-2 is formed from a precursor during incubation of the *in vitro* reaction mixture with the *V. harveyi* BB170 biosensor. The reaction also yields homocysteine, which was identified by HPLC analysis coupled to electrospray mass spectrometry. Homocysteine, as well as adenine and ribose, had no significant activity in BB170-based AI-2 bioassays, indicating that the AI-2 molecule is derived from the ribosyl moiety of SRH (Schauder *et al.*, 2001).

A similar approach was initiated by Winzer *et al.* (2002a) after it was found that in *Porphyromonas gingivalis, pfs* and *luxS* are contained within a single operon (Section VII; Burgess *et al.*, 2002), and that in *Clostridium difficile*, the *metH* gene is separated from *luxS* by only one open reading frame. *metH* encodes the cobalamin-dependent N^5-methyltetrahydrofolate–homocysteine methyltransferase (methionine synthase; cobalamin is also known as vitamin B_{12}), which catalyzes the formation of methionine from homocysteine. This study confirmed the pathway presented by Schauder *et al.* (2001) and verified that a reaction mixture containing only SRH and LuxS is sufficient to generate AI-2 activity *in vitro*. Both studies illustrate the value of comparative genomics for the elucidation of biochemical and physiological problems.

The putative *luxS* homologues identified in the genomes of gram-positive and gram-negative bacteria appear to be functionally equivalent. Schauder *et al.* (2001) demonstrated the *in vitro* synthesis of AI-2 with purified LuxS proteins from five different species, namely, *E. coli, S. typhimurium, V. harveyi, Vibrio cholerae*, and *Enterococcus faecalis*, whereas Winzer *et al.* (2002a) used the LuxS proteins of *E. coli, P. gingivalis, Neisseria meningitides*, and *S. aureus* in equivalent experiments.

IV. The Structure of AI-2

A. AI-2: A Furanone Derivative

The possibility of performing the LuxS reaction *in vitro* has provided the means to generate large amounts of comparatively pure AI-2 which can be used to elucidate its chemical properties and its structure. The known components of the *in vitro* reaction include SRH, adenine, and homocysteine. These compounds have been detected and quantified by conventional methods (Schauder *et al.*, 2001; Winzer *et al.*, 2002a). For instance, as a standard assay for LuxS activity, the amount of homocysteine liberated from SRH can be measured spectrophotometrically

using the SH group-specific Ellman's reagent (5,5′-dithiobis-[2-nitrobenzoic acid]). The fate of the ribosyl moiety of SRH, however, could not be established by electrospray mass spectrometry (Schauder *et al.*, 2001). Instead, further research was aided by information already available in the literature for decades, which described a chemical conversion very similar to the one catalyzed by LuxS. In the 1960s, an enzymatic activity in *E. coli* was shown to convert SRH to homocysteine and a carbohydrate like compound, later identified as 4,5-dihydroxy-2,3-pentanedione (DPD; Duerre and Miller, 1966; Miller and Duerre, 1968; Duerre *et al.*, 1971). DPD is not stable in aqueous solutions and is known to form furanone derivatives via cyclization and dehydration, particularly 4-hydroxy-5-methyl-3(*2H*)-furanone (MHF) (see Fig 4; Feather, 1981; Nedvidek *et al.*, 1992; for a review on biologically relevant furanones, see Slaughter, 1999). Indeed, when tested for AI-2 activity, MHF and two related compounds, 2,5-dimethyl-4-hydroxy-3(*2H*)-furanone (DMHF) and 4-hydroxy-2-ethyl-5-methyl-3(*2H*)-furanone (EMHF), were capable of inducing bioluminescence in the *V. harveyi* bioassay (Schauder *et al.*, 2001; Winzer *et al.*, 2002a). Furthermore, MHF was identified in methanol extracts of the *in vitro* reaction mixture, which also contained significant amounts of AI-2 activity (Winzer *et al.*, 2002a).

However, although capable of fully activating the BB170 biosensor at high concentrations, MHF was shown to exhibit at least a 1000-fold lower activity than AI-2. The maximal theoretical concentration of *in vitro* synthesized AI-2 can be estimated by assuming a 1:1 ratio between homocysteine and AI-2 formation. Schauder *et al.* (2001) reported that MHF concentrations of approximately 100 μM were required for half-maximal activation in the *V. harveyi* bioassay, whereas AI-2 gave the same activity at a concentration of 10 to 100 nM. In contrast, Winzer *et al.* (2002a) reported approximately 10-fold higher values (1 mM for MHF and 1 to 3 μM for AI-2). Despite these discrepancies, which resulted from differences in data interpretation (Schauder *et al.*, [2001] used the midpoint of a logarithmic plot to estimate half maximal activation, whereas Winzer *et al.* [2002a] used a linear scale), it is clear from both studies that LuxS *in vitro* reaction mixtures contain at least one molecule with a much higher activity than MHF.

B. AI-2 Is a Furanosyl Borate Diester

Interestingly, it was not the purification and chemical characterization of AI-2 isolated from LuxS *in vitro* assays or culture supernatants but a crystallographic study which finally identified AI-2 as a furanosyl

borate diester (Chen *et al.*, 2002; referred to as 3A-methyl-5,6-dihydrofuro [2,3-D][1,3,2] dioxa-borole-2,2,6,6A tetraol by Mok *et al.*, 2003). In *V. harveyi* and *V. cholerae*, the periplasmic binding protein LuxP is required for AI-2 detection, apparently by acting as the primary acceptor for this molecule. AI-2 binds so tightly to LuxP that it remains associated with the protein during chromatographic purification (Chen *et al.*, 2002). The structure of recombinant *V. harveyi* LuxP, overproduced in *E. coli*, was solved by x-ray crystallography (Chen *et al.*, 2002). It consists, like other periplasmic binding proteins, of two similar domains connected by a hinge. Interestingly, the deep cleft between these domains contained additional electron density corresponding to the AI-2 ligand. The final resolution of 1.5 Å enabled the authors to propose a structure for AI-2, which apparently consists of two fused five-membered rings, one of which is formed by a diester bridge. The molecule is stabilized within the LuxP binding site by numerous polar interactions (Fig. 3). Although not distinguishable from carbon on the basis of electron density at this resolution, boron was identified as the most likely candidate for the atom bridging the diester on the basis of chemical considerations and additional experimental evidence. A carbon atom at this position would result in a highly unstable orthocarbonate moiety, whereas heavier atoms such as sulfur or phosphorus would produce a higher electron density. Furthermore, ^{11}B NMR spectroscopy of the LuxP–AI-2 complex revealed a chemical shift within the range observed for the borate esters of carbohydrate 1,2-diols but different from those of 1,3 diols. This shift was not observed in LuxP isolated from an *E. coli luxS* mutant. Finally, electrospray mass spectrometry revealed a mass difference between LuxS and the LuxS–AI-2 complex very close to that of the proposed AI-2 structure (194.2 $\pm$ 3 daltons and 192.9 daltons, respectively). However, attempts to capture the mass of free AI-2 were not successful (Chen *et al.*, 2002).

C. Formation of AI-2 From DPD and Borate

The presence of boron in a bacterial signal molecule is remarkable. Although boron is an essential element in plants, only a few other microbial secondary metabolites are currently known to contain boron (see Section X.D). The data presented by Chen *et al.* (2002) suggest that this element may also play an important role in bacterial communication. Based on the proposed structure, the authors suggested a chemical route for the conversion of DPD to AI-2 (Fig. 4). Cyclization of DPD gives rise to a furanone derivative termed

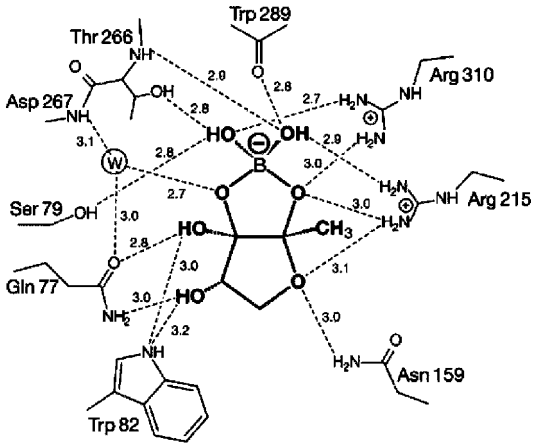


FIG. 3. Furanosyl borate diester and the hydrogen bond network that stabilizes it in the LuxP binding site. O–O or O–N distances for potential hydrogen bonds are shown in ångstroms. Reproduced from Chen *et al.* (2002).

pro-AI-2 (2,4-dihydroxy-2-methyl-4,5-dihydrofuran-3-one), which in turn converts to AI-2 through reaction with borate.

Chen *et al.* (2002) demonstrated that addition of 1 mM boric acid to *V. harveyi* BB170 resulted in approximately 1000-fold induction of light, a response which was not observed in *luxS* or *luxP* mutants and therefore was dependent on the presence of AI-2 and its cognate signal transduction machinery. Even the addition of only 10 μM boric acid resulted in a 10-fold induction of bioluminescence. It was therefore suggested that boric acid (or the corresponding borate anion), while available in varying concentrations in nature, is a limiting factor for AI-2 formation in the culture medium. Addition of boric acid may simply result in the spontaneous conversion of pro-AI-2 into AI-2. It is also possible that this reaction is promoted by a presently unknown enzymatic activity (Chen *et al.*, 2002; see also Section X.D). Whether boron addition takes place immediately after DPD synthesis in the cytoplasm, the periplasm, or extracellularly is not known (Chen *et al.*, 2002). In this context, it is important to note that DPD purification by Duerre *et al.* (1971) was achieved by chromatography on Dowex 1-X8 resin with a sodium borate concentration gradient as the eluting agent (according to Duerre and Walker, 1977). Thus, the mere presence of borate was obviously not sufficient to convert the reactive DPD quantitatively to other products either by reacting directly with its diol

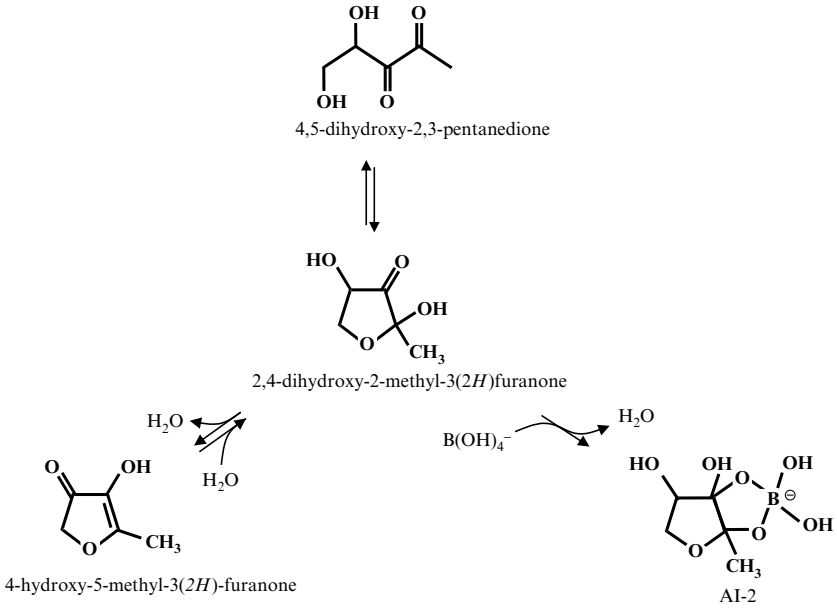


FIG. 4. Scheme to show the proposed chemical conversion of DPD to AI-2. Although some conversions between the intermediates shown may occur in multiple steps, the main steps are likely to be cyclization of 4,5-dihydroxy-2,3-pentanedione to form 2,4-dihydroxy-2-methyl-3(2*H*)furanone followed by either dehydration to MHF or formation of the furanosyl borate diester (AI-2) predicted by Chen *et al.* (2002). It is possible that AI-2 and MHF are also interconverted. The relative stability of the intermediates *in vivo* is uncertain, as is the availability of boric acid and thus the position of the equilibrium in any of the intermediate steps. Adapted from Winzer *et al.* (2002b).

structure or by shifting the equilibrium of the DPD cyclization reaction through ester formation with pro-AI-2.

The data presented by Chen *et al.* (2002) also suggest that the furanosyl borate diester is, if not the only, then at least the most potent molecule with AI-2 activity. Other pro-AI-2 derivatives could be formed, depending on the environmental conditions, and serve as signal molecules (Chen *et al.*, 2002). Indeed, the discovery of MHF in methanol extracts of LuxS *in vitro* reaction mixtures (Winzer *et al.*, 2002a) was not unexpected, given the fact that DPD is a known precursor. At least under *in vitro* conditions, where the boric acid concentration is limiting, most of the DPD formed may convert spontaneously to MHF. Another possibility is that MHF formation from DPD (or pro-AI-2) was favored during the purification procedure, which involved

concentration and removal of water through evaporation under vacuum and resuspension of the resulting residue in methanol before GC-MS analysis. The weak activity of MHF may result from poor interaction with LuxP. Alternatively, in watery solutions, MHF may be in equilibrium with pro-AI-2 or with other compounds exhibiting ''AI-2 activity.'' The chemistry of DPD in biological systems certainly requires further attention.

D. AI-2: A Collective Term?

If the availability of boric acid is a limiting factor for AI-2 formation in *V. harveyi* BB170 cultures, this should also apply to the formation of synthetic AI-2 under defined conditions in a test tube. LuxS *in vitro* reaction mixtures should only contain trace amounts of boron (originating from the glassware used to store the reagents and also depending on the purity of the chemicals added) in contrast to DPD, which is derived from the ribosyl-moiety of SRH and therefore present in millimolar concentrations. Under these conditions, only a fraction of DPD can be converted to the furanosyl borate diester. Nevertheless, addition of 20 μM ''synthetic AI-2'' was reported to induce light production approximately 7000-fold (Chen *et al.*, 2002; this concentration was calculated by assuming a 1:1 ratio between homocysteine and AI-2 formation). It is possible that a low concentration of boric acid still allows sufficient quantities of highly active AI-2 to be formed (either directly in the reaction mixture or after its addition to the bioassay) to fully induce light production. However, an alternative explanation would be that pro-AI-2 itself, or derivatives other than AI-2, interact with LuxP, but with low affinity. Addition of the LuxS *in vitro* reaction mixture (consisting mainly of pro-AI-2) would then shift the equilibrium toward the LuxP-pro-AI-2 complex, resulting in induction of bioluminescence. Addition of boric acid would give rise to the formation of furanosyl borate diester, which has a much higher affinity for LuxP and therefore saturates the available binding sites at a lower concentration than pro-AI-2. Therefore, strong induction of bioluminescence would occur in the presence of boric acid, even at low cell densities, when only small amounts of pro-AI-2 have accumulated. According to this view, the term ''AI-2 activity'' describes the collective activity of at least two different molecules, which are capable of stimulating bioluminescence via binding to LuxP.

Regardless, whether pro-AI-2 can bind to LuxP or not, the activity of the quorum-sensing system 2 in *V. harveyi* (and perhaps other *Vibrio* Spp. containing a highly conserved LuxP homologue) is clearly

influenced by the concentration of boric acid. Thus, in the natural environment, this system could serve as a boric acid sensor (see Section X.D).

V. LuxS: Crystal Structure and Enzymatic Properties

A. LuxS Is Identical to the Ribosylhomocysteine Cleavage Enzyme

The enzymatic cleavage of SRH into homocysteine and a carbohydrate-like compound was first described for *E. coli* in the 1960s, and the enzyme responsible was termed the SRH-cleavage enzyme (Duerre and Miller, 1966; Miller and Duerre, 1968). Although absent from eukaryotes, the reaction was later confirmed for other bacteria (Walker and Duerre, 1975; Shimizu *et al.*, 1984). Duerre *et al.* (1971) identified the carbohydrate-like compound as DPD, a known precursor in MHF formation (Nedvidek *et al.*, 1992). MHF was shown to be present in methanol extracts of the LuxS *in vitro* reaction (Winzer *et al.*, 2002a), and it is therefore reasonable to assume that LuxS is identical to the SRH-cleavage enzyme (Lewis *et al.*, 2001; Ruzheinikov *et al.*, 2001; Winzer *et al.*, 2002a). DPD formation in the LuxS reaction has recently been demonstrated by Zhu *et al.*, 2003. This designation is also in agreement with the observation that *E. coli* grown in the presence of radiolabled SRH (^{3}H incorporated either into the homocysteine or the ribose moiety) converted significant amounts of SRH-derived homocysteine into protein methionine, but did not use the ribose moiety to the same extent (Duerre and Bowden, 1964), probably because under the conditions used it was excluded from the cell in the form of AI-2 (or another DPD derivative).

None of the recently purified LuxS proteins have been characterized biochemically in great detail. Although a recent biochemical analysis of $LuxS_{Bs}$ is now available, see Zhu *et al.*, 2003. However, some information is available for the SRH cleavage enzyme, which will be presented.

B. Biochemical Properties of the SRH Cleavage Enzyme

Initially, the SRH cleavage enzyme was characterized using cell lysates which had been subjected to Sephadex G-25 chromatography to remove low molecular weight compounds (Duerre and Miller, 1966). The enzymatic activity in this preparation was found to be unstable in solutions of low ionic strength, during dialysis, and in the presence of low protein concentrations. The enzyme was also reported to be

more stable and to show higher activities in Tris buffer compared with phosphate buffer. The optimal substrate concentration for homocysteine formation is around 2 mM. As with more recent studies using purified LuxS, no cofactor requirement was noted. For further studies, the enzyme was partially purified (15-fold) in four steps (Miller and Duerre, 1968). However, the resulting preparation was found to be extremely unstable. Therefore, characterization was carried out after the third purification step (approximately 8-fold purification). In this preparation, the enzyme was most stable at around pH 7.5 and at protein concentration above 5 mg/ml. At a pH below 7, the enzyme was inactivated immediately. A rapid loss of activity was also observed when the enzyme was incubated at pH values above 8.2. In the *in vitro* reaction, the highest enzymatic activities were observed between pH 7.9 and 8.1 and no activity was measurable at pH values below 7.0 and above 8.6. For SRH a K_m value of 1.94×10^{-3} M was reported. A linear relationship between the disappearance of the substrate and appearance of homocysteine indicated stoichiometric cleavage of SRH.

C. Identification of DPD

Originally, the cleavage of SRH had been assumed to yield homocysteine and ribose (Duerre and Miller, 1966; this is the only reason why an enzyme assumed to catalyze the respective reaction is still listed in some databases under EC 3.2.1.148). However, when SRH (labeled with tritium in its ribosyl moiety) was incubated with the partially purified enzyme, no ribose was recovered. Instead, the radioactivity was associated with 2,4-dinitrophenylhydrazine–positive material, which could be separated from the other compounds in the reaction mixture by chromatography on Dowex 1-X8 using a sodium borate gradient. When ribose alone was incubated with the enzyme preparation, this material was not formed, indicating that ribose was not a free intermediate of the reaction. The 2,4-dinitrophenylhydrazine–positive material did not give the typical reaction for pentoses in the orcinol test and also did not react as a typical ketopentose in the cysteine–carbazole assay for keto sugars.

After purification of sufficient quantities, the unknown compound was chemically analyzed in more detail (Duerre *et al.*, 1971). The reaction with 2,4-dinitrophenylhydrazine had already indicated the presence of a free aldehyde or ketone and Rothlin modification of the iodoform test as well as the proton magnetic resonance (PMR) spectrum of the uncrystallized compound indicated a methyl ketone moiety.

Elemental analysis and mass spectrometry revealed a molecular formula of $C_{17}H_{18}O_6N_6$ for the pure 2-nitrophenyl-hydrazine derivative, while the UV spectrum showed it to be an osazone. Breakdown products in the mass spectrum also suggested the presence of a methyl group and two hydroxyl groups. Furthermore, the PMR spectrum of the osazone also indicated the presence of methyl group and a—CH_2OH group. Thus, the osazone was characterized as pentane-1,2-diol-3,4-bis(*o*-nitrophenylhydrazone), suggesting that the ribosyl moiety of SRH is converted to 4,5-dihydroxy-2,3-pentanedione (DPD).

D. LuxS Crystal Structure

1. *Overview*

After the identification of LuxS as the ''AI-2-synthase,'' efforts have been undertaken to understand its structure and catalytic mechanism, not least because of the prospect to develop antibacterial agents which interfere with AI-2 production. Presently, the structures of four different LuxS homologues have been resolved by x-ray crystallography at resolutions varying from 1.2 to 2.4 Å. All of these proteins were overproduced in *E. coli* before purification and crystallization. The LuxS proteins of *D. radiodurans* ($LuxS_{Dr}$), *H. influenzae* ($LuxS_{Hi}$), and *H. pylori* ($LuxS_{Hp}$) contained an additional C-terminal histidine tag (Lewis *et al.*, 2001), which was not removed prior to crystallization, whereas the protein of *B. subtilis* ($LuxS_{Bs}$), was purified in two independent studies without the use of affinity tags (Hilgers and Ludwig, 2001; Das *et al.*, 2001; Ruzheinikov *et al.*, 2001).

The overall folding pattern of the different LuxS homologues is very similar. The proteins from all four species were found to be homodimeric enzymes which contain two Zn^{2+} atoms in their catalytic sites and a novel alpha–beta fold.

2. *Overall structure*

Each LuxS subunit is made of a single polypeptide chain folded into a domain essentially consisting of a four-stranded antiparallel β sheet flanked on one side by three α-helices and on the other by a short 3_{10} helix, which is formed by four residues of the N-terminus (Fig. 5, see color insert). The primary differences between the four LuxS homologues are located in the N- and C-terminal regions and include a 3_{10} helical extension in the third α-helix of $LuxS_{Hp}$, and three additional 3_{10} helices as well as a fifth strand in the beta sheet of $LuxS_{Hi}$ (Lewis *et al.*, 2001). For $LuxS_{Bs}$, a short additional helix (4 residues) close to

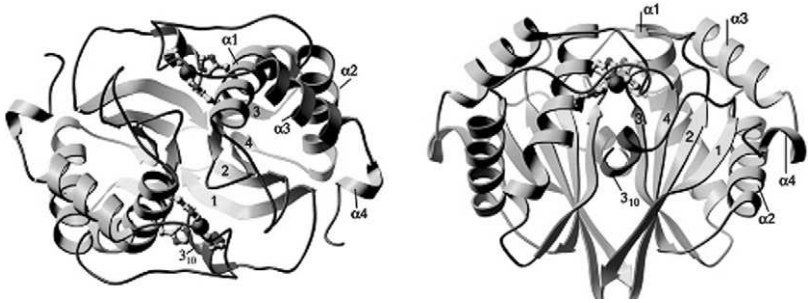


FIG. 5. Structure of *B. subtilis* LuxS. The dimer of LuxS is shown in two orientations, viewed down the two-fold axis (left) and perpendicular to the two-fold axis (right). The helices are labeled and sheet strands are numbered according to their order in the sequence. Zn is represented as a large sphere, and the protein ligands, His-54, His-58, and Cys-126, are drawn in ball-and-stick mode. Reproduced from Hilgers and Ludwig, 2001.

the C-terminus has been described by Hilgers and Ludwig (2001) but was not observed by Ruzheinikov *et al.* (2001).

In the crystal, LuxS is present as a symmetric homodimer. Dynamic light-scattering studies, cross-linking, and gel filtration experiments indicate that this is also true in solution (Das *et al.*, 2001; Hilgers and Ludwig, 2001; Lewis *et al.*, 2001; Hardie *et al.*, 2003). The dimer interface is extensive—it buries nearly a quarter of the solvent-accessible surface area of each monomer—and involves a complex network of hydrogen bonding, ionic interactions, and hydrophobic contacts. The central part of the interface is formed by the open faces of the β-sheets of the two monomers, which pack together in a face-to-face arrangement (Lewis *et al.*, 2001; Hilgers and Ludwig, 2001; Ruzheinikov *et al.*, 2001). As a consequence of dimerization, two deep and symmetry-related pockets are formed, which contain the active sites of the enzyme.

The LuxS fold appears to be novel, as structural similarity searches using the DALI server or the TOPS algorithm failed to identify proteins with significant overall similarity (Lewis *et al.*, 2001; Hilgers and Ludwig, 2001; Ruzheinikov *et al.*, 2001).

3. *LuxS Is a Zinc-containing Metalloenzyme*

All LuxS homologues crystallized so far contain two Zn^{2+} ions per dimer, which are buried deeply at the bottom of the tunnel-like pockets formed between the two subunits. A recent study by Zhu *et al.* (2003), suggests that the native $LuxS_{BS}$ contains Fe^{2+} and not Zn^{2+}, which may account for the rapid loss of activity observed. Each ion is coordinated with tetrahedral geometry through ion–dipole

interactions with three amino acid side chains and one water molecule, an arrangement that suggests an enzymatic role for this metal. The coordinating side chains, two conserved histidine residues and one conserved cysteine (His-54, His-58, and Cys-126 in $LuxS_{Bs}$) are derived from a single monomer and bind Zn^{2+} via their Nε and Sγ atoms, respectively. The histidine side chains are located in the central region of α helix 1, whereas the cysteine residue is located in the loop between the α helices 2 and 3.

Interestingly, Hilgers and Ludwig (2001) and Ruzheinikov *et al.* (2001) independently reported the presence of an oxidized cysteine residue in close proximity to the Zn atom in $LuxS_{Bs}$. This residue (Cys84), which is conserved among the LuxS homologues, derives from the second subunit of the dimer. The electron density map clearly indicated the presence of three additional atoms bound to the sulfur, suggesting its complete oxidization to cysteine sulfenate. Two conserved neighboring residues (His11 and Arg39) provide a favorable environment for the negative charge of this residue. However, oxidation of the corresponding cysteine was not reported by Lewis *et al.* (2001) for $LuxS_{Dr}$, $LuxS_{Hi}$, or $LuxS_{Hp}$ and it is presently not known whether this modification in $LuxS_{Bs}$ is an artefact or plays an important role in substrate binding and catalysis of SRH cleavage.

4. *The Active Site of LuxS*

Ruzheinikov *et al.* (2001) co-crystallized $LuxS_{Bs}$ in the presence of either SRH (0.1 mM) or homocysteine (5 mM) and the resulting binary complexes were resolved at a resolution of 2.2 and 2.3 Å, respectively. SRH was found to bind in the deep pockets formed between the subunits, with the ribose moiety being located adjacent to Zn^{2+}. Residues known to be conserved in LuxS proteins are clustered tightly around this region.

The homocysteine moiety of SRH is bound in an extended conformation, with its α-amino and carboxy groups stabilized through hydrogen bonds with five neighboring residues (Arg65, Asp78, Ile79, and Ser80, as well as Lys35 of the second subunit). Its ribose moiety appears to be in the c2′-endo conformation. The O_2 and O_3 hydroxyl groups of this moiety are involved in a long ion–dipole interaction with Zn^{2+} and also appear to be stabilized by hydrogen bonds with the oxygen atoms of the cysteine sulfenate described earlier. O_1 of the ribose hydroxyl group is probably in the β-conformation, stabilized by hydrogen bonds to a serine (Ser6) of the second subunit. The sulfur atom of SRH makes van der Waals contacts with two side chains, phenylalanine (Phe7) and alanine (Ala61), respectively. Thus, amino

acid residues from both subunits contribute to substrate binding. Of the 12 residues that make interactions of less than 3.5 Å with SRH, only one is not conserved (Ruzheinikov *et al.*, 2001).

Overall, the binding of homocysteine to the active site is very similar to that of SRH. Amino and carboxyl groups are in equivalent positions and the position of the α carbon of homocysteine is identical to that of SRH. Although there appear to be some minor differences in the position of the side chains, the position of sulfur atoms is approximately the same for both complexes.

Interestingly, there is strong evidence for the presence of a second homocysteine molecule, which is present in both the SRH–LuxS and the homocysteine–LuxS binary complexes. This molecule appears to be covalently bound to a cysteine residue (Cys41) via a disulfide bond. Furthermore, weak electron density associated with another cysteine (Cys22) suggested that this residue is, at least partially, linked with a third homocysteine molecule in a homocysteine–LuxS complex. Since neither of these cysteine residues is conserved among LuxS homologues, these modifications may have no functional significance.

Interestingly, Lewis *et al.* (2001) obtained $LuxS_{Hp}$, $LuxS_{Hi}$, and $LuxS_{Dr}$ crystals with methionine incorporated into the active site of the proteins. This amino acid had been added as an oxidative sink during protein purification. Methionine is not a substrate for LuxS, but its structural similarity to homocysteine has probably allowed the formation of the respective binary complexes. Again, similar interactions to those described for homocysteine and SRH were observed between the protein and the amino and carboxyl groups of methionine.

There appears to be no major conformational rearrangement of the $LuxS_{Bs}$ protein on SRH binding (Ruzheinikov *et al.*, 2001). However, the substrate binding site appears to be buried in the free enzyme and the SRH–$LuxS_{Bs}$ binary complex. The walls of the tunnel-like binding pockets are formed, in part, by the residues 125 to 129 and residues at the N-terminus of the symmetry-related subunit. In the ''closed conformation'' of the free enzyme and the SRH–$LuxS_{Bs}$ binary complex, the tight packing of these residues seems to prevent SRH from entering or leaving the active site (Ruzheinikov *et al.*, 2001; Hilgers and Ludwig, 2001). However, the $LuxS_{Bs}$ dimer does not appear to be a static structure, because in the homocysteine–$LuxS_{Bs}$ binary complex the N-terminal residues are disordered and the residues 125 to 129 lie in a region with high *B*-factors, which is indicative of high mobility. Thus, the tight grip on the homocysteine moiety may be released and the N-terminal region may function as a flexible gate that permits access of the substrate and exit of the products (Ruzheinikov *et al.*, 2001; Hilgers

and Ludwig, 2001). It is possible that the deeply buried active site provides a closed environment to retain the reactive DPD product. Overproduced and purified LuxS protein is still associated with a compound that exhibits activity in the A1-2 bioassay (unpublished data, this laboratory), as is LuxP (Chen *et al.*, 2002). The structure of $LuxS_{Dr}$ was reported to be unaltered upon methionine binding (Lewis *et al.*, 2001).

5. *The Catalytic Site of LuxS Resembles Those of Zinc-dependent Hydrolases*

LuxS is assumed to catalyze the cleavage of SRH to homocysteine and DPD. This reaction is remarkable because it does not involve net hydrolysis—in contrast to the reaction catalyzed by SAH hydrolase, which yields homocysteine and adenosine from SAH in many prokaryotes and most eukaryotes (Walker and Duerre, 1975; Shimizu *et al.*, 1984). Nevertheless, the metal binding site of LuxS resembles the active site of some zinc-dependent hydrolases (Hilgers and Ludwig, 2001). These hydrolases form a subgroup within the group of class IV mononuclear zinc proteins, a class which is defined by the presence of Zn^{2+} tetrahedrally coordinated with two histidine residues, a water molecule, and a glutamate (Karlin and Zhu, 1997). The sequences that bind the metal ion in LuxS are related to those found in thermolysin and other zinc-dependent hydrolases. These hydrolases are characterized by the classic zinc protease-binding motif HEXXH. However, in LuxS this motif is inverted (HXXEH). Despite the large differences in the overall fold, the metal–ligand clusters of LuxS and thermolysin can be superimposed, which, intriguingly, also aligns the absolutely conserved glutamate residue of the HXXEH motif of $LuxS_{Bs}$ (Glu57) with a catalytic glutamate residue contained in the HEXXH motif of thermolysin (Hilgers and Ludwig, 2001). In thermolysin, this glutamate residue accepts a proton from the Zn^{2+}-bound water, thereby generating a hydroxide ion that initiates hydrolysis of the substrate through nucleophilic attack (this glutamate residue is different from the glutamate residue that coordinates the zinc atom). However, LuxS differs from thermolysin and other zinc-dependent proteases in that it employs cysteine 126 rather than a glutamate as a ligand for Zn^{2+}. Furthermore, the overall architecture of the active site is markedly different. According to Hilgers and Ludwig (2001), only three other crystal structures are presently known to posses a ligand cluster composed of two histidine residues, a water molecule, and a cysteine residue, namely, T7 lysozyme, peptide deformylase, and threonyl-tRNA synthetase. Structural similarities between the latter and the active site of LuxS have also been pointed out by Lewis *et al.* (2001). T7 lysozyme and peptide deformylase

are both hydrolases (amidases) and their metal clusters are involved in catalysis, but that of threonyl-tRNA synthetase is implicated in amino acid recognition. Peptide deformylase also contains an HEXXH motif. Whether the cleavage of SRH involves a hydrolytic step as suggested by the nature of its metal center and the HXXEH motif remains to be seen.

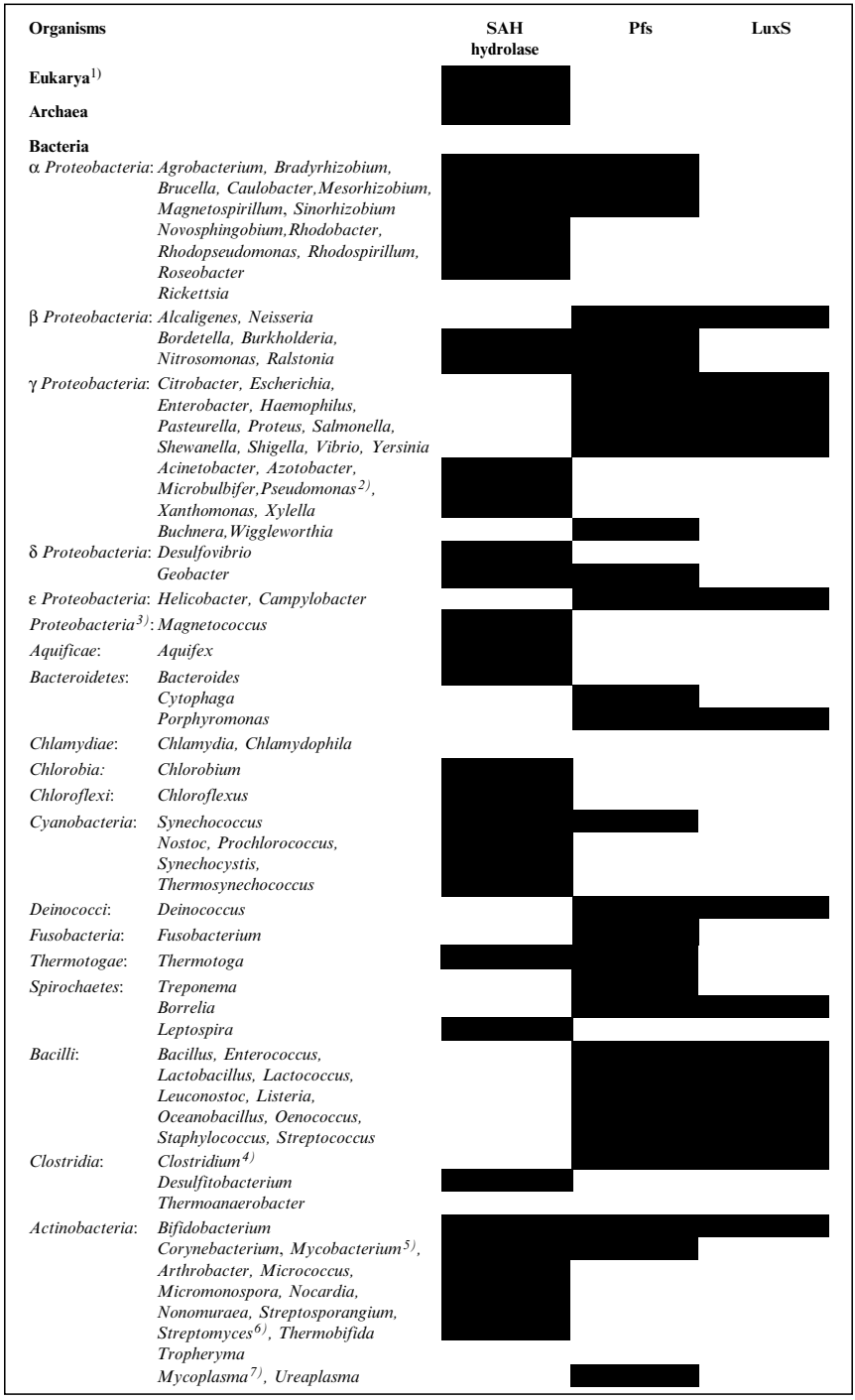
VI. The Metabolic Role of LuxS

In most publications to date, LuxS is assumed to be dedicated to AI-2 production and cell-to-cell communication, and further physiological functions have rarely been taken into consideration. Only a few reports have pointed out that LuxS plays a role in a recycling pathway linked to methionine metabolism, which may, in fact, be its most important function in many organisms (Winans, 2002; Winzer *et al.*, 2002a,b). Indeed, when Miller and Duerre (1968) first analyzed this enzyme in *E. coli* more than three decades ago, it was in the context of SAH degradation and methionine biosynthesis. It is now clear that LuxS fulfills a role in a metabolic cycle termed the ''activated methyl cycle,'' which is described in the following section.

A. The Activated Methyl Cycle

The activated methyl cycle generates methyl groups with a high transfer potential. Methyl groups enter the cycle in the conversion of homocysteine to methionine. The groups are then made highly reactive through the generation of SAM, which is used to methylate a wide variety of acceptors. Finally, homocysteine is regenerated (Fig. 2).

SAM is produced from methionine and ATP in a reaction catalyzed by SAM synthetase which involves the transfer of an adenosyl group to the sulfur atom of methionine. The methyl group of the methionine moiety is activated by the resulting positive charge on the adjacent sulfur atom, which enables it to be transferred to a large number of different substrates, including DNA and RNA, as well as proteins and various metabolites. These reactions, which are carried out by SAM-dependent methyltransferases, also create SAH as a co-product. SAH is further metabolized for two different reasons. First, accumulation of SAH is likely to be toxic, as it has been shown to function as a potent feedback inhibitor of the SAM-dependent methyltransferase reactions (Deguchi and Barchas, 1971; Hildesheim *et al.*, 1973; Duerre and Walker, 1977; Crooks *et al.*, 1984; Bechthold and Floss, 1994). Furthermore, it is advantageous for the cell to recycle the ''building blocks'' of SAH. Interestingly, two variants of the activated methyl cycle exist.


Organisms
SAH hydrolase
Pfs
LuxS
Eukarya[1)]
Archaea
Bacteria
α Proteobacteria: Agrobacterium, Bradyrhizobium, Brucella, Caulobacter, Mesorhizobium, Magnetospirillum, Sinorhizobium Novosphingobium, Rhodobacter, Rhodopseudomonas, Rhodospirillum, Roseobacter Rickettsia
β Proteobacteria: Alcaligenes, Neisseria Bordetella, Burkholderia, Nitrosomonas, Ralstonia
γ Proteobacteria: Citrobacter, Escherichia, Enterobacter, Haemophilus, Pasteurella, Proteus, Salmonella, Shewanella, Shigella, Vibrio, Yersinia Acinetobacter, Azotobacter, Microbulbifer, Pseudomonas[2)], Xanthomonas, Xylella Buchnera, Wigglesworthia
δ Proteobacteria: Desulfovibrio Geobacter
ε Proteobacteria: Helicobacter, Campylobacter
Proteobacteria[3)]: Magnetococcus
Aquificae: Aquifex
Bacteroidetes: Bacteroides Cytophaga Porphyromonas
Chlamydiae: Chlamydia, Chlamydophila
Chlorobia: Chlorobium
Chloroflexi: Chloroflexus
Cyanobacteria: Synechococcus Nostoc, Prochlorococcus, Synechocystis, Thermosynechococcus
Deinococci: Deinococcus
Fusobacteria: Fusobacterium
Thermotogae: Thermotoga
Spirochaetes: Treponema Borrelia Leptospira
Bacilli: Bacillus, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Listeria, Oceanobacillus, Oenococcus, Staphylococcus, Streptococcus
Clostridia: Clostridium[4)] Desulfitobacterium Thermoanaerobacter
Actinobacteria: Bifidobacterium Corynebacterium, Mycobacterium[5)], Arthrobacter, Micrococcus, Micromonospora, Nocardia, Nonomuraea, Streptosporangium, Streptomyces[6)], Thermobifida Tropheryma
Mycoplasma[7)], Ureaplasma

Eukaryotes, archaebacteria, and some eubacteria remove SAH in a single step catalyzed by SAH hydrolase, producing adenosine and homocysteine (Fig. 6). Other eubacteria employ Pfs and the SRH cleavage enzyme (LuxS) for the conversion of SAH to homocysteine, adenine, and DPD (Walker and Duerre, 1975; Shimizu *et al.*, 1984; Winzer *et al.*, 2002a). Homocysteine is then converted back to methionine, which is then available for further rounds of the cycle.

The importance of SAH conversion is demonstrated by the fact that the genomes of most organisms analyzed to date contain homologues for one of the two pathways (Fig. 6). Exceptions include some obligately host-associated bacteria (see following). This suggests that the ability to metabolize SAH is an important capacity, which contributes to the overall fitness of an organism in its natural habitat. It is also remarkable that the vast majority of bacteria do not contain both a SAH hydrolase and a LuxS homologue (*Bifidobacterium longum* is presently the only known exception). Organisms apparently do not gain a competitive advantage by acquiring the capacity for parallel use of both SAH-degrading pathways. This is consistent with the idea that together with Pfs, LuxS fulfills primarily a metabolic function which is analogous to that of the SAH hydrolase reaction.

Fig. 6. Comparison of the distribution of LuxS, Pfs, and SAH hydrolase among different organisms. SAH hydrolase catalyzes the conversion of SAH to homocysteine and adenosine in a single step while Pfs (MTA/SAH-ase) acts together with LuxS (SRH cleavage enzyme) to generate homocysteine, adenine, and 4,5-dihydroxy-2,3-pentanedione. Most organisms contain one of these pathways, however, there are a few which contain only *pfs* since this enzyme is important for the detoxification of SAH and MTA (see Fig. 7), or lack all components. Members of the latter two groups are often host associated. The figure is based on biochemical evidence (Walker and Duerre, 1975; Shimizu *et al.*, 1984) and the presence of putative homologues identified by genome analysis. It is not known whether identified homologues are functional, particularly *pfs* homologues, since some are truncated (e.g., *Ureaplasma urealyticum*) or considerably longer (e.g., *Caulobacter cresentus* and *Bradyrhizobium japonicum*). Only genomes completed by April 2003 have been considered. Positive results are marked with black boxes. Updated from Winzer *et al.* (2002a). 1) A putative *pfs* homologue is present in the *Entamoeba histolytica* genome and possibly other eukaryotic genomes. 2) *Pseudomonas putida* KT2440 genome sequence contains a putative Pfs homologue rather than SAH hydrolase; however, biochemical evidence suggests the contrary for *Ps. putida* IF012996. 3) *Magnetococcus sp.* MC-1 is an unclassified proteobacterium. 4) *Clostridium thermocellum* contains an SAH hydrolase gene, unlike other members of this genus which use the Pfs/LuxS pathway. *Clostrium tetani* E88 possesses a *pfs* homologue but does not appear to contain *luxS*. 5) No *pfs* homologue has been identified in *Mycobacterium leprae*. 6) Although not annotated as *pfs, Streptomyces coelicolor* A3(2) has a gene with low similarity to *E. coli pfs*. However, *Streptomyces hydroscopicus* does not exhibit Pfs activity. 7) *Mycoplasma penetrans* and *Mycoplasma pulmonis* are the only members of this genus to contain a *pfs* homologue currently.

The location of *luxS* in the genome of some bacteria also points toward a function in the activated methyl cycle (Fig. 7, see color insert). As described earlier, *luxS* forms a putative operon with *pfs* and the SAM synthetase gene *metK* in *B. burgdorferi*. In *C. difficile, luxS* is separated from the putative cobalamin-dependent methionine synthase gene *metH* by only one open reading frame. Furthermore, *pfs* and *luxS* are linked in the periodontal pathogens *P. gingivalis* and *Prevotella intermedia*, and in some lactic acid bacteria *luxS* is linked to putative *metE* genes (encoding a probable cobalamin-independent methionine synthase).[2]

There is also *in vivo* evidence that the flux of metabolites through the activated methyl cycle determines the rate of AI-2 formation. Intracellular SAM levels can be manipulated with the help of specific enzymes. Production of the bacteriophage T3 SAM hydrolase, which catalyzes the conversion of SAM to methylthioadenosine and homoserine, leads to a significant reduction of the SAM pool in *E. coli* (Posnick and Samson, 1999). When the corresponding gene was expressed in *E. coli* MG1655, a significant reduction of AI-2 levels in the culture supernatant was observed (Winzer *et al.*, 2002a). Conversely, overexpression of a SAM synthetase gene, which is known to increase the intracellular SAM concentration in *E. coli* (Hughes *et al.*, 1987; Posnick and Samson, 1999), also increased AI-2 levels (Winzer *et al.*, 2002a).

The possibility remains that SRH is also formed via a different pathway, thus partially uncoupling DPD formation from the activated methyl cycle. However, this does not seem to be the case, at least in *E. coli* and *Neisseria meningitidis*, where the culture supernatants of *pfs* mutants have been shown to be devoid of AI-2 (unpublished data this laboratory; Winzer *et al.*, 2002c).

Why some bacteria employ two enzymatic steps to achieve what other organisms can accomplish in a single step is intriguing. It has been suggested by Schauder *et al.* (2001) that the Pfs/LuxS pathway is used because in addition to recycling and detoxification, it also leads to the production of the signal molecule AI-2. However, an alternative explanation is that DPD or one of its derivatives fulfills other functions not necessarily connected with cell-to-cell communication (see Section X). Furthermore, the SAH hydrolase reaction and the Pfs/LuxS pathway are not entirely equivalent. The former reaction is reversible and the

[2] In some bacteria, the gene encoding SAH hydrolase is also located next to genes required for the activated methyl cycle, notably *metF* and *metK. MetF* encodes the 5,10-methylenetetrahydrofolate reductase, which catalyzes the formation of the methyl donor required for methionine synthesis.

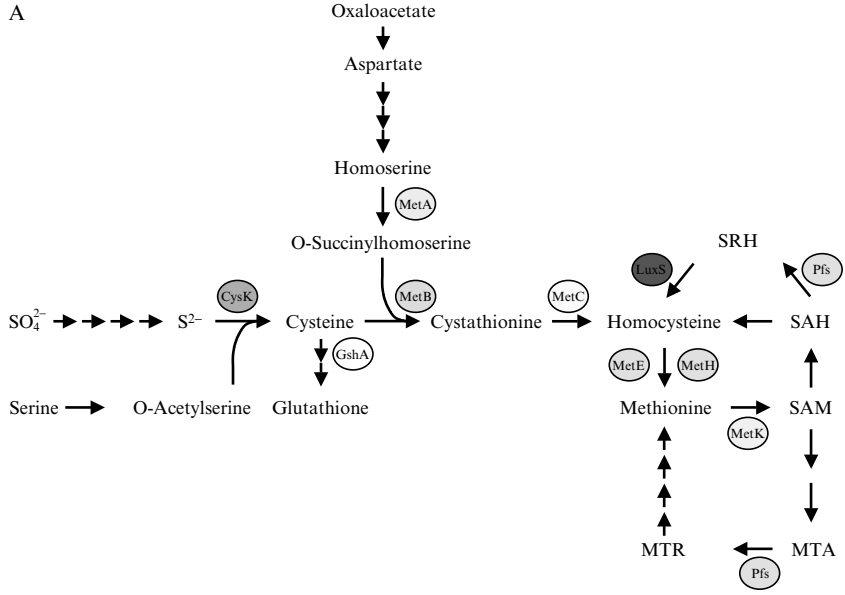
chemical equilibrium lies far in the direction of condensation (Duerre and Walker, 1977). Thus, efficient *S*-adenosylhomocysteine hydrolysis relies on the efficient removal of the products homocysteine and adenosine. In contrast, SAH is metabolized irreversibly in the two-step reaction catalyzed by Pfs and LuxS. The use of either one of these pathways could therefore have further metabolic implications.

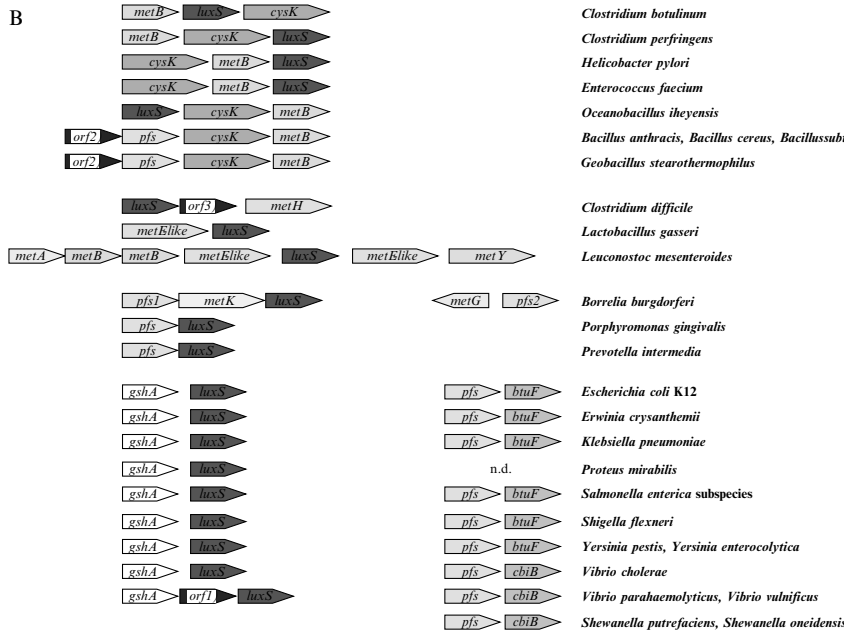
B. The Activated Methyl Cycle and Sulfur Metabolism

Phenotypes linked to *luxS* inactivation (Section IX.B) have often been attributed to a defect in cell-to-cell communication. However, it is important to point out that some of these phenotypes could also have their origin in metabolic changes caused by the disruption of the activated methyl cycle. In this section, we will briefly discuss the contribution of this cycle to bacterial fitness, as well as potential metabolic effects of *pfs* and *luxS* inactivation.

The disruption of a metabolic pathway which serves to recycle a metabolite will increase the need for the uptake of this compound from the surrounding medium, or, if not available, *de novo* biosynthesis. Accordingly, in the absence of *luxS* or *pfs*, bacteria will have to increase either methionine uptake or synthesis to maintain optimal growth. In the presence of nonlimiting external concentrations of methionine, cysteine, or other suitable sulfur sources, this is not likely to cause major problems. However, under limiting conditions, growth is likely to be affected. *De novo* synthesis of methionine (Fig. 7A), particularly from unreduced sulfur sources such as sulfate, is energetically expensive. Bacteria that are capable of recycling methionine efficiently will have an advantage in a competitive environment because they can use limited resources more economically. Metabolic cycles in general have probably evolved for this very reason (Baldwin and Krebs, 1981). On the other hand, obligately host-associated bacteria with small genomes have often discarded certain metabolic genes because they can rely on the consistent availability of important intermediates in the host cytoplasm or tissue. It is not a coincidence that many bacteria presently known to possess an incomplete activated methyl cycle, such as most members of the genera *Mycoplasma, Ureaplasma, Chlamydia, Chlamydophila, Rickettsia, Buchnera, Tropheryma, Treponema*, and *Wigglesworthia*, belong to this group of organisms.[3]

[3] Some free-living organisms with sizable genomes may also possess an incomplete activated methyl cycle, e.g., *Ps. putida, Clostridium tetani*, and *Thermonanaerobacter tengcongensis*.


A
Oxaloacetate
Aspartate
Homoserine
MetA
O-Succinylhomoserine
SRH
LuxS
Pfs
CysK
MetB
MetC
SO_4^{2-}
S^{2-}
Cysteine
Cystathionine
Homocysteine
SAH
GshA
MetE
MetH
Serine
O-Acetylserine
Glutathione
Methionine
SAM
MetK
MTR
MTA
Pfs


B
metB luxS cysK Clostridium botulinum
metB cysK luxS Clostridium perfringens
cysK metB luxS Helicobacter pylori
cysK metB luxS Enterococcus faecium
luxS cysK metB Oceanobacillus iheyensis
orf2 pfs cysK metB Bacillus anthracis, Bacillus cereus, Bacillussubtilis
orf2 pfs cysK metB Geobacillus stearothermophilus
luxS orf3 metH Clostridium difficile
metElike luxS Lactobacillus gasseri
metA metB metB metElike luxS metElike metY Leuconostoc mesenteroides
pfs1 metK luxS metG pfs2 Borrelia burgdorferi
pfs luxS Porphyromonas gingivalis
pfs luxS Prevotella intermedia
gshA luxS pfs btuF Escherichia coli K12
gshA luxS pfs btuF Erwinia crysanthemii
gshA luxS pfs btuF Klebsiella pneumoniae
gshA luxS n.d. Proteus mirabilis
gshA luxS pfs btuF Salmonella enterica subspecies
gshA luxS pfs btuF Shigella flexneri
gshA luxS pfs btuF Yersinia pestis, Yersinia enterocolytica
gshA luxS pfs cbiB Vibrio cholerae
gshA orf1 luxS pfs cbiB Vibrio parahaemolyticus, Vibrio vulnificus
pfs cbiB Shewanella putrefaciens, Shewanella oneidensis

The impact of *pfs* or *luxS* disruption on methionine metabolism has not been studied and, therefore, there is only little evidence that some of the phenotypes observed in the respective mutants are caused either directly or indirectly by perturbing metabolism. In summary,

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Fig. 7. Sulfur amino acid metabolism in bacteria (A) and proximity of *luxS* and *pfs* to the genes involved (B). (A) illustrates the *de novo* synthesis of cysteine and methionine with sulfate as the principal source of sulfur. After uptake, sulfate is reduced to sulfide, and the sulfur incorporated into cysteine. The latter reaction is catalyzed by CysK. In many bacteria, cysteine is also used for methionine *de novo* synthesis. Cysteine reacts with *O*-succinylhomoserine to form cystathionine, which is then cleaved to yield homocysteine. These reactions are catalyzed by MetB and MetC, respectively. MetB can also catalyze the direct formation of homocysteine from *O*-succinylhomoserine and sulfide. The conversion of homocysteine to methionine is catalyzed by either MetE or MetH (see Fig. 2 for more details; not all bacteria contain both enzymes). These reactions are also part of the activated methyl cycle. The function of this cycle is the generation of the methyl group donor SAM and the regeneration of methionine from SAH (see Fig. 2). In addition to its function as a methyl donor, SAM is also required for the synthesis of spermidine. After the decarboxylation of SAM, an aminopropyl group is transferred to putrescine, resulting in the formation of spermidine and methylthioadenosine (MTA). The second function of Pfs is to convert MTA to methylthioribose (MTR) and adenine. The further metabolism of MTR is not known in *E. coli*, but in *Klebsiella pneumoniae* and *B. subtilis* MTR is converted back to methionine. For a review of methionine and sulfur metabolism, see Greene (1996), Kredich (1996), and Sekowska *et al.* (2002). The colors used to mark individual enzymes in (A) are also used to indicate their encoding genes in (B). (A) is simplified and does not show the reverse transulfurylation pathway present in some bacteria (leading from methionnine and homocysteine to cysteine) or the utilization of alternative organic and anorganic sulfur sources. The reaction catalyzed by MetY (see following text) and the synthesis of N^5-methyltetrahydrofolate derivatives required for the methylation of homocysteine are also not shown. Some bacteria use *O*-acetylhomoserine instead of *O*-succinylhomoserine in the MetB reaction. (B). Note that in a considerable number of bacteria, *luxS* and *pfs* are located next to genes involved in cysteine and methionine *de novo* synthesis (particularly *cysK* and *metB*), methionine recycling (the activated methyl cycle), or genes directly or indirectly linked to these pathways. Note, however, that not all bacteria shown in this figure contain a complete activated methyl cycle. The listed genes encode the following enzymes: *cysK*: *O*-acetylserine (thiol)-lyase-A; *metA*: homoserine *O*-succinyltransferase; *metB*: cystathionine-γ-synthase; *metC*: cystathionine-β-lyase; *metE*: cobalamin-independent methionine synthase; *metG*: methionyl-tRNA synthetase; *metH*: cobalamin (vitamin B_{12}) -dependent methionine synthase; *metK*: SAM synthetase; *metY*: *O*-acetylhomoserine (thiol) lyase (also named *cysD*, catalyzing the reaction of *O*-acetylhomoserine with either methaethiol or H_2S to form methionine or homocysteine, respectively). This should not be confused with the *metY* of *E. coli* and *Salmonella* coding for methionine tRNA; *gshA*: γ-glutamylcysteine synthetase; *btuF*: periplasmic cobalamin (vitamin B_{12}) binding protein (cobalamin is required as a cofactor by MetH and other enzymes); *cbiB* (*cobD*): involved in cobalamin synthesis. The genome of *Borrelia burgdorferi* contains two *pfs* homologues, both located next to genes involved in methionine metabolism. The term ''*metE*-like'' indicates a group of genes wich exhibit similarity to *metE* but are significantly smaller. *orf*$^{1)}$ and *orf*$^{3)}$ possess no significant similarity to known genes; *orf*$^{2)}$ shows similarity to methyl transferases.

disruption of the cycle could (i) lead to the accumulation of toxic intermediates, (ii) reduce the methionine pool, and (iii) cause additional expenditure of energy due to increased methionine *de novo* synthesis. As a consequence, other metabolic conversions could be influenced, resulting in further phenotypical changes.

C. Metabolic Effects of *pfs* Inactivation

In vitro, SAH has been shown to inhibit methyltransferase reactions, but presently it is not clear whether the concentrations in *pfs* mutants are sufficiently high to influence SAH levels *in vivo*. Nevertheless, *E. coli pfs* mutants are known to be strongly impaired in growth (Cadieux *et al.*, 2002). On LB medium, the colonies of *pfs* mutants were much smaller than those of the parent strains. Growth was even more strongly reduced on minimal medium supplemented with methionine and was undetectable in its absence. Interestingly, growth on minimal medium could not be further improved through the addition of products of SAM metabolism (such as spermidine), nucleosides, or a mixture of all amino acids but was restored almost to wild-type levels by biotin at concentrations as low as 1 ng/ml. This is remarkable in two respects. First, the fact that methionine allowed weak growth on minimal medium supports the idea that disruption of the activated methyl cycle depletes the methionine pool. Second, the restoration of growth in the presence of biotin may indicate that in *pfs* mutants the SAH concentration indeed increases to a level where it inhibits enzymatic reactions *in vivo*. SAM is required for biotin synthesis as a substrate in the 7,8-diaminopelargonic acid synthase reaction, although, in this case, not as a methyl but as an amino group donor (Sekowska *et al.*, 2000). Two SAM molecules are also required in the biotin synthase reaction (for 5′-deoxyadenosyl radical formation; Shaw *et al.*, 1998). It is therefore tempting to speculate that these steps are inhibited in the presence of high SAH concentrations. However, further analysis is required since Pfs also catalyzes the degradation of MTA (Fig. 7A), which itself is a powerful inhibitor of several enzymes, including spermidine synthase and transmethylases[4] (Pajula and Raina, 1979; Raina *et al.*, 1982; Riscoe *et al.*, 1984).

In minimal medium, the methionine pool of *pfs* mutants could be reduced either because SAH could not be recycled or because the inhibition of SAM-dependent methylation led to artificially high

[4] Note, however, that most studies were undertaken in eukaryotic systems.

SAM levels, which inhibited methionine *de novo* synthesis. (After overexpression of SAM synthetase, high SAM levels were thought to cause partial methionine auxotrophy in *B. subtilis* and *E. coli* [Yocum *et al.*, 1996; Posnick and Samson, 1999]).

D. Metabolic Effects of *luxS* Inactivation

In *luxS* mutants, toxic accumulation of SAH is not likely to occur due to its rapid and irreversible conversion to SRH in the Pfs reaction. It is not known, however, whether SRH itself has inhibitory effects on other metabolic conversions at high concentrations. Metabolic changes linked to *luxS* inactivation are likely to vary, depending on growth conditions and the metabolic capabilities of the species in question. Some bacteria, e.g., *Klebsiella aerogenes*, are capable of using methionine as the sole source of sulfur, an ability which was lost by the *E. coli/Salmonella* lineage (Seiflein and Lawrence, 2001). In the presence of methionine, *K. aerogenes* appears to use the reverse transulfurylation pathway for the generation of cysteine, which involves the conversion of homocysteine to cysteine via a cystathionine intermediate. Since the activated methyl cycle is required for the conversion of methionine to homocysteine, inactivation of *luxS* is predicted to affect the growth of bacteria using the reverse transulfurylation pathway in habitats where methionine represents a major sulfur source.

Methionine pool sizes in *luxS* mutants have not yet been determined. However, there is indirect evidence for homocysteine (and thus methionine) depletion in *S. typhimurium*. Taga *et al.* (2001) reported down-regulation of the *metE* gene in a *S. typhimurium luxS* mutant. *metE* encodes the cobalamin-independent methionine synthase, which is known to be positively regulated by MetR (Urbanowski *et al.*, 1987). Homocysteine is known to considerably enhance MetR stimulation of *metE* expression in both *S. typhimurium* and *E. coli* (Cai *et al.*, 1989; Urbanowski and Stauffer, 1989), suggesting that its depletion was responsible for the down-regulation of *metE* (Taga *et al.*, 2001).

What are the potential consequences of homocysteine and methionine pool reduction? Obviously, protein biosynthesis is likely to suffer, resulting in reduced growth. Furthermore, methionine and SAM synthesis are closely linked in many bacteria. For instance, in *E. coli* and *S. typhimurium*, the genes of the methionine biosynthesis pathway are repressed not by sensing high levels of methionine directly but by the resulting increased formation of SAM, which acts as a co-repressor of the regulator protein MetJ (Weissbach and Brot, 1991). A reduction in methionine pool size is therefore also likely to influence numerous

SAM-dependent processes. Apart from being the major methyl donor in the cell, SAM provides amino, aminopropyl, aminobutyryl, adenosyl, and ribosyl moieties for a variety of physiologically important conversions. Only a few of these will be discussed (see Sekowska *et al.* (2000) for a comprehensive review, which also discusses other aspects of sulfur metabolism in more detail).

In *E. coli*, reduction of SAM levels by T3 SAM hydrolase has been shown to inhibit *dam* and *dcm*-dependent DNA methylation (Gefter *et al.*, 1966; Hughes *et al.*, 1987; Collier *et al.*, 1994; Posnick and Samson, 1999; Macintyre *et al.*, 2001). Under certain growth conditions, this may have serious consequences, as *dam*-dependent methylation is required for such fundamental processes as the control of chromosome replication and strand-specific mismatch repair. *dam*-dependent methylation has also been shown to regulate numerous genes in *E. coli, S. typhimurium*, and other bacteria (Low *et al.*, 2001; Oshima *et al.*, 2002b). *S. typhimurium dam* mutants are avirulent and in certain α *Proteobacteria*, another methyltransferase, CcrM, is essential for viability (Reisenauer *et al.*, 1999; Low *et al.*, 2001). These few examples should suffice to illustrate the importance of SAM-dependent DNA methylation. However, whether *luxS* inactivation reduces SAM concentrations sufficiently to influence these processes significantly remains to be seen. Other important substrates for methylation include rRNA, tRNA, and ribosomal proteins (Holmes *et al.*, 1995; Björk, 1996; Arnold and Reilly, 1999; Caldas *et al.*, 2000), all of which are present in the cell in large numbers.

SAM is also a precursor in spermidine synthesis (reviewed in Sekowska *et al.*, 2000). This compound, together with Mg^{2+} and other polyamines, such as putrescine, is one of the major polycations in the bacterial cell. Spermidine can bind to polyanions such as nucleic acids, nucleotides, and negatively charged proteins, thereby modulating their function (Igarashi and Kashiwagi, 2000). For spermidine synthesis, SAM is first decarboxylated. In the subsequent reaction, catalyzed by spermidine synthase, an aminopropyl group is transferred to putrescine, resulting in the formation of spermidine and methylthioadenosine (MTA). As described previously, the toxic MTA is converted to methylthioribose (MTR) under the direction of Pfs. Many organisms recycle MTR back to methionine, e.g., *Klebsiella pneumoniae* and *B. subtilis*, but interestingly that is not the case in *E. coli*, which excretes MTR into the medium. Expression of the T3 SAM hydrolase gene in *E. coli* has been shown to impair the synthesis of spermidine (Hughes *et al.*, 1987).

SAM provides an aminobutyryl group for *N*-acyl homoserine lactone (AHL) synthesis. A reduction of the intracellular SAM concentration

in *luxS* mutants could therefore indirectly effect AHL-based cell-to-cell communication. Indeed, when Val and Cronan (1998) expressed the *tral* gene from *Agrobacterium tumefaciens* in *E. coli*, they found that comparatively small changes in intracellular SAM levels, again achieved by T3 SAM hydrolase gene expression, produced proportionally larger changes in TraI-dependent AHL production. Furthermore, cycloleucine, an inhibitor of SAM synthesis, was shown to reduce autoinducer synthesis in *Photobacterium fischeri* (Hanzelka and Greenberg, 1996).

Finally, SAM also has a role as a free radical initiator. An increasing number of SAM-dependent reactions have been found to involve a one-electron reduction of this cofactor, resulting in its homolytic cleavage to methionine and a 5′-deoxyadenosyl radical. This radical plays a role in a number of important reactions, including those catalyzed by biotin and lipoate synthases, as well as anaerobic ribonucleotide reductase and pyruvate formate lyase (Frey, 2001).

From the examples given, it is clear that the metabolism of SAM is complex. It is therefore not surprising that a lack of SAM results in growth and cell division defects (Hughes *et al.*, 1987; Newman *et al.*, 1998; Posnick and Samson, 1999), which are more severe in minimal medium (Posnick and Samson, 1999). In a nutrient-poor environment, cells may have an increased requirement for SAM (because they cannot acquire important building blocks from their surroundings) and at the same time fewer resources to produce it.

In the context of this chapter, the most important and yet unsolved question is whether *luxS* inactivation indeed causes a significant change in the SAM pool under certain growth conditions. Intracellular methionine and SAM concentrations have not yet been determined in *luxS* mutants and may not differ substantially from those of the wild-type in nutrient-rich environments. It will be interesting to determine whether some of the phenotypes reported for *luxS* mutants depend on the nutritional status of the cells and whether they can be restored through the addition of methionine.

VII. The Location of *luxS* and *pfs* in Bacterial Genomes

It has been argued that the Pfs/LuxS pathway is primarily used for the recycling of SAH, thus enabling the cells to make economic use of methionine as a methyl donor (Winzer *et al.*, 2002a). Interestingly, this link with methionine metabolism is also reflected in the organization of the *pfs* and *luxS* genes on some bacterial chromosomes (Fig. 7). Here, *luxS* or *pfs* are often located next to genes involved in methionine and cysteine *de novo* biosynthesis (cysteine provides the sulfur atom

found in methionine). In *H. pylori, Enterococcus faecium, Clostridium perfringens, Clostridium botulinum*, and the deep sea bacterium *Oceanobacillus iheyensis, luxS* is located next to genes annotated as *cysK* and *metB*, which encode *O*-acetylserine (thiol)-lyase-A and cystathionine-γ-synthase, respectively.[5] *O*-Acetylserine (thiol)-lyase-A catalyzes the formation of cysteine from sulfide and *O*-acetylserine. Cystathionine-γ-synthase, in turn, uses cysteine and *O*-succinylserine to form cystathionine, another intermediate in methionine formation (Fig. 7A). Although it has only been shown for *C. perfringens* that *luxS, cysK*, and *metB* form a transcriptional unit (Banu *et al.*, 2000), such coordinated expression makes sense. In the absence of exogenous cysteine or methionine, cells have to synthesize these compounds *de novo*. At the same time, it is particularly important to make economic use of the methionine already produced.

A similar gene arrangement is present in *B. subtilis, Bacillus anthracis, Bacillus cereus*, and *Geobacillus stearothermophilus*, where *pfs* is located upstream of *cysK* and a gene encoding a homologue of either cystathionine-γ-synthase or cystathionine-β-lyase.[6] (Note: Most of the genes linked to putative *pfs* and *luxS* homologues have been annotated on the basis of sequence similarity only. In some cases, genes were annotated as *metB* but also show high similarity to experimentally confirmed *metC* genes and *vice versa*. For example, the gene clustered with *pfs* in *B. subtilis* has independently been annotated as cystathionine-γ-synthase or cystathionine-β-lyase due to the sequence similarity of gene products; according to the criteria presented by Messerschmidt *et al.* (2003), the genes next to *pfs* in *Bacillus* sp. should be annotated as *metB*. Some of the bacteria mentioned, e.g., members of the genus *Bacillus* and *O. iheyensis*, contain several potential *metB* or *metC* homologues, but only one of these homologues is located next to *luxS* or *pfs*.

The link of *luxS* to *metK* and *metH* in *B. burgdorferi* and *C. difficile*, respectively, has already been noted (see Section VI. A). Even in some lactic acid bacteria, which often have ''degenerate'' metabolic capabilities, *luxS* is located next to genes with putative functions in methionine metabolism. In *Lactobacillus gasseri*, a putative *luxS* homologue

[5] In *Oceanobacillus iheyensis*, the gene downstream of *luxS* and *cysK* has been annotated *metC*, but the conserved glutamate in position 321 (glutamate 325 is the corresponding residue in *E. coli MetB*) indicates that the correct annotation is likely to be *metB* (see Messerschmidt *et al.*, 2003).

[6] *metB* and *metC*, respectively, according to the *E. coli* nomenclature. The genes originally annotated as *B. subtilis metB* and *metC* genes, encode homoserine transsuccinylase and colabamin-independent methionine synthase, respectively.

is located downstream of a gene encoding a putative protein with similarity to the cobalamin-independent methionine synthase (MetE) and in *Leuconostoc mesenteroides, luxS* is part of a gene cluster putatively encoding MetA (homoserine O-succinyltransferase), two MetE-like proteins, as well as several enzymes of the transulfurylation family with similarity to MetB, MetC, and *O*-acetylhomoserine (thiol) lyase (the latter catalyzing the formation of homocysteine from *O*-acetylhomoserine and sulfide or methionine from methanthiol, respectively). However, for some bacteria, the discussed association between *luxS*, *pfs*, and methionine *de novo* synthesis genes may reflect only their evolutionary past rather than a present-day function, as sometimes the activated methyl cycle appears to be incomplete (e.g., *H. pylori, Steptococcus pyogenes, C. perfringens*, and *P. gingivalis*) or the putative homologues are truncated and may be nonfunctional. Alternatively, the produced homocysteine may serve different purposes.

Finally, almost all enterobacterial genomes presently analyzed are similar in their *luxS* and *pfs* gene arrangements. In *E. coli* K12, presently analyzed *Salmonella enterica* serovars, *Shigella flexneri, Yersinia pestis, Yersinia enterocolitica, Proteus mirabilis, Erwinia chrysanthemii, K. pneumoniae*, and the related bacterium *V. cholerae, luxS* is located downstream of the *gshA* gene, which encodes γ-glutamylcysteine synthetase. This enzyme also uses cysteine as a substrate and catalyzes the first step in glutathione biosynthesis. The isotripeptide glutathione is the major intracellular thiol compound in *E. coli* and many other Gram-negative bacteria (Fahey *et al.*, 1978). Glutathione can accumulate to high concentrations and is important for maintaining the cytoplasm in a reduced state. It plays a role in osmoregulation (McLaggan *et al.*, 1990), protects against a number of toxic compounds, and is part of the glutaredoxin system (Holmgren, 1989; Carmel-Harel and Storz, 2000). Although *luxS* and *gshA* may not form a single transcriptional unit, it is still intriguing that *gshA* is so consistently located next to *luxS* in enterobacterial genomes— thus linking a pathway that uses considerable amounts of cysteine to one that functions to preserve it. It is also interesting that thiol compounds, at least *in vitro*, have been shown to prevent the oxidative damage caused by MHF (Hiramoto *et al.*, 1996), one of the potential products of the LuxS reaction.

The location of *pfs* is similarly maintained in enterobacterial genomes. In *E. coli, S. enterica* subspecies, *Shi. flexneri, Y. pestis, Y. enterocolitica, P. mirabilis, E. chrysanthemii*, and *K. pneumoniae, pfs* is located upstream of *btuF*, which encodes the periplasmic cobalamin (vitamin B_{12}) binding protein (Van Bibber *et al.*, 1999; Cadieux *et al.*,

2002). In *E. coli*, the disruption of *pfs* with a kanamycin resistance cassette had a polar effect on *btuF* function, suggesting that the two genes form an operon (Cadieux *et al.*, 2002). Again, such co-regulation between methionine recycling and cobalamin uptake makes sense from a metabolic perspective: cobalamin is a cofactor of the methionine synthase MetH, which is over 100-fold more active than the cobalamin-independent methionine synthase MetE (Greene, 1996). However, there are many other cobalamin-dependent enzymes. Therefore, it is intriguing that in *V. cholerae, V. vulnificus, V. parahaemolyticus, Shewanella putrefaciens*, and *Shewanella oneidensis, pfs* is located immediately upstream of a putative cobalamin biosynthesis gene (*cbiB/cobD*).

VIII. Regulation of *luxS* Expression and AI-2 Production

The accumulation of AI-2 during growth and, in some cases, its subsequent removal from the culture fluid has been well documented. However, little is known about the regulation of the Pfs/LuxS pathway itself and the parameters which govern export and uptake of AI-2. Although metabolic flux through the activated methyl cycle is an absolute requirement for AI-2 production, it is not necessarily always linked with the exclusion of AI-2 from the cell. Therefore, the regulation of *pfs* and *luxS* expression will be discussed, insofar as possible, separately from AI-2 export and uptake.

A. Overview of AI-2 Production and Uptake

Many bacteria are known to produce high levels of extracellular AI-2 activity. However, others, e.g., *S. aureus, Pasteurella trehalosi, Actinobacillus pleuropneumoniae*, and *P. gingivalis*, seem to produce comparatively little, at least under the conditions tested (Chung *et al.*, 2001; Frias *et al.*, 2001; Burgess *et al.*, 2002; Doherty *et al.*, 2002; Malott and Lo, 2002). It is not yet clear whether this is due to culture conditions, differences in AI-2 export, or the production of less active AI-2 derivatives.

AI-2 activity, which is often not detectable during the early exponential stage, accumulates during growth and usually reaches a maximal level during mid- to late exponential phase (*E. coli, S. typhimurium, H. pylori, C. perfringens, Campylobacter jejuni, Streptococcus mutans*), the transition to stationary phase (*N. meningitidis*), or stationary phase (*S. pyogenes, Vibrio vulnificus*). The accumulation of AI-2 in

many bacterial cultures is transient because levels often decrease considerably during late logarithmic growth or after entry into stationary phase. Temporary accumulation of AI-2 has been observed in the culture supernatants of *Actinobacillus actinomycetemcomitans, E. coli, S. typhimurium, H. pylori, C. jejuni, S. pyogenes, S. aureus, N. meningitidis, C. perfringens, Proteus mirabilis, P. gingivalis*, and *V. vulnificus* (Surette and Bassler, 1998; Forsyth and Cover, 2000; Joyce *et al.*, 2000; Chung *et al.*, 2001; Fong *et al.*, 2001, Lyon *et al.*, 2001; McDougald *et al.*, 2001; Burgess *et al.*, 2002; Doherty *et al.*, 2002; Elvers and Park, 2002; Ohtani *et al.*, 2002; Schneider *et al.*, 2002; Winzer *et al.*, 2002d; Hardie *et al.*, 2003). However, AI-2 production and degradation in some species depends on the growth conditions. For instance, AI-2 production profiles of *S. typhimurium* and *E. coli* are dramatically influenced by the presence of glucose. In LB medium, in the presence of 0.5% glucose, significant amounts of AI-2 activity are still detectable after 8 h of growth, whereas in the absence of glucose at the same timepoint, no activity remains (Surette and Bassler, 1998). Furthermore, AI-2 accumulation by *S. typhimurium* and *E. coli* is strongly influenced by additional parameters such as pH, temperature, and osmolarity (Surette and Bassler, 1998; Surette and Bassler, 1999; DeLisa *et al.*, 2001a; Cloak *et al.*, 2002). For *C. jejuni*, temporary accumulation of AI-2 has been reported for some conditions, e.g., in milk or chicken broth (Cloak *et al.*, 2002), but not in Mueller-Hinton broth (Elvers and Park, 2002). Similarly, *P. gingivalis* did not produce detectable levels of AI-2 when grown in BHI medium but did in a defined medium (Burgess *et al.*, 2002).

It is also clear that extracellular AI-2 accumulation is not necessarily coupled to the levels of LuxS protein. Although increased production of LuxS protein was reported to increase extracellular AI-2 levels in *E. coli* O157:H7 cultures, this was not the case for *S. typhimurium* and *E. coli* K12 strains (Beeston and Surette, 2002; Burgess *et al.*, 2002; Hardie *et al.*, 2003). Furthermore, LuxS protein levels are maintained or even increased in *S. typhimurium* and *P. gingivalis* when cells enter stationary phase, despite decreasing levels of extracellular AI-2 levels (Beeston and Surette, 2002; Burgess *et al.*, 2002).

In some cases, the transient buildup of detectable AI-2 levels has been observed for only a comparatively short period of time, e.g., in *S. typhimurium* under glucose limitation (Surette and Bassler, 1999) or in *Proteus mirabilis* during swarming on L agar (Schneider *et al.*, 2002). Although certain stimuli have been shown to influence extracellular AI-2 activity, it is not clear whether this is achieved through changes in production, export, degradation or different combinations

of these activities. An ABC transporter system appears to be responsible for AI-2 uptake in *S. typhimurium* (Taga *et al.*, 2001) and homologous systems appear to be present in some but not all AI-2 producing bacteria.

In the following sections, terms such as ''increased AI-2 production'' or ''decreased AI-2 activity'' will be used to mean that a net change in extracellular activity (i.e., stimulation of bioluminescence) was observed with the *V. harveyi* bioassay (see Section III.A). Again, one should bear in mind that depending on bacterial species and growth conditions, different structurally related DPD derivatives may be formed, which could be responsible for this activity.

B. AI-2 Production in *S. typhimurium*

In the absence of glucose or under glucose-limiting conditions (0.1%), AI-2 activity in *S. typhimurium* supernatants is only detectable for a short period of time during mid-exponential growth (Surette and Bassler, 1998, 1999; Beeston and Surette, 2002). In the presence of non-limiting amounts of glucose, AI-2 is not produced earlier but is made in larger amounts and for a longer period of time, and peaks at the end of exponential growth (0.5%; Surette and Bassler, 1998; Beeston and Surette, 2002) or during entry into stationary phase (1%; Surette and Bassler, 1999). Furthermore, AI-2 activity, although drastically reduced, can still be detected after at least 20 h of growth.

Other carbohydrates produce similar effects. These include sugars or sugar derivatives taken up by the phosphotransferase system (PTS), such as fructose, mannitol, or glucosamine, as well as non-PTS sugars such as galactose or arabinose. No significant effects were observed in the presence of acetate, glycerol, citrate, and serine (Surette and Bassler, 1998, 1999).

To study the influence of other environmental parameters on AI-2 production, *S. typhimurium* was first grown for 6 h in the presence of 0.5% glucose to generate cells actively producing the AI-2 molecule and then resuspended in fresh LB under a number of different conditions. At low osmolarity (hypotonic conditions), in the absence of glucose, or at 43°C in the presence of glucose, no or only little net production of AI-2 was observed after 2 h. However, the presence of glucose, high osmolarity (hypertonic conditions), or at pH 5, significant AI-2 accumulation occurred (Surette and Bassler, 1998).

Interestingly, the temporary accumulation of AI-2 in cultures limited for glucose coincided with a transient drop in pH and the almost complete consumption of glucose. At the same timepoint in the presence of

1% glucose, glucose was only partially consumed and from that point on, the continued drop in pH was mirrored by AI-2 accumulation. Furthermore, when the pH of the culture was maintained at pH 7.2 in the presence of glucose or at pH 5 to 5.2 in the absence of glucose, a transient accumulation of AI-2 was observed. Surette and Bassler (1999) concluded from these results that glucose metabolism and pH independently induce AI-2 production in *S. typhimurium*. However, in contrast to glucose-containing cultures without pH control (Surette and Bassler, 1998), AI-2 peaks were reduced and no AI-2 activity was detectable after 6 h of growth, suggesting that both glucose and a low pH are required for maximal and extended AI-2 accumulation. A possible link between these parameters is discussed in Section VIII.E.

C. Mechanisms of AI-2 Degradation

The transient appearance of AI-2 in bacterial culture supernatants is intriguing. Theoretically, there are several possibilities for the removal of active AI-2. The molecule could be enzymatically degraded or modified, either in the supernatant, on the cell surface, or after reuptake into the cytoplasm. Degradation could also occur nonenzymatically—AI-2 activity has been shown to be base labile but is stable under acidic conditions (Surette and Bassler, 1998). Degradation of AI-2 could serve different purposes. Some bacteria may need to ''reset'' their signaling systems while others may use the molecule as a nutrient. For *S. typhimurium*, there is now convincing evidence for at least one uptake system that takes part in the removal of AI-2 from the culture supernatant (Taga *et al.*, 2001). Homologous systems are present in *E. coli* and a number of other bacteria. It will be interesting to see whether alternative systems have evolved in other organisms.

Surette and Bassler (1999) provided evidence for a cell-associated system. They reported that AI-2 activity was stable for at least 24 h at 30°C in cell-free supernatant, even when culture fluids from actively producing cells were mixed with culture supernatants where AI-2 activity had already started to decline. When cells actively producing AI-2 were resuspended in either 0.4 M or 0.1 M NaCl, similar amounts of AI-2 activity were generated during early timepoints. However, at high osmolarity, AI-2 activity began to decline after 3 h whereas at low osmolarity, all AI-2 activity was lost within 2 h. In contrast, similarly processed cells which had already commenced AI-2 activity removal rapidly reduced AI-2 activity even at high osmolarity. Inhibition of protein biosynthesis by chloramphenicol prevented AI-2 activity loss at high osmolarity and allowed only partial removal at

low osmolarity. Taken together, these results indicated the presence of a cell-associated and protein-based AI-2 turnover system, which is induced at low osmolarity.

This system was identified by Taga *et al.* (2001), who screened a library of transposon-generated transcriptional *lacZ* fusions for genes differentially expressed in an *S. typhimurium luxS* mutant. With one exception, all fusions resided within a single putative operon, which was named the *lsr* (*luxS*-regulated) operon. This operon, *lsrACDBFGE*, consists of seven genes and encodes a putative ABC transporter (*lsrACDB*), two genes of unknown function (*lsrF* and *lsrG*), and a gene with similarity to ribulose phosphate epimerase (*lsrE*). *lsrA* encodes the putative ATPase of the ABC transporter, *lsrB* a putative sugar-binding protein, and *lsrC* and *lsrD* appear to encode homologous proteins probably forming the heterodimeric membrane channel of the system. A similar operon, termed the *b1513* operon, also exists in *E. coli* but does not contain the *lsrE* gene.

The *lacZ* fusions obtained were not only downregulated in an *S. typhimuium luxS*-negative backgound but also responded to the addition of *in vitro*–synthesized AI-2. This response was not observed with controls containing SRH, adenine, and homocysteine, indicating that this operon was clearly regulated by AI-2 (or another DPD derivative). A putative DNA binding protein, LsrR, is also involved in the regulation. LsrR is encoded by a gene located immediately upstream of *lsr* but transcribed in the opposite direction. This protein shows similarity to SorC, a regulator of sorbose metabolism in *K. pneumoniae* (Wohrl *et al.*, 1990). A deletion of *lsrR* resulted in the upregulation of *lsrC-lacZ* transcription, in both wild-type and *luxS* mutants, indicating that LsrR acts as a repressor of the *lsr* operon. This role was further confirmed through LsrR overproduction in a *luxS*-positive background, which resulted in a drastic repression of *lsrC-lacZ* expression, indicating that the AI-2 levels produced were insufficient to relieve repression under these conditions.

Thus, the *lsr* operon is clearly involved in the elimination of extracellular AI-2. Various *lsr-luxS* double mutants were analyzed for their capacity to degrade *in vitro*–synthesized AI-2. *lsrA, lsrC*, and *lsrB* mutants were all unable to remove AI-2 from the culture supernatant, whereas the mutation of *lsrF* and *lsrE* had no effect on AI-2 uptake (Taga *et al.*, 2001). Furthermore, deletion of the *lsrR* repressor gene resulted in rapid and complete elimination of AI-2, probably because in this strain the *lsr* operon is maximally expressed.

Based on these results, Taga *et al.* (2001) presented a model for the regulation and function of the *lsrR* operon. According to this model,

AI-2 is produced in the cytoplasm and released to the external environment via an unidentified route. Extracellular AI-2 is sensed by an unknown mechanism, which signals the information to LsrR, resulting in a relief of *lsr* operon repression. The *lsr* encoded ABC transporter then imports AI-2 back into the cell. However, the authors point out that AI-2 sensing could also occur in the cytoplasm. In that case, AI-2 has to be imported by an *lsr*-independent mechanism, as *lsr* mutants impaired in uptake still induced the operon in the presence of AI-2. Indeed, there is evidence for a second AI-2 removal system in *S. typhimurium* because AI-2 activity also decreased in cultures of *lsrA*, *lsrB*, and *lsrC* mutants after long periods of incubation (Taga *et al.*, 2001).

The subsequent fate of AI-2 in the cell is not known, but it appears likely that it is converted back into a usable metabolite. After internalization, no activity was detectable in cell lysates, indicating that it is modified upon entry into the cell (Taga *et al.*, 2001). The LsrE protein could be involved in such a pathway. The very transient production of extracellular AI-2 in the absence of glucose suggests that cells have the metabolic capability to recycle this compound not only after reuptake but also during other growth stages where no AI-2 export occurs.

LsrB has been suggested to function as the AI-2 binding protein of *S. typhimurium*, to deliver AI-2 to the membrane components of the Lsr transporter (Taga *et al.*, 2001). AI-2 is derived from ribose and both LsrB and LuxP share some similarity in their C-terminal domains with the *E. coli* ribose binding protein RbsB and other periplasmic binding proteins of the LacI/Rbs family. However, LsrB shares only very little sequence similarity with LuxP and is, in fact, more similar to RbsB, suggesting that the two proteins have evolved independently. None of the residues known to interact with the furanosyl–borate diester in LuxP are conserved in LsrB. It will be interesting to see whether LsrB binds the furanosyl–borate diester or a different DPD derivative with ''AI-2 activity'' (see Section IV).

D. AI-2 Production in *E. coli*

The stimulatory effect of glucose on AI-2 production by *E. coli* grown in batch culture has already been described. Other carbohydrates, such as mannose, fructose, and galactose, show similar effects, whereas TCA cycle intermediates do not stimulate accumulation of AI-2 (Surette and Bassler, 1998). Contradictory data have been published with regard to glycerol (Surette and Bassler, 1998; Hardie *et al.*, 2003) and it remains to be seen whether these differences are strain dependent. The early induction of AI-2 uptake in *E. coli* and *S. typhimurium* in the absence of

certain carbohydrates suggests a regulatory mechanism that resembles catabolite repression. However, neither uptake via the PTS system nor CRP/cAMP is required: the presence of both PTS and non-PTS sugars is known to delay AI-2-degradation and mutants defective in *ptsI, ptsII, ptsH, crp*, or *cya* (encoding adenylate cyclase) are no different in this respect (Hardie *et al.*, 2003).

DeLisa *et al.* (2001a) used chemostat-cultivated *E. coli* K12 to monitor AI-2 production in response to growth rate, intracellular stress, and a variety of environmental stimuli. In contrast to batch cultures, where parameters such as nutrient availability, dissolved oxygen, pH, or the concentration of toxic products are changing continuously, chemostat cultivation offers the advantage of a reproducible and well-defined growth environment, where AI-2 production in response to distinct environmental changes can be studied.

In this study, AI-2 production was found to be directly proportional to the growth rate of the bacterial culture, at least between 0.45 and 1.1 h^{-1} in LB medium supplemented with 50 mM glucose. A similar correlation between growth rate and AI-2 production was later described for steady fed-batch cultures at high cell densities (DeLisa *et al.*, 2001b). It was also noted that glucose was not required for AI-2 production in *E. coli*, a result confirmed for batch cultures and growth in defined minimal medium (DeLisa *et al.*, 2001b). In the absence of glucose, AI-2 production by chemostat-cultivated *E. coli* also increased with increasing growth rate but at a lower overall level.

AI-2 levels in chemostat cultures also exhibited an interesting dynamic profile during upshifts and downshifts in the dilution rate, resulting in a transient overshoot or sluggish lag, respectively, prior to reaching the final steady level. Furthermore, in LB medium, a single pulse of glucose resulted in a biphasic upshift of AI-2 levels. Again, this response was greater at higher growth rates. After the pulse, AI-2 accumulated temporarily, declined to prestimulus levels, and then peaked again at an even higher level. This second peak occurred when the glucose concentration fell below 10 mM. This is interesting because batch culture experiments with *S. typhimurium* have also shown that a transient increase in AI-2 levels coincides with glucose depletion (Surette and Bassler, 1999). However, data by Beeston and Surette (2002) for *S. typhimurium* have demonstrated that this transient peak also occurs in the absence of glucose.

In addition to glucose, several other intra- and extracellular stimuli were shown to perturb the AI-2 signal in chemostat cultures. A pulse of Fe (III), high osmolarity (35 g/l NaCl), or a decreased culture redox potential (1g/l DTT) was followed by an increase in AI-2 activity within

the first hour. The activity remained elevated for another 2 to 3 h before returning to the prestimulus level. A pulse of serine hydroxamate (100 mg/l), known to induce the stringent response, resulted in rapid fall in AI-2 activity. Interestingly, AI-2 levels remained low for at least 4 h, even after serine hydroxamate had been washed out of the reactor.

Other stimuli, some of which are known to induce σ^{32} or σ^{S}-mediated responses, gave rise to more complex responses. For example, heat shock and ethanol (4%) reduced AI-2 levels. In both cases, this reduction was followed by an oscillatory response before steady state was reestablished. For the heat-shocked culture, AI-2 production remained low because the temperature was maintained at 42°C for the entire length of the experiment. Exposure to sodium acetate reduced AI-2 production initially; subsequently, a rapid increase of AI-2 activity followed and AI-2 levels remained slightly elevated for more than 1 h before falling back below the prestimulus level.

A hydrogen peroxide pulse (2 mM) led to a rapid decrease in AI-2 activity over the first 30 min, but then activity increased again and peaked at a level of 1.5-fold before returning to steady-state level. When the oxygen concentration was increased for 4 h, AI-2 levels were reduced for the entire length of the experiment. However, when the additional oxygen supply was switched off after 2 h, AI-2 activity increased, before settling back toward the steady state.

Finally, overproduction of a recombinant protein in response to a pulse of IPTG was shown to decrease AI-2 production initially, but then AI-2 activity returned to steady-state level in a dampened oscillatory manner. A reduction of AI-2 activity in response to protein overexpression has also been shown for batch cultures (DeLisa *et al.*, 2001b). Although the observed reduction was protein-specific, it was linearly related to the relative accumulation of most proteins.

DeLisa *et al.* (2001a) hypothesized that the observed changes in AI-2 production were not specific to each individual stress but indicative of a shift in metabolic activity (or state) of the cells caused by this stress. More specifically, they suggested that it is this change in the metabolic state, manifested as a change in growth rate but possibly including other phenomena, which is responsible for variations in AI-2 production. This hypothesis is not only in agreement with the observed correlation between growth rate and AI-2 production, but it also explains the downshift of AI-2 levels after addition of sodium acetate and serine hydroxamate (DeLisa *et al.*, 2001a) or during protein overexpression (DeLisa *et al.*, 2001b), conditions which are all known to decrease the metabolic activity and/or capacity of *E. coli* cells. Furthermore, this hypothesis is also compatible with the idea that AI-2 production

is linked to the metabolic flux through the activated methyl cycle (Winans, 2002; Winzer *et al.*, 2002a,b). However, it is important to keep in mind that changes in extracellular AI-2 accumulation are not necessarily directly linked with metabolic activity but could also be achieved through the regulation of export, uptake, or inactivation. Indeed, there is evidence for the active removal of AI-2 from *E. coli* chemostat culture fluids, as AI-2 activity in response to inhibitory stimuli decreased faster than predicted from the washout in the absence of continued synthesis (DeLisa *et al.*, 2001a).

As a final comment, it has also been suggested that AI-2 production is indirectly coupled to membrane synthesis, and thus growth, because most SAM is used for the synthesis of the choline moiety of phospholipids (Winans, 2002). Generally, however, this does not seem to be the case. While eukaryotic cells indeed require large amounts of SAM for the synthesis of phosphatidylcholine, this compound is only formed by a small number of bacteria and is not synthesized, for instance, by *E. coli*, which, like most other bacteria, employs phosphatidylethanolamine as the major membrane-forming phospholipid. Bacteria known to use SAM for the conversion of phosphatidylethanolamine into phosphatidylcholine include *Sinorhizobium meliloti* and *Agrobacterium tumefacines* (Kaneshiro and Law, 1964; Goldfine, 1982; de Rudder *et al.*, 1997), both of which do not possess a *luxS* gene. Furthermore, some bacteria only utilize host-derived choline for phospholipid synthesis (Lopez-Lara and Geiger, 2001). However, many bacteria require SAM for the synthesis of other lipid components such as *N*-methylethanolamine (e.g., *Proteus vulgaris*) or cyclopropane rings (Goldfine and Ellis, 1964; Goldfine, 1972; Cronan *et al.*, 1979). Like phosphatidylcholine, the proportion of the latter compounds is often significantly increased when cells enter stationary phase (Goldfine, 1972; Taylor and Cronan, 1976; Goldfine, 1982; Wang and Cronan, 1994), which may contribute to the peak in AI-2 accumulation observed for some bacteria during this stage of growth (see Section VIII. A). However, this does not seem to be the case for *E. coli* grown in LB medium without glucose, where AI-2 levels decline after the mid-exponential phase but cyclopropane fatty acid biosynthesis increases dramatically during the transition into stationary phase (Wang and Cronan, 1994).

E. Regulation of *PFS* and *LUXS* Genes in *E. coli* and *S. typhimurium*

Relatively little information is available on the regulation of *pfs* and *luxS* in different bacteria and under different environment conditions. A study by Beeston and Surette (2002) suggests that in *S. typhimurium*

in the presence of glucose, *pfs* but not *luxS* transcription correlates with AI-2 production. Expression of *luxS* and *pfs* was monitored using reporter fusions and RT-PCR for bacteria grown in LB medium with or without 0.5% glucose, conditions previously reported to produce major differences in AI-2 activity (Surette and Bassler, 1999). Interestingly, *luxS* was constitutively transcribed at approximately the same level at all timepoints and no changes were observed in the presence or absence of glucose. In contrast, glucose had a drastic effect on *pfs* expression. In the absence of glucose, AI-2 levels peaked after 3 to 4 h and disappeared completely after 5 h, whereas *pfs* expression levels, after a small initial increase, remained largely unchanged during this period and only started to increase significantly after 5 h (which incidently coincides with the transition into stationary phase). In the presence of glucose, however, *pfs* expression peaked after 4 h and then rapidly declined, a pattern which was reflected by the AI-2 levels in the culture supernatant. After 4 h, both *pfs* expression and AI-2 accumulation were about three times higher in the presence of glucose than in its absence. Furthermore, in the presence of other mono-, di-, or trisaccharides as well as glycerol, a very similar *pfs* expression pattern to that observed in the presence of glucose was noted. Again, *pfs* expression levels appeared to correlate with the accumulation of AI-2. Overexpression of *luxS* did not affect AI-2 production, and addition of exogenous AI-2 had no effect on *pfs* or *luxS* expression. On the basis of these results, Beeston and Surette (2002) suggested that the tight regulation of AI-2 production may be mainly at the level of *pfs* transcription and that AI-2 production is therefore regulated by the availability of SRH. However, seeming correlations between changes in gene expression and metabolite levels should be interpreted with caution. Depending on the stability of the enzyme in question, a decrease in gene expression is not necessarily correlated with a decrease in the corresponding enzymatic activity. The availability of the Pfs substrate, SAH, is another issue (Beeston and Surette, 2002; Winzer *et al.*, 2002a,b). In the presence of glucose, a decrease in *pfs* expression and AI-2 levels coincides with the entry into stationary phase, where the activated methyl cycle might be used to a much lesser extent. It is therefore possible that the regulation of *pfs* does not serve to limit AI-2 production but merely reflects the given metabolic requirement for complete substrate conversion. Furthermore, Pfs activity must also satisfy its role in MTA detoxification.

The addition of 50 mM acetate to *E. coli* cultures grown in buffered LKB broth at pH 6.7 increases the steady-state level of the LuxS protein, but addition of formate failed to show the same effect (Kirkpatrick *et al.*,

2001). The LuxS protein was also elevated at low pH, probably because fermentation products such as acetate can reenter the cells in their membrane-permeable, protonated form and accumulate in the cytoplasm (Stancik *et al.*, 2002). It has been suggested that in glucose-containing medium, *luxS* transcription responds to the production of acetate, which peaks in midlog phase and then declines due to reuptake and degradation via the TCA cycle (Stancik *et al.*, 2002). This is an interesting hypothesis because certain environmental stresses, including acetate accumulation, seem to perturb methionine biosynthesis in *E. coli*. This results in a reduced growth rate due to partial methionine auxotrophy (Roe *et al.*, 2002). In the case of acetate, growth inhibition can be relieved through the addition of methionine (Han *et al.*, 1993). Experimental evidence suggests that acetate accumulation inhibits a biosynthetic step required for the conversion of homocysteine to methionine, resulting in the depletion of the methionine pool and toxic accumulation of homocysteine (Roe *et al.*, 2002). The transient reduction of AI-2 activity in response to sodium acetate in a continuous culture of *E. coli* (DeLisa *et al.*, 2001a) may well have been caused by a reduced flux through the activated methyl cycle due to methionine pool depletion. It is tempting to speculate that cells respond to methionine pool depletion by the upregulation of enzymes required for salvage pathways such LuxS. As a further example, temperatures above 40°C inhibit growth of *E. coli* and other enteric bacteria, at least in minimal medium, and this inhibition can be overcome by the addition of methionine (Ron and Davis, 1971; Ron, 1975). Indeed, the requirement for methionine at elevated temperatures has been associated with a decrease in homoserine transuccinylase (MetA) activity (the first step in methinonine biosynthesis; see Fig. 7A) due to MetA aggregation (Ron and Shani, 1971; Ron, 1975; Biran *et al.*, 2000; Gur *et al.*, 2002). Perhaps depletion of this amino acid contributes to the observed reduction in AI-2 activity when *E. coli* cultures are grown at 42°C (DeLisa *et al.*, 2001a) and *S. typhimurium* undergoes heat shock (Surette and Bassler, 1999).

DeLisa *et al.* (2001a) reported that *luxS* transcript levels in response to the recombinant protein overexpression were highly correlated with those of stress and starvation-induced genes, namely, *rpoS, groEL, dnaK, grpE, clpP, ftsA, ftsJ, ftsQ*, and *recA*. As an initial response, the reduction of AI-2 and concomitant increase of *luxS* transcript levels was reported. DeLisa *et al.* (2001a) suggested an overlap between quorum-sensing and stress-related circuitries, but it is also possible that *luxS* (like the other genes) responded to the metabolic and physiological consequences of protein overexpression, such as amino acid

pool depletion and protein misfolding. Methionine pool depletion, and thus a reduction in the metabolic flux through the activated methyl cycle as indicated by the reduction in AI-2 levels, could result in increased expression of *luxS* and other genes involved in methionine salvage pathways.

F. Regulation of AI-2 Production in the Gut

Because of the genetic organization of *pfs* and *btuF* in enteric bacteria (see Section VII), Beeston and Surette (2002) suggested that AI-2 production may be tightly linked to cobalamin uptake in the intestinal tract, a habitat where anaerobic conditions prevail and cobalamin is readily available for colonizing or infecting bacteria. Many enterics, but not *E. coli*, can also synthesize cobalamin *de novo* (Roth *et al.*, 1996) and, interestingly, in other facultative anaerobic γ *Proteobacteria* such as *V. cholerae, S. putrefaciens*, and *S. oneidensis pfs* is not linked to *btuF* but to homologues of the cobalamin biosynthesis gene *cobD* (see Section VII). The suggested coregulation of *pfs* and cobalamin transport/synthesis genes is therefore more likely to reflect an increased requirement for cobalamin and the recycling of methionine under anaerobic conditions rather than a direct link between cobalamin uptake and signal molecule generation. *S. typhimurium*, for instance, synthesizes cobalamin only under anaerobic conditions (Roth *et al.*, 1996). Many of the known cobalamin-dependent reactions support anaerobic fermentation of small molecules (Roth *et al.*, 1996). Cobalamin is also part of class II ribonucleotide reductases and methionine synthesis by MetH. In *E. coli* and *S. typhimurium*, the cobalamin-dependent methionine synthase MetH is probably only functional during growth in the gut, where cobalamin represses the expression of *metE* (Wu *et al.*, 1992; Matthews, 1996). That said, the suggested coordinated regulation of cobalamin acquisition and SAH catabolism in enteric bacteria is intriguing and certainly argues in favor of increased AI-2 production in the gut. There are other observations that suggest that AI-2 production may be maximal in the intestinal lumen. For example, it has been pointed out that this environment is rich in nutrients (including carbohydrates) and high in osmolarity, conditions known to favor extracellular AI-2 accumulation (Surette and Bassler, 1999; Beeston and Surette, 2002).

Furthermore, it might be interesting to determine whether for *E. coli* and *S. typhimurium* there is a link between extracellular AI-2 accumulation and the production of short-chain fatty acids (SCFAs) and acetate in particular. Accumulation of these compounds can have severe

physiological effects because SCFAs in their undissociated form are membrane permeable and can reenter and, depending on the pH gradient across the cytoplasmic membrane, accumulate within the cell (Russell and Diez-Gonzalez, 1998). As was described in more detail (Section VIII. D), the short-lived accumulation of AI-2 under glucose-limiting conditions coincides not only with the depletion of glucose but also with a temporary drop in pH (Surette and Bassler, 1998), which, at least for *E. coli*, has been associated with the buildup of acetate (Stancik *et al.*, 2002). However, the inclusion of unlimited glucose results in steady acidification without recovery (Surette and Bassler, 1999; Stancik *et al.*, 2002), resulting first in increased AI-2 accumulation and also a delayed degradation. This, together with the fact that LuxS protein levels are increased at low pH and under acetate stress, led to the suggestion that in the presence of glucose, the *luxS* gene may respond to the production of acetate (Stancik *et al.*, 2002). To take this hypothesis a step further, is it possible that acetate stress, rather than a drop in pH, is one of the signals that leads to AI-2 accumulation? The fact that high concentrations of acetate somewhat reduced the rate of AI-2 production in chemostat-grown *E. coli* (DeLisa *et al.*, 2001a) does not necessarily mitigate against this suggestion.

High concentrations of SCFAs, fermentation products of the local microflora, are also present in the intestine (Cummings *et al.*, 1987) and although the pH of the gut lumen is nearly neutral, it is sufficiently low in some parts (Cummings *et al.*, 1987; Fallingborg, 1999), particularly on the surface of the intestinal lining (Bugaut, 1987), to promote the accumulation of SCFAs in bacteria unable to reduce their intracellular pH. Other signals must exist, however, because temporary AI-2 production is also observed for *S. typhimurium* grown at pH 7.4 (Surette and Bassler, 1999), where reentry of acetate is not likely to be a major undertaking due to the small pH gradient and a low amount of undissociated acid.

G. Regulation of *pfs* and *luxS* Genes in Other Bacteria

In *P. gingivalis, luxS* transcript levels were more than three times higher in cells from the late log phase than in those from early log phase (Chung *et al.*, 2001). LuxS protein levels also increased throughout growth (Burgess *et al.*, 2002). The concentration of NaCl affected *luxS* transcript levels significantly, whereas changes in pH (6.5 and 8.5) or temperature had no effect (Chung *et al.*, 2001). Highest transcript levels were observed in the presence of 80 mM NaCl. At a concentration

of 160 and 240 mM NaCl, expression was significantly reduced and absent, respectively.

In *S. pyogenes*, amino acid starvation, i.e., the absence of isoleucine and valine, was reported to increase *luxS* transcript levels approximately threefold (Steiner and Malke, 2001). This response, which is *relA*-independent, makes sense from a metabolic perspective. As has been mentioned, under conditions of starvation, cells may upregulate recycling pathways such as the activated methyl cycle. In organisms such as *E. coli* and S. *typhimurium*, the biosynthetic pathways leading to isoleucine and methionine formation share a common precursor, homoserine. However, *S. pyogenes* lacks many of the essential genes required for methionine and isoleucine *de novo* biosynthesis including *metE* and *metH*, and consequently, the efficient generation of homocysteine cannot free up resources for isoleucine and methionine formation. Although upregulation of *luxS* appears to be part of a general starvation response in this organism, its role under these conditions remains unclear.

During long-term starvation, the activated methyl cycle, like other metabolic processes, would be predicted to be downregulated. Indeed, differential regulation of *luxS* was reported for *B. burgdorferi* inhabiting the gut of ticks (*Ixodes scapularis*) which were either fed to repletion on mice or left unfed (Narasimhan *et al.*, 2002). In fed ticks, a significant increase of *luxS*-specific transcripts was observed. Although it was suggested that *luxS* may be part of a signaling circuit mediating responses to environmental stimuli, the observed changes in transcription make more sense from a metabolic point of view. In the presence of nutrients, the enzymes of the activated methyl cycle are induced, because the bacteria require SAM for growth.

In *S. aureus*, the *luxS* gene is transcribed as a single transcriptional unit from a σ70-dependent promoter (Doherty *et al.*, 2002). Transcript levels are highest during exponential growth and decrease moderately during the transition to stationary phase. In common with *P. gingivalis* and *S. typhimurium*, overexpression of *luxS* in *S. aureus* does not result in increased AI-2 production, again suggesting that the metabolic flux through the activated methyl cycle is not limited by LuxS activity. Furthermore, expression of the *luxS* gene is not influenced by the *agr* quorum sensing system (Doherty *et al.*, 2002). Walker and Duerre (1975) reported only trace amounts of SRH-cleaving activity in *S. aureus* cell-free extracts, which could explain the low amounts of AI-2 produced by this organism. However, Doherty *et al.* (2002) found that the specific activities of LuxS in cell extracts of *S. aureus* RN6390B and *E. coli* MG1655 entering stationary phase were comparable.

For *C. perfringens* (Banu *et al.*, 2000; Ohtani *et al.*, 2002), it has been proposed that the operon containing *luxS*, *metB*, *cysK*, and a gene of unknown function, *ycgJ*, is regulated by the VirR/VirS two-component system, originally identified as a regulator of toxin, sialidase, protease, and hemagglutinin production. Two different transcripts appear to be present, a 4.9 kb transcript encoding the entire operon and a 2 kb transcript encoding *cysK* and *luxS* (Banu *et al.*, 2000). The 4.9 kb transcript was moderately increased (twofold) in a *virR*-negative background, whereas the 2 kb transcript appeared to be *virR* independent. The 4.9 kb transcript was only detectable at the midexponential phase of growth and disappeared at the late exponential phase. Whether the twofold increase of the 4.9 kb transcript is a direct or indirect effect of *virR* inactivation remains to be seen, especially since this is the first example of negative regulation by the system, but it could represent a mechanism to coordinate virulence factor production with metabolic requirements during infection.

IX. Mechanisms of Bacterial Cell-to-Cell Communication

Section II describes the variety of bacterial cell–cell communication (quorum-sensing) mechanisms discovered to date. Although LuxS clearly has an important role within the activated methyl cycle (as discussed in detail in preceding sections), the molecule it generates (AI-2) appears to function as a diffusible signal molecule in some bacteria. Indeed, it was in this context that it was first discoved in *V. harveyi* (Bassler *et al.*, 1994). As such, AI-2-dependent quorum sensing would be unique since it is shared by diverse gram-negative and gram-positive bacteria. The role of AI-2 in cell-to-cell communication, particularly the regulation of bioluminescence in *V. harveyi*, is described in detail in the following section.

A. Quorum Sensing in *Vibrio* Species: A Role for AI-2

1. *Quorum Sensing in* Vibrio harveyi *and* Vibrio cholerae

V. harveyi and *V. cholerae* both possess highly sophisticated quorum-sensing systems. Information from multiple channels is integrated with the help of a complex phosphorelay system and determines, together with other physiological and environmental parameters, the expression of specific target genes. Owing to the complexity of these systems, a short general description will be given first in the relevant sections (IX. A.2.a and IX. A.3.a), followed by a more detailed discussion of

individual components. *V. harveyi* and *V. cholerae* are presently the only species, where a role of AI-2 in quorum sensing has been convincingly demonstrated.

2. *The Quorum-Sensing System of* V. harveyi

a. Overview. It has been known for a long time that the marine bacterium *V. harveyi* (formerly *Beneckea harveyi*) regulates bioluminescence via the production of a ''substance'' that accumulates in the medium and induces the production of luciferase (Greenberg *et al.*, 1979). It is now clear that the expression of the *luxCDABEGH* operon, which encodes the enzymatic functions for light production, is controlled in a complex manner involving not one but two different signal molecules and two converging signal transduction pathways (Fig. 8).

The first signal molecule to be isolated was identified as *N*-(3-hydroxybutyryl)-L-homoserine lactone (Cao and Meighen, 1989; 1993), whereas the second molecule, autoinducer-2 (AI-2), appears to be a furanosyl borate diester (Chen *et al.*, 2002, see Section IV for details). Synthesis of *N*-(3-hydroxybutyryl)-L-homoserine lactone (3OH-C4-HSL also termed AI-1) is directed by the LuxM protein (originally designated LuxLM by Bassler *et al.* (1993) but subsequently found to be a single ORF (Milton *et al.*, 2001)). After its synthesis, AI-1 is released into the medium where it accumulates. In contrast to most of the known AHL-based signaling systems, however, this molecule is not recognized by an intracellular LuxR-type regulator, but a histidine protein kinase, LuxN, which is located in the cell membrane (Bassler *et al.*, 1993).

The production of a second autoinducer, AI-2, became apparent after Bassler *et al.* (1993) discovered that the addition of spent culture fluid from a *luxM* mutant culture was still capable of inducing bioluminescence in a *luxN* mutant prematurely, whereas synthetic AI-1 failed to show the same effect. Furthermore, the *luxN* mutant, although defective in AI-1 signal transduction, still produced light in a cell density-dependent manner. Synthesis of AI-2 depends on the LuxS protein (see Section III). It is presently not known how this comparatively polar signal molecule is released into the medium but, in common with AI-1, it accumulates during growth of a *V. harveyi* culture. AI-2 binds to a periplasmic binding protein, LuxP, which then, in turn, is thought to interact with a membrane-bound histidine protein kinase termed LuxQ (Bassler *et al.*, 1994; Chen *et al.*, 2002).

LuxN and LuxQ belong to the family of so-called hybrid sensor kinases because they contain a signaling domain as well as a receiver

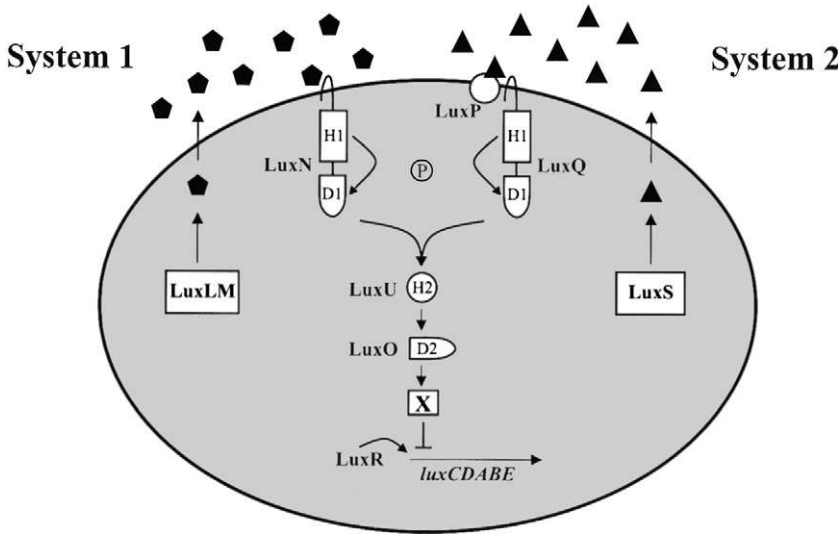

FIG. 8. Proposed quorum-sensing cascade of *V. harveyi*. Two parallel quorum-sensing circuits regulate light production in *V. harveyi*. System 1 involves the LuxM-dependent production of AI-1 (pentagons), which is sensed by the histidine kinase LuxN. In system 2, AI-2 (triangles) is produced by LuxS and binds to the periplasmic binding protein LuxP. The LuxP–AI-2 complex then interacts with the histidine kinase LuxQ. In the absence of AI-1 or AI-2, LuxN and LuxQ act as kinases which, after autophosphorylation, transfer the phosphate (''P'' in a circle) to the phosphorelay protein LuxU. From here, the phosphate is transferred to the transcriptional regulator LuxO. Phospho-LuxO is hypothesized to activate the unidentified repressor (X), preventing action of the transcriptional activator $LuxR_{Vh}$ and switching off light production. LuxO also represses $luxR_{Vh}$ transcription (Miyamoto *et al.*, 2003; not shown), possibly via the repressor X. Repressor X has been postulated to directly influence *luxCDABE* expression, but it is also possible that this effect is mediated by $LuxR_{Vh}$. In the presence of AI-1 and AI-2, LuxN and LuxQ convert from kinases to phosphatases, leading to the dephosphorylation of LuxO. Dephospho-LuxO is inactive, no expression of the repressor X occurs, and $LuxR_{Vh}$ activates transcription of the luciferase operon to generate light. Reproduced from Miller *et al.* (2002).

domain within a single polypeptide. Both kinases apparently autophosphorylate (Freeman and Bassler, 1999a,b; Freeman *et al.*, 2000) in the absence of signal molecule (low cell densities) but function as phosphatases at high signal molecule concentrations (high cell densities). A model of quorum sensing in *V. harveyi* has been presented where, at low cell densities the phosphate is transferred from the autophosphorylated LuxN and LuxQ proteins to a two-component phosphorelay protein termed LuxU, which in turn phosphorylates the response regulator protein LuxO (Freeman and Bassler, 1999a,b; Freeman *et al.*,

2000). Thus, the sensory inputs from LuxN/AI-1 and LuxQ/LuxP/AI-2 are integrated. LuxO in its phosphorylated form is known to repress bioluminescence (Bassler *et al.*, 1994; Freeman and Bassler, 1999a), probably indirectly by activating the production of a repressor of the *luxCDABEGH* operon. At a critical autoinducer concentration, the binding of AI-1 and AI-2 to LuxN and LuxP, respectively, is thought to lead to a conformational change of LuxN and LuxQ, converting these proteins from kinases into phosphatases (Freeman *et al.*, 2000). Consequently, the flow of phosphates is reversed, now going from LuxO via LuxU to LuxN and LuxQ where dephosphorylation takes place. Dephosphorylated LuxO can no longer activate the transcription of the hypothetical repressor gene and, eventually, *luxCDABE* is transcribed. Not surprisingly, LuxO and, therefore, signal molecule accumulation also controls other phenotypes in *V. harveyi*, such as siderophore production and colony morphology (Lilley and Bassler, 2000), as well as genes encoding putative type III secretion proteins and a metalloprotease (Mok *et al.*, 2003). *luxCDABE* is also controlled by an activator protein termed $LuxR_{Vh}$ (not to be confused with the LuxR protein of *Photobacterium fischeri*), a homologue of a family of proteins which includes: HapR in *V. cholerae*, VanT in *V. angularum*, and LitR in *V. fischeri.* $luxR_{Vh}$ is also negatively regulated by LuxO (Miyamoto *et al.*, 2003).

b. The phosphorelay system. It should be noted that the phosphorylation events described here have not yet been demonstrated directly, but have been deduced from a series of elegant experiments including the systematic exchange of amino acids in LuxN, LuxU, and LuxO. Some of these changes were based on results obtained for similar studies with CheY, NtrC, and EnvZ, and resulted in the formation of mutant proteins mimicking either the phosphorylated or dephosphorylated form of the respective protein. These experiments have contributed substantially to our understanding of the *V. harveyi* quorum-sensing signal transduction cascade and will therefore be described in more detail. No such experiments have been undertaken with LuxQ. However, this protein is thought to function in a manner analogous to LuxN due to the phenotypes observed for *luxQ*, *luxN*, or *luxNQ* double mutants (see Section X.A.4).

c. luxN. While a conserved histidine in the sensor kinase domain is required solely for LuxN kinase activity, a conserved aspartate localized in the response regulator domain is required for both kinase and phosphatase activity (Freeman *et al.*, 2000). LuxN kinase activity is regulated by AI-1, whereas its phosphatase activity appears to be

constitutive. The elimination of the proposed autophosphorylation site (the histidine in position 471 [H471] was exchanged against glutamine [H471Q]) resulted in constitutive light production, similar to the phenotype of the wild-type in the presence of exogenous AI-1. The phenotype associated with the H471Q mutation is consistent with a loss of LuxN kinase activity but unaltered phosphatase activity. In the *E. coli* sensor kinase EnvZ, all mutations of the equivalent conserved histidine residue had been reported to eliminate kinase activity but only some of these changes also affected phosphatase activity (Hsing and Silhavy, 1997). Consequently, H471 was exchanged for a number of other amino acids, but none of the resulting proteins was altered in phosphatase activity (elimination of both kinase and phosphatase activity would have been expected to produce the phenotype of a *luxN* deletion mutant).

Random mutagenesis of *luxN* created an allele where leucine in position 166 (L166) was exchanged for arginine (R) in the encoded protein (L166R) (Freeman *et al.*, 2000). This replacement resulted in a constitutively dark strain, just like the phenotype observed with *luxM* mutations. Because L166 is located within the domain of LuxN which spans the membrane and periplasm, the amino acid exchange in this position was interpreted to render the protein unable to respond to the presence of AI-1, effectively locking it in the kinase mode. As expected a strain containing a *luxN* gene with both mutations (L166R and H471Q) exhibited constitutive bioluminescence: Although the enzyme is locked in the kinase state, it cannot autophosphorylate but still exhibits a constitutive phosphatase activity (which apparently is strong enough to efficiently override LuxQ kinase activity).

Since none of the sensor kinase module mutations affected phosphatase activity, the location of this activity was speculated to be in the response regulator domain. The predicted phosphorylation site of this domain, an aspartate residue in position 771 (D771), was changed into alanine (D771A). A strain producing this protein, just like a *luxN* deletion mutant, showed cell density-dependent production of light which could not be advanced by the addition of exogenous AI-1. This phenotype is consistent with the loss of both kinase and phosphatase activity. In order to demonstrate a function for D771 in the kinase reaction, the luxN L166R mutation was combined with a luxN D771A mutation. Since L166R locks luxN in the kinase mode, the ''dark'' phenotype conferred by this mutation should also depend on D771. Indeed, it was found that a strain carrying this double mutation produced wild-type levels of light, confirming that D771 is required for the transfer of the phospho group to LuxU.

Furthermore, an in-frame deletion mutant of *luxN*, expressing only the response regulator domain, displayed maximal constitutive bioluminescence, a phenotype consistent with a lack of kinase activity and the presence of constitutive phosphatase activity. D771 is required for this activity, since its exchange against alanine in the expressed response regulator domain resulted in cell density-dependent light production, just as the corresponding mutation in the complete protein or a complete *luxN* deletion.

d. luxU. LuxU is a small phosphorelay protein which appears to receive phosphates from both LuxN and LuxQ and to transfer them subsequently to LuxO (and vice versa). A histidine residue (His 58) is essential for this transfer (Freeman and Bassler, 199b), because its exchange against alanine prevents the protein from becoming phophorylated and therefore confers, like the deletion of *luxU*, a constitutive bioluminescent phenotype (LuxO remains unphosphorylated). The same phenotype was also observed for a *luxU–luxO* double mutant. Evidence for the position of LuxU in the signal transduction cascade was obtained by combining a *luxU* deletion with alleles of either *luxO* or *luxN* carrying defined point mutations. When the *luxO* allele D47E (encoding a Phospho–LuxO mimic) was combined with a *luxU* deletion, the resulting strain was constitutively dark, placing LuxU upstream of LuxO in the signal transduction hierarchy. Moreover, a strain carrying the *luxN* allele L166R (the encoded protein is locked in the kinase state; see preceding section) showed a constitutively dark phenotype (due to continuous phosphorylation of LuxO), but when combined with a *luxU* deletion the resulting strain was constitutively bright, confirming that LuxU works downstream of LuxN and—by analogy—LuxQ (Freeman and Bassler, 1999b). It is, however, possible that LuxU is also phosphorylated by other sensors kinases, and it may be the point where other environmental signal input converges. The role of LuxU and LuxO in signal integration is further described in Section IX. A. 4.

e. luxO. Freeman and Bassler (1999a) constructed a set of *luxO* alleles, which replaced the wild-type gene on the chromosome and encoded proteins mimicking either the unphosphorylated (constructs D47A, D47N, K97A) or phosphorylated protein (D47E, F94W). Production of proteins mimicking the unphosphorylated state of LuxO resulted in constitutive light production, whereas production of Phospho–LuxO mimics resulted in a dark phenotype, suggesting that an unphosphorylated response regulator domain inhibits the activity of the protein (DNA binding and activation of transcription of the

putative *lux* operon repressor gene). This was further supported by the observation that a strain containing a truncated *luxO* gene, which only encoded the N-terminal DNA-binding domain, was dark at high cell densities, probably because this domain was still capable of binding to its DNA target site and activating repressor transcription.

f. The role of rpoN. Since LuxO is a NtrC homologue which contains a conserved σ54 (RpoN) activation domain, Lilley and Bassler (2000) studied the role of RpoN in cell density-dependent regulation of bioluminescence. Indeed, it was confirmed that RpoN is required for the cell density-dependent regulation: An *rpoN* mutant is constitutively bright, just like a *luxO* mutant. Furthermore, when the *rpoN* mutation was transferred into the constitutively dark LuxO mutant D47E, which is locked in a form mimicking phosphorylated LuxO, the resulting double mutant also exhibited constitutive light production, indicating that LuxO does not act downstream of LuxN in the signal transduction hierarchy. *luxO–lacZ* fusion analysis also demonstrated that RpoN is not required for *luxO* expression. Conversely, LuxO is not required for *rpoN* expression because *rpoN* mutants are nonmotile in contrast to *luxO* mutants. Taken together, these results confirmed that phosphorylated LuxO is required to activate the RpoN-dependent transcription of a gene encoding a repressor of *luxCDABE*.

Lilley and Bassler (2000) also showed that LuxO, and, therefore, signal molecule accumulation, also controls other phenotypes in *V. harveyi*, such as siderophore production, colony morphology, and pellicle formation in liquid culture. The LuxO D47E mutation significantly increased siderophore amounts in AB medium, but additional mutation of *rpoN* was shown to reduce amounts to wild-type level. The colonies of the D47E strain, as well as those of strain L166R, where LuxN is locked in the kinase state, displayed a morphology similar to the rugose morphology described for *V. cholerae* El Tor biotype *hapR* mutants (see Section IX. A. 3) and the opaque morphology of *V. parahaemolyticus*. The D47E and L166R strains also formed pellicles in liquid culture.

g. $luxR_{vh}$. The $LuxR_{vh}$ protein of *V. harveyi* is required for the expression of the *luxCDABE* operon (Showalter *et al.*, 1990), but it is not a member of the well-known family of LuxR-type proteins found in other bacteria, such as *P. fischeri* or *Pseudomonas aeruginosa*. Instead, it belongs to a family of proteins presently only described in members of the genus *Vibrio* and closely related bacteria. Known examples include HapR, SmcR, OpaR, LitR, and VanT, in *V. cholerae, Vibrio*

vulnificus, Vibrio parahaenolyticus, P. fischeri, and *Vibrio anguillarum*, respectively. Alignments indicate that these proteins contain a helix-turn-helix domain with high similarity to the helix-turn-helix region typical for the TetR family of transcriptional regulators (Jobling and Holmes, 1997; McDougald *et al.*, 2000).

V. harveyi $LuxR_{vh}$ is known to interact directly with the operator region of the *lux* operon (Swartzman *et al.*, 1992; Miyamoto *et al.*, 1994) and it also directly regulates its own expression by functioning as an autorepressor (Chatterjee *et al.*, 1996). Interestingly, reporter gene studies indicated that transcription of $luxR_{Vh}$ can be stimulated, albeit only two-fold, through the addition of exogenous AI-1, suggesting that the AI-1 signal can also be transduced through $LuxR_{vh}$. However, these findings were made with two undefined mutants thought to be impaired in AI-1 synthesis[7] and response, respectively, and no stimulation was observed for the wild-type (Miyamoto *et al.*, 1996). The precise mechanism and the significance of these observations are not clear and require further experimentation. A paper by Miyamoto *et al.* (2003) suggests that LuxO negatively regulates $luxR_{vh}$ (a *luxO* mutant contains three times more $luxR_{vh}$ mRNA at low cell density compared to the wild-type and expression of a plasmid-borne copy of $luxR_{Vh}$ in a *luxO* mutant significantly stimulates bioluminescence).

Homologues of *V. harveyi* $LuxR_{Vh}$ have been shown to control a number of different phenotypes, including bioluminescence in *P. fischeri* (Fidopiastis *et al.*, 2002; LitR regulates transcription of the *P. fischeri luxR* gene), metalloprotease production, pigment production, and biofilm formation in *V. anguillarum* (Croxatto *et al.*, 2002), and a number of virulence genes in *V. cholerae* (see Section X. A. 3). Miyamoto *et al.* (2003) suggest that LuxO-dependent control of $luxR_{Vh}$ homologues is a common regulatory component of quorum-sensing systems in *Vibrio sp.*

h. Metabolic control of bioluminescence. Bioluminescence in *V. harveyi* is not only controlled by quorum sensing but is also linked to the nutritional status of the cell (Nealson and Hastings, 1979; Meighen, 1991). Chatterjee *et al.* (2002) demonstrated that the global regulator CRP (cAMP receptor protein) is absolutely required for *luxCDABE* expression, whereas the LysR-type regulator MetR plays a more modulatory role. Bioluminescence of *V. harveyi* was 1000-fold reduced in a *crp* mutant but 10-fold increased in a *metR* mutant. Both proteins interact directly with the $luxR_{Vh}$ and *luxCDABE* promoter.

[7] This mutant is also deficient in AI-2 production (Bassler *et al.*, 1997).

Bioluminescence appears to be subject to catabolite repression, because the binding of CRP to the $luxR_{Vh}$ and *luxCDABE* promoter requires cAMP, and light production of *V. harveyi* is reduced in the presence of glucose. Homocysteine, known to act as the MetR coregulator in *E. coli*, reduced bioluminescence when added to the growth medium. Thus, it appears that *luxCDABE* expression is also linked to methionine metabolism.

3. *The Quorum-Sensing System of* Vibrio cholerae

a. Overview. The detection of an AI-2–like signal molecule in spent culture supernatants of a number of *V. cholerae* strains (Bassler *et al.*, 1997) as well as the presence of putative homologues for all components of the *V. harveyi* AI-2 signal transduction cascade in the *V. cholerae* genome (*luxS, luxP, luxQ, luxU, luxO*) suggested that this bacterium uses a quorum-sensing system similar to that of *V. harveyi* to regulate target genes in a cell density-dependent manner. Indeed, gene array studies by Zhu *et al.* (2002) indicated that the entire ToxR regulon is repressed in a *V. cholerae luxO* mutant (E1 Tor biotype strain C6706), and that this repression is mediated by the regulator HapR, a homologue of $LuxR_{Vh}$, originally identified as a positive regulator of the HA protease (Jobling and Holmes, 1997). Gene array analysis of *V. cholerae* constitutively expressing *hapR* revealed a transcriptional profile very similar to that of a *luxO* mutant, identifying HapR itself as a negative regulator of virulence gene expression. Further experiments by Zhu *et al.* (2002) suggested that HapR negatively regulates *tcpP*, via direct inhibition of *ahpA* expression (Kovacikova and Skorupski, 2002), thereby preventing the production of the positive regulator ToxT, which is required for virulence gene expression (Fig. 9).

Zhu *et al.* (2002) demonstrated that LuxO represses *hapR* transcription during early logarithmic growth, thereby allowing TcpP-dependent virulence genes to be expressed. The authors also demonstrated that LuxO and HapR are involved in the regulation of protease production and biofilm formation. In addition, *V. cholerae* LuxO positively regulates motility. In 2002, the unusually complex quorum-sensing system of *V. cholerae* has been unraveled by Miller *et al.* Like *V. harveyi*, two parallel quorum-sensing systems converge to regulate specific target genes in *V. cholerae*. Using the *luxCDABE* gene cluster of *V. harveyi* as a reporter, a new signal molecule of unknown structure, CAI-1, was discovered which appears to interact with a hybrid sensor kinase termed CqsS. A protein with similarity to aminotransferases, CqsA, is required for CAI-1 production. CqsA, CqsS, and CAI-1 represent the first quorum-sensing system of *V. cholerae*. The second system corresponds

to the AI-2–based system of *V. harveyi*, consisting of the periplasmic protein LuxP, the sensor kinase LuxQ, AI-2, and the AI-2 signal molecule generator LuxS. Analysis of various *V. cholerae* mutants suggests that CqsS and LuxQ, by analogy with *V. harveyi*, also act as kinases at low cell density but switch to phosphatase activity upon the binding of their cognate signal molecules at high cell density. Furthermore, the sensory input from both systems is also integrated on the level of LuxU, which mediates the exchange of phosphate groups between the sensor kinases and the response regulator LuxO. However, in contrast to what has been described for *V. harveyi*, a third signaling circuit, possibly responding to an intracellular signal, feeds into the *V. cholerae* system at the level of LuxO, adding yet another layer of complexity (Fig 9A).

Phospho–LuxO, which is only present at low autoinducer concentrations, negatively regulates the production of HapR, thereby allowing TcpP-dependent virulence gene expression to take place. Thus, in contrast to many other plant or animal pathogens, quorum sensing in *V. cholerae* serves to repress rather than activate virulence gene expression at high cell densities, at least *in vitro* (Miller *et al.*, 2002; Zhu *et al.*, 2002).

b. Analysis of V. cholerae *quorum-sensing using a* luxCDABE *reporter.* Although closely related to *V. harveyi*, the vast majority

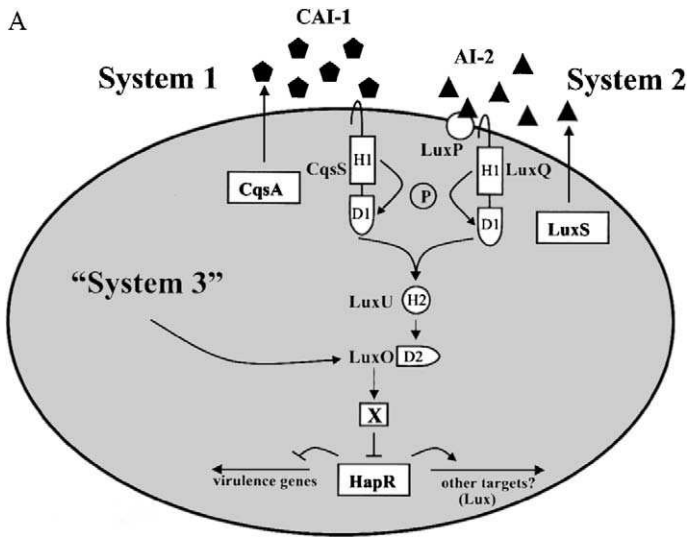

FIG. 9 (*continued*)

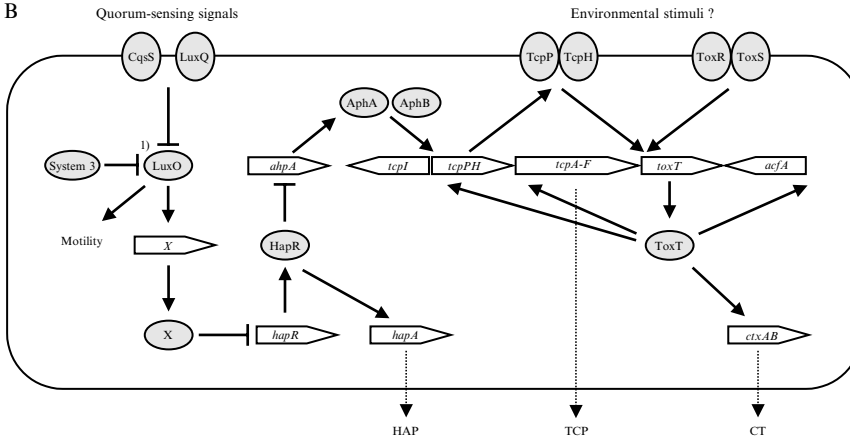

FIG. 9. (A) Proposed quorum-sensing cascade of *V. cholerae*. At least three sensory circuits function in parallel in *V. cholerae*. System 1 is composed of the CqsA-dependent autoinducer (CAI-1, pentagons) and its sensor, the histidine kinase CqsS. System 2 is composed of the LuxS-dependent autoinducer (AI-2, triangles) and its sensor LuxPQ. Information from both systems is channelled through LuxU to LuxO by phosphorelay (''P'' in a circle) in a similar manner to that described for *V. harveyi* in Fig. 8. An additional intracellular regulatory system (System 3) is thought to influence LuxO phosphorylation. Phospho-LuxO is suggested to repress *hapR* expression by activating the repressor (X). HapR then regulates the expression of genes including the virulence regulon encoding cholera toxin. Reproduced from Miller *et al.* (2002). (B) Regulation of *V. cholerae* virulence. Solid arrows denote positive effects while solid T-bars denote negative effects. At low cell density, phospho-LuxO is active and induces motility in addition to repressing *hapR* expression via activation of the repressor X. HapR represses *ahpA*; thus, in the absence of HapR, AphA is made which together with AphB activates *tcpPH* expression. In concert with ToxRS, TcpPH activates *toxT*, resulting in expression of the virulence factors TCP and CT. Activation of *toxT* is also thought to be influenced by environmental signals sensed by ToxRS or TcpPH. In contrast, at high cell density, LuxO is dephosphorylated and inactive due to the accumulation of autoinducer signals. This leads to *hapR* expression and, in turn, repression of *tcpP* alongside activation of *hapA*, resulting in production of the Hap protease. (1) indicates that the system 3, although reported to be intracellular, acts in a fashion similar to systems 1 and 2. Based on information presented by Cotter and DiRita (2000), Kovacikova and Skorupski (2002), Miller *et al.* (2002), and Zhu *et al.* (2002).

of *V. cholerae* strains are nonluminescent. Nevertheless, light production has been reported for some nonpathogenic isolates, and some nonluminescent strains contain remnants of luciferase genes (Palmer and Colwell, 1991). This, and the presence of most of the components required for *luxCDABE* expression, makes the *V. harveyi* luciferase operon an ideal reporter for the analysis of quorum-sensing–dependent gene expression in *V. cholerae*. Indeed, the cosmid pBB1 which carries

the *V. harveyi luxCDABE* operon has proven an invaluable tool (Miller *et al.*, 2002). Initial experiments established that *V. cholerae* containing this cosmid expresses this operon in a cell density-dependent manner, as expected for a reporter controlled by quorum sensing. Furthermore, *V. cholerae luxO* and *hapR* mutants display constitutively bright and dark phenotypes, respectively, just as their corresponding *V. harveyi* counterparts, demonstrating that the *luxCDABE* operon is controlled by elements already known from the *V. harveyi* quorum-sensing system.

The subsequent analysis of quorum sensing in *V. cholerae* by Miller *et al.* (2002) involved the construction of various single, double, or triple mutants as well as random mutagenesis. However, comparisons of quorum sensing in *V. harveyi* and *V. cholerae* should consider the possibility that differences in the way the *lux* operon is controlled in both species may exist. Production of HapR, which activates the *lux* operon, is negatively regulated by LuxO in *V. cholerae*. Given the fact that phospho–LuxO acts as an activator of RpoN-dependent promoters in *V. harveyi* (Lilley and Bassler, 2000), it can be postulated that *V. cholerae* LuxO is required for the expression of an as yet unknown repressor of *hapR* expression. Interestingly, LuxO-dependent regulation of $luxR_{Vh}$ has also been demonstrated for *V. harveyi* (Miyamoto *et al.*, 2003). However, whether this is a major regulatory mechanism for *luxCDABE* expression in this organism remains unclear, given that in the *V. harveyi* wild-type, addition of exogenous AI-1 has a dramatic effect on (LuxO-mediated) bioluminescence (Cao and Meighen, 1989; Bassler, 1999) but no effect on $luxR_{Vh}$ transcription (Miyamoto *et al.*, 1996).

c. The V. cholerae *quorum-sensing system 1.* The *V. cholerae* genome contains a putative hybrid sensor kinase gene, originally annotated ''*luxN*'' (Heidelberg *et al.*, 2000; see supplementary data www.nature.com; www.tigr.org) but now renamed CqsS (*C*holerae *q*uorum-*s*ensing *s*ensor; Miller *et al.*, 2002). CqsS shows no similarity to the N-terminal sensory domain of the *V. harveyi* LuxN protein. Furthermore, no *luxM* homologue is present in the *V. cholerae* genome. This raised the question whether CqsS senses a signal different from AI-1. The enzyme responsible for the production of this signal has been identified and termed *CqsA* (*C*holerae *q*uorum-*s*ensing *a*utoinducer). Miller *et al.* (2002) reasoned that if *cqsS* is involved in the quorum-sensing control of the *luxCDABE* reporter, a *luxS–cqsA* double mutant should be dark, because in the absence of autoinducers, both sensors, CqsS and LuxQ, should remain in the kinase state, leading to the phosphorylation of

LuxO and therefore repression of HapR. To isolate the postulated autoinducer synthase, the authors first mutagenized a *V. cholerae luxS* mutant with Tn5 and then introduced the *luxCDABE* reporter operon. Among the isolated dark strains, those responding to a diffusible compound produced by the AI-2–negative parent strain were further analyzed and shown to contain transposon insertions within the *cqsA* gene, originally annotated as VCA0523 in the *V. cholerae* genome database (www.tigr.org). Interestingly, *cqsA* is located adjacent to *cqsS* but transcribed in the opposite direction.

Cross-feeding experiments using *cqsA–luxS* and *cqsA–luxQ* double mutants as sensors and culture supernatants from wild-type *luxS* and *cqsA* mutants as a source for the signal molecules verified that *cqsA* encodes an enzyme responsible for the production of a diffusible signal, termed CAI-1 (*C*holerae *a*uto*i*nducer 1). Interestingly, the cross-feeding experiments also revealed that the response of *V. cholerae* to CAI-1 is approximately 100 times stronger than to AI-2 (although this result may reflect the actual concentrations of CAI-1 and AI-2 in the culture fluid). Analysis of *cqsS* and *cqsA* single and double mutants exhibited the same phenotype as the corresponding *V. harveyi* mutants (*luxM* and *luxN*), confirming that CqsS represents the CAI-1 sensor: Inactivation of *cqsA* results in a constitutively dark phenotype, because in the absence of CAI-1, CqsS is locked in the kinase state which overrides the phosphatase activity of system 2 (and also system 3; see Section IX.A.3.e) at high cell densities. In contrast, a *cqsS* and also a *cqsS–cqsA* double mutant display a cell density-dependent expression of the *lux* reporter, because system 2 (and also system 3; see following) is still functional and light production is not affected by signals from either CqsA or CqsS.

The chemical structure of CAI-1 is still unknown. Miller *et al.* (2002) (and also the annotation of VCA0523 in the genome database) suggests that CqsA acts as an aminotransferase. Aminotransferases (or transaminases) are enzymes which transfer amino groups from a donor, generally an amino acid, to an acceptor, generally a 2-oxo acid. However, further analysis revealed that CqsA is more similar to α-oxoamine synthases (Winzer *et al.*, unpublished), a small group of PLP-dependent enzymes that catalyze decarboxylative condensations of amino acids and CoA-activated acyl moieties (Alexeev *et al.*, 1998). Members of this group share certain features with aminotransferases, particularly with the subgroups (classes) I and II. Thus, it is not surprising that attempts to synthesize CAI-1 *in vitro* using cell extracts and SAM as a substrate have failed (Miller *et al.*, 2002). Nevertheless, the reaction is likely to require common precursors since expression of *cqsA* in *E. coli* leads to the production of abundant CAI-1.

d. Multiple signal transduction circuits. In addition to system 1, Miller *et al.* (2002) also analyzed *lux* operon expression in *V. cholerae* mutants of system 2, carrying defects in *luxS*, *luxP*, *luxQ*. As for *V. harveyi*, inactivation of *luxP* and *luxQ* in *V. cholerae* does not abolish cell density–dependent regulation (i.e., system 1 is dominant). The same is true for a *luxS* mutant. However, in comparison to the wild-type, light production in *luxQ* and *luxP* mutants is induced at lower and higher cell densities, respectively, suggesting that the relative ratio of kinase and phosphatase activity has been altered.

LuxU is required for signal transduction from CqsS and LuxQ, since the constitutive dark phenotype of *cqsA–luxQ* and *csqS–luxP* double mutants changes to cell density–dependent light production in the respective *luxU* triple mutants (*luxU–cqsA–luxQ* and *luxU–cqsS–luxP*, respectively). CqsS and LuxQ, respectively, are locked in the kinase mode in the *cqsA–luxQ* and *cqsS–luxP* double mutants, leading to the constitutive phosphorylation of LuxO and thus repression of the *lux* operon. However, the *luxU* mutation prevents LuxO phosphorylation in the triple mutants. Furthermore, the cell density–dependent light production observed for a single *luxU* mutant is nearly identical to those observed with the triple mutants and as has been stated, a *luxO* mutant is constitutively light. The cell density–dependent bioluminescence of *luxU* mutants therefore indicates that, in contrast to the situation in *V. harveyi*, at least one additional *V. cholerae* signaling circuit regulates the *luxCDABE* reporter in a cell density–dependent manner via LuxU-independent phophorylation of LuxO. Further evidence for at least one more signaling system comes from system 1 and system 2 double mutants. For instance, a *cqsS–luxQ* double mutant is not constitutively bright as expected (due to the lack of LuxO phosphorylation) but still exhibits density-dependent light production. Miller *et al.* (2002) hypothesized that the sensory input to system 3 in *V. cholerae* is an intracellular signal (but see Section IX.A.3.e).

One aspect that requires more attention in the future is the interaction of LuxP with LuxQ. A *cqsS–luxP* double mutant is dark, probably because LuxQ is locked in the kinase mode and therefore, in the absence of CqsS, overrides the influence of system 3 on LuxO. However, a *cqsS–luxS* double mutant, which is expected to produce the same phenotype, exhibits a phenotype very similar to that of the wild-type, indicating that LuxQ kinase activity cannot override the input of system 3 in this background. So it appears that the presence of LuxP, even in the absence of AI-2, has some effect on LuxQ kinase activity. It is therefore possible that LuxP recognizes ligands different from AI-2 (Fig. 9).

e. Quorum sensing and virulence. Zhu *et al.* (2002) demonstrated that LuxO-mediated repression of *hapR* is required for the expression of TcpP-dependent virulence genes. Since the quorum-sensing systems 1 and 2 both influence the level of Phospho–LuxO, Miller *et al.* (2002) determined the impact of these systems on the virulence of *V. cholerae* in infant mouse colonization assays, as well as the *in vitro* production of cholera toxin (CT) and toxin-coregulated pilus (TCP). Various mutants carrying defects in either system 1 or system 2 or in both systems showed no significant differences to the wild-type in these assays, whereas a *luxO* mutant is significantly attenuated in its ability to colonize infant mice and does not produce detectable levels of CT or TCP. However, at least one system remains operational in the system 1 and 2 mutants and, therefore, it was argued that all three signaling circuits must be inactivated in order to establish the *luxO* phenotype.

Interestingly, HapR-mediated repression of CT production was only observed when *hapR* expression was (artificially) induced early during growth (up to 2 h) but not after late induction (4 h and later) (Zhu *et al.*, 2002). As has been stated, the level of phospho–LuxO *in vivo*, and thus the level of *hapR* repression, is mediated via all three identified signal circuits, and as a result depends on the extracellular concentrations of CAI-1 and AI-2, and possibly other external and internal parameters. Therefore, it can be speculated that the timing of HapR production *in vivo* may be an important factor in the modulation of virulence gene expression.

Miller *et al.* (2002) suggested that the function of the *V. cholerae* quorum-sensing system is to repress virulence gene expression at high autoinducer concentrations. Indeed, the addition of spent culture fluid from the wild-type prevented the production of TCP and CT by a *cqsA–luxS* mutant (which is defective for both autoinducers) and reduced the expression of a *tcp–lacZ* fusion approximately 10-fold. To provide further evidence that this repression was caused by the autoinducers present in the wild-type culture fluid, the effect of wild-type and *cqsA–luxS* supernatants was compared. *tcp-lacZ* expression in a *cqsA–luxS* mutant was approximately 4-fold reduced in the presence of wild-type culture fluid, but this effect was not observed in a *cqsA–luxS–hapR*-negative background. This further supports the idea that the presence of autoinducers (and not other compounds present in the culture fluid) represses virulence factor production mediated via HapR. However, the possibility remains that other molecules, the production of which depends on the presence of CAI-1 or AI-2 (and which therefore are absent in a *cqsA–luxS*-negative background), are responsible for repressing virulence factor production

via HapR. Pure preparations of CAI-1 and AI-2 are required to confirm these findings.

These experiments were interpreted to support the hypothesis that the sensory input to system 3 is an intracellular signal (Miller *et al.*, 2002). The level of *tcp-lacZ* expression in the presence of culture fluid derived from a *cqsA–luxS* double mutant is the same in a *cqsA–luxS* double mutant and a *cqsA–luxS–hapR* triple mutant. It has been argued that if the system 3 signal was present in this supernatant, the double mutant should have been downregulated in *tcp-lacZ* expression due to the changes in LuxO phosphorylation state (compared with the *hapR*-negative background, where *tcp-lacZ* expression is expected to be maximal). However, this interpretation contradicts the idea that in the absence of their cognate autoinducers, CqsS and LuxQ are both locked in the kinase state, which should override any influence exerted by system 3. Specifically, it has been demonstrated by Miller *et al.* (2002) that a *cqsA* mutant containing the *luxCDABE* reporter is dark, which means that even system 2 and system 3 together are not strong enough to override the effects of constitutive LuxN kinase activity. Therefore, the absence of a third extracellular signal molecule cannot be ruled out.

4. *Signal Integration and AI-2 Function*

What is the advantage of using two different diffusible quorum-sensing signal molecules and converging signal transduction pathways? It has been suggested that accumulation of AI-1 enables *V. harveyi* to ''count'' its own numbers, whereas AI-2, a molecule known to be produced by many other bacteria, may be employed for interspecies communication (Bassler, 1999; Bassler, 2002). The same function has been proposed for CA-1 and AI-2 produced by *V. cholerae* (Miller *et al.*, 2002). For instance, by monitoring intra- and interspecies signals, bacteria may be able to ''estimate'' its proportion in a mixed population (Bassler *et al.*, 1997). However, since the signal transduction pathways of *V. harveyi* and *V. cholerae* converge, the inputs from both autoinducers are integrated and, at the level of LuxO, information about their individual concentrations is lost (with the possible exception of extreme conditions; see following). In other words, although both autoinducers contribute to LuxO dephosphorylation, their relative contributions are not known to the cell at this level and, therefore, specific responses to a given concentration of either one of the two autoinducers cannot be mediated via LuxO. However, the possibility remains that LuxN and/or LuxQ specifically interact with other phosphorelay systems.

Mok *et al.* (2003) have recently suggested that ''the *V. harveyi* quorum sensing circuit may function as a 'coincidence detector'[8] that discriminates between conditions in which both autoinducers are present and all other conditions.'' The authors used random *lacZ* insertions in a *V. harveyi luxS* mutant to identify genes which are regulated by AI-2. A metalloprotease gene was found to be positively regulated by AI-2, whereas nine other genes, including several putative type III secretion genes, were repressed. Interestingly, all these genes were also controlled by AI-1, and the regulation was mediated via LuxO. Using various ratios of AI-1– and AI-2–containing culture supernatants, obtained from *luxS* and *luxLM* mutants, respectively, Mok *et al.* (2003) demonstrated that both autoinducers act synergistically: In a *luxLM–luxS* double mutant, strong activation of metalloprotease transcription and bioluminescence as well as significant inhibition of the other responsive genes only occurred if both autoinducers were added together. In the presence of just one of the two autoinducers, only comparatively small effects were observed, although the influence of AI-1 on metalloprotease and bioluminescence expression was stronger than that of AI-2. When culture supernatants containing either AI-1 or AI-2 were mixed in various ratios, significant changes in transcription were observed for ratios ranging from 9:1 to 1:9. Finally, synthetic AI-1 and *in vitro* synthesized AI-2 were used to mimic autoinducer concentrations equal or higher to those achieved in wild-type cultures. Again, individually, each autoinducer had only a small effect on gene expression, whereas in combination strong changes were observed. Interestingly, one exception was the effect of AI-1 on bioluminescence, which was induced to 33% of the maximal level in the presence of synthetic AI-1 alone, but only to 0.01% in the presence of *in vitro*–synthesized AI-2. In conclusion, Mok *et al.* (2003) suggested that the *V. harveyi* quorum-sensing system can discriminate between no autoinducer, AI-1 only, AI-2 only, and AI-1 plus AI-2 together, and that these four conditions must be represented by four widely separated levels of phosphorylated LuxO. However, the authors pointed out that not all four test conditions are necessarily meaningful and that *V. harveyi* may use its quorum sensing system only to distinguishing the ''coincidence state'' (both autoinducers present, luxO completely dephosphorylated) from all other conditions, where the cells still contain an active fraction of phosphorylated LuxP.

So how do these observations affect the concept of AI-2–dependent cross-species communication? The data presented by Mok *et al.*

[8] An analogy to those described for neural networks.

(2003) clearly demonstrate that the organism can still respond to changes in the ratio of AI-1 and AI-2. For instance, transcriptional activation of the metalloprotease-*lacZ* fusion increased from 1 unit in the presence of AI-1 alone to 5 units in the presence of a 9:1 ratio of supernatants containing only AI-1 and AI-2, respectively, before reaching a plateau of 10 units at a 7:3 ratio. However, similar levels of gene expression can result from different ratios (e.g., activation of the metalloprotease-*lacZ* fusion was approximately half-maximal when AI-1– and AI-2–containing supernatants were either mixed in a 9:1 or a 1:9 ratio), supporting the idea that this is the consequence of comparable LuxO phosphorylation and that information on the individual concentration of AI-1 and AI-2 is lost at this level. AI-2–based interspecies communication could therefore be limited to conditions where the required minimal AI-2 concentration to reach the coincidence state (or maximal expression/repression within this state) is present.

It is also interesting that in the case of the bioluminescence, high expression levels (33%) were already reached if AI-1 was present, whereas AI-2 failed to show the same effect. Earlier work from Bonnie Bassler's laboratory had already indicated that in *V. harveyi*, timing and potency of the quorum-sensing system 1 and 2 are likely to be different, and several observations suggested that LuxN/AI-1 exert a much greater effect on the phosphorylation level of LuxO and thus bioluminescence than LuxQ-LuxP-AI-2 (Freeman and Bassler 1999b; Freeman *et al.*, 2000). For example, a *luxN* mutant which is ''locked'' in the phosphatase state exhibits constitutive light production, apparently by overriding LuxQ kinase activity completely. Similarly, a *luxN* mutant locked in the kinase conformation is constitutively dark because LuxN kinase activity efficiently overrides LuxQ phosphatase activity. Furthermore, a *luxQ* deletion mutant exhibits light production at a lower population density when compared with the wild-type, whereas a *luxN* deletion mutant requires a higher cell density. This indicates that under the conditions used, LuxN and LuxQ switch from kinase to phosphatase activity at different cell densities. Hence, it is possible that in the natural habitat of *V. harveyi*, expression of bioluminescence and other quorum-sensing regulated phenotypes, although mainly determined by AI-1, may be modulated by the variable AI-2 concentration produced by a consortium of bacteria. A similar situation is present in *V. cholerae*, where the dominance of system 1 has also been demonstrated (Miller *et al.*, 2002), and CAI-1 appears to be 100 times more potent than AI-2 in activation of *hapR*-dependent *luxCDABE* expression. However, it must be kept in mind that these conclusions are based on the analysis of mutants, where, due to the inactivation or modification of either

one of the sensor kinases, the signal transduction process has been perturbed. Although these data imply a major role for system 1 at least for the expression of bioluminescence, AI-2 may play a more important role in the natural habitats of *V. harveyi* and *V. cholerae*. At least for *V. harveyi*, it has been shown that addition of boric acid leads to a significant increase in bioluminescence (Chen *et al.*, 2002). This effect is dependent on the presence of *luxS* and *luxP*, suggesting that increased formation of AI-2 (a furanosylborate diester) is responsible for *luxCDABE* activation (see Section IV. C for more details). Thus, it is possible that in the presence of borate, which is present at high concentrations in seawater, the AI-2 system is far more potent than under standard laboratory conditions. An alternative interpretation, namely, that the AI-2 system acts as a ''borate sensor,'' will be discussed in Section X.D.

The concentration of autoinducer is just one of many signals that a bacterium receives in a given environment and it makes sense for the cells to integrate all relevant information before initiating a specific response (Withers *et al.*, 2001). Although the autoinducer concentration may signal an appropriate cell density, other environmental parameters, such as temperature and pH, osmolarity as well as the nutritional status have to be considered. The quorum-sensing regulated phenotypes of *V. harveyi* and *V. cholerae* are no exception. As has been described, the expression of *luxCDABE* in *V. harveyi* is influenced by physiological parameters, in particular, the nutritional status of the cell, as represented by cAMP levels and methionine metabolism. In *V. cholerae*, a third system influences the LuxO phosphorylation status. If this system is indeed dependent on an intracellular signal as suggested by Miller *et al.* (2002), it may convey similar information. In addition, CRP/cAMP is known to negatively regulate the genes encoding CT and TCP (Skorupski and Taylor, 1997; Kovacikova and Skorupski, 2001) and the ToxR–ToxS and the TcpP–TcpH signaling circuits (Fig. 9B) are thought to detect and respond to environmental stimuli (Cotter and DiRita, 2000). Nevertheless, it is noteworthy that bioluminescence in *V. harveyi* can be induced prematurely through the addition of exogenous autoinducer, whereas this is not possible for some quorum sensing-regulated genes in other bacteria, e.g., *P. aeruginosa*, which are strictly controlled in growth phase-dependent fashion (Diggle *et al.*, 2002).

5. *The Quorum-Sensing Systems of* Vibrio vulnificus *and* Vibrio parahemolyticus

AI-2 production in *V. vulnificus* has been observed in LB medium, minimal medium, at room temperature or 37°C (McDougald *et al.*, 2001). AI-2 activity in the culture supernatant is maximal when cells

enter the stationary phase. Addition of glucose inhibits accumulation of AI-2 activity, whereas production is stimulated under conditions of starvation.

The genome sequences of *V. vulnificus* and *Vibrio parahemolyticus* have recently become available (http://genome.nhri.org.tw/VV/index.html and http://www.gen-info. osaka-u.ac.jp). They contain homologues of all genes known to be required for AI-2 signal transduction, (organized as in *V. harveyi* and *V. cholerae* with *luxP* upstream of *luxQ*, and the phosphorelay protein *luxU* downstream of *luxO*). This suggests that AI-2 may play a role in *V. vulnificus* quorum sensing and possibly cross-species communication with other members of the genus *Vibrio*. Indeed, Kim *et al*. (2003) demonstrated that *V. vulnificus luxS* mutants are attenuated in mice and that the *luxS*/AI-2 system appears to play a role in virulence gene regulation.

B. AI-2–Dependent Signaling in Other Bacteria

Evidence that AI-2–based quorum sensing is not restricted to members of the genus *Vibrio* has been provided by a number of publications. In most of these studies, potential target genes or phenotypes regulated by AI-2 have been identified either through the analysis of *luxS* mutants or after the incubation of cells with conditioned medium (CM) either containing or lacking AI-2 activity. In principle, these are valid approaches which have been used successfully in the past to identify components of various quorum-sensing systems. In the case of AI-2, however, these procedures are complicated by the fact that inactivation of *luxS* will not only abolish the production of a potential signal molecule, but also, depending on growth condition and species tested, influence the metabolism of key intermediates to a greater or lesser extent. As a consequence, gene expression and culture fluid composition are likely to change, a complication which should be kept in mind when assessing the data presented below (Winzer *et al.*, 2002b). Final proof for a cell-to-cell signaling system always requires the use of a purified, defined signal molecule, as well as the identification of its receptor and the events that link signal perception with gene expression. For most bacteria where a role of AI-2 in cell-to-cell signaling has been proposed, none of these requirements has yet been satisfied.

1. *Enteric Bacteria*

a. Enterohemorrhagic and enteropathogenic E. coli. AI-2–based signaling has been reported by Sperandio *et al*. (1999) to control gene expression in enterohemorrhagic (EHEC) and enteropathogenic (EPEC)

E. coli. In these organisms, the LEE pathogenicity island is required for the formation of the ''attaching and effacing'' (A/E) lesions on gut enterocytes. The LEE pathogenicity island consists of five major operons encoding a type III secretion system, intimin adhesin, intimin receptor (*tir*), as well as other secreted proteins and proteins of regulatory function. Evidence provided by Sperandio *et al*. (1999) suggests that the *LEE1, LEE2, LEE3*, and *tir* operons are directly or indirectly activated when AI-2 accumulates in the culture supernatant. *lacZ* reporter fusions were created for all five operons and introduced either as single chromosomal copies into the *E. coli* K12 strain TE2680 or on multicopy plasmids into EHEC 86-24h-11 and the *E. coli* K12 strain MC4100. *LEE1*- and *LEE2-lacZ* fusions in all backgrounds were induced between 2- to 4-fold after the addition of CM from *E. coli* O157:H7 when compared with fresh LB medium. *LEE3-lacZ* was also induced 2-fold in EHEC 86-24h-11. However, induction of *LEE3-lacZ* and *tir-LacZ* in the *E. coli* K12 background was only observed in the presence of heterologously produced Ler, a regulatory protein encoded by the first gene of the *LEE1* operon. These results suggested the presence of an inducing signal in the CM from *E. coli* O157:H7, which acted directly on *LEE1* and *LEE2* but required Ler for the activation of *LEE3* and *tir*. More importantly, induction of the *LEE1*- and *LEE2-lacZ* fusions in the K12 background was not observed with CM from *E. coli* DH5α, a strain that cannot produce AI-2 due to a frameshift in *luxS* (Surette *et al*., 1999), but induction was observed when the same strain expressed the *luxS* gene from EHEC 86-24h-11 and produced AI-2 activity. These data suggested that AI-2 was responsible for the induction of the *lacZ* fusions in the presence of CM. Furthermore, a *luxS* mutant of the EPEC strain E2348/69 did not produce AI-2 activity and CM from this strain, in contrast to that of the parent strain, did not activate the *LEE1* and *LEE2* operons. Consistent with *lacZ* reporter results, this strain also showed decreased levels of secreted protein.

In EPEC, *ler* is regulated by a protein termed Per (Mellies *et al*., 1999), the gene coding for which was also shown to be 2-fold upregulated in the presence of CM (Sperandio *et al*., 1999). In EHEC, a LysR-type regulator termed QseA acts directly or indirectly on the distal of the two LEE1 promoters (Sperandio *et al*., 2002a). In a *luxS* mutant, *qseA* transcript levels were 23-fold reduced in comparison to the parent strain, whereas a *qseA-lacZ* fusion showed the same trend but was only 3.5-fold downregulated (the discrepancy in the levels was attributed to multicopy effects). These fusions were 2- and 2.5-fold activated by CM from parent strain and complemented *luxS* mutant, respectively, but not by CM derived from the *luxS* mutant, again suggesting that

AI-2 may be responsible for this activation. A similar system appears to operate in EPEC, because a *qseA* mutation in this background like its EHEC counterpart was drastically reduced in the secretion of Tir, EspA, and EspB, and showed reduced transcription of chromosomal *LEE1* and *LEE2-lacZ* fusions (Sperandio *et al.*, 2002a).

The data summarized here are all consistent with a role of AI-2 in LEE operon activation. It is important to note, however, that the experiments by Sperandio *et al.* (1999) also demonstrated the significant influence of CM composition: while the *V. harveyi* BB170 bioassay indicated that CM from *E. coli* MC4100 contained nearly four times more AI-2 activity than that of *E. coli* DH5α expressing the O157:H7 *luxS* gene, the latter was still more effective in the stimulation of a single chromosomal *LEE1-lacZ* fusion in TE2680 (Sperandio *et al*, 2001).

It has also been suggested that AI-2–based quorum sensing is involved not only in type III secretion but acts as a global regulatory mechanism in EHEC O157:H7 (Sperandio *et al.*, 2001). A comparison of RNA profiles from this strain and its isogenic *luxS* mutant by gene array analysis revealed that 404 transcripts, representing approximately 10% of the genes on the array, were changed at least 5-fold in their levels. Fifty-eight percent of these genes were upregulated in the wild-type and 42% were downregulated (Sperandio *et al.*, 2001). Seven hundred and thirty-six genes were changed at least 2-fold in their expression. Also, because an *E. coli* K12 gene array which lacks 1.34 Mb of DNA found in EHEC was used for this study, many more genes might be affected.

As might be anticipated for changes on this scale, many of the differentially expressed genes are known to fulfill important functions, for instance, in growth and the regulation of cell division, SOS response, motility, or metabolism. However, for the vast majority of the observed changes, it is not clear whether they originated from a metabolic or a signaling defect. Sperandio *et al.* (2001) pointed out that their gene array study was not a definite analysis of quorum sensing in *E. coli*, because the data were obtained from a *single* analysis at a *single* timepoint in the growth phase and only for a single set of conditions. Instead, the results were used to identify a set of promising candidate genes, which were further analyzed through the application of independent techniques, such as the use of *lacZ*-fusions, Northern and Western blots, or phenotypic assays, which were generally in agreement with the gene array experiment. Furthermore, complementation of the chromosomal *luxS* mutation by a plasmid-borne copy of the gene restored the characteristics of the parent strain to a greater or lesser degree, indicating that the observed changes were not caused by second site mutations.

Several genes involved in cell division such as *ftsQ* or *ftsE* were upregulated in the *luxS* mutant, whereas *minE* (involved in cell division topology) and *sulA* (a cell division inhibitor) were significantly downregulated (13- and 25-fold, respectively). Together with the observed upregulation of genes important for cell growth, e.g., several genes encoding ribosomal proteins, this could explain the increased growth rate of the EHEC *luxS* mutant in DMEM medium (the same medium was used for the gene array analysis). The *luxS* mutant had an average generation time of only 32 min, whereas the parent strain and the complemented *luxS* mutant grew much slower, showing generation times of 55 min and 101 min, respectively. Interestingly, the growth rate of the *luxS* mutant was significantly reduced in the presence of CM from the parent strain but not the mutant, suggesting that AI-2 was responsible for this effect. The very slow growth observed for the complemented mutant was explained by AI-2 overproduction in the presence of multicopy *luxS* (Sperandio *et al.*, 2001). However, overproduction of LuxS does not increase AI-2 levels significantly in *S. typhimurium* (Beeston and Surette, 2002) or *E. coli* stains MG1655 or DH5α (Hardie *et al.*, 2003).

The gene array data also indicated that genes required for the SOS response were drastically downregulated in the *luxS* mutant (e.g., *recA*, *uvrA*, and *sulA*). In agreement with this observation, Sperandio *et al.* (2001) found that the H-19B phage encoded *stx2* toxin, known to be induced together with the late genes of the phage upon stimulation of the SOS response, was downregulated in a *luxS* mutant.

The effect of *luxS* mutation on motility was also examined in more detail, as the gene array data indicated that the expression of genes required for flagellar biosynthesis and chemotaxis were downregulated in the EHEC *luxS* mutant. In *E. coli* and *S. typhimurium*, the genes required for flagella synthesis are transcribed in hierarchical order, and accordingly, genes have been grouped into three major classes. Northern blots for *fliA* and *fliF* and *lacZ* fusions with promoter regions of flhD (class I), fliA (class II), fliC (class III), and motA (class III) confirmed the observed downregulation, which, when tested for the *lacZ* fusions, was reversed in the presence of plasmid-borne *luxS*.

In the EHEC *luxS* mutant, addition of AI-2 containing CM from wild-type, but not AI-2 free CM of the mutant, stimulated *motA–lacZ* transcription. A similar result was obtained with motility plates, where both wild-type and complemented *luxS* mutant formed small halos on DMEM semi-solid plates, whereas no halo was detectable for the *luxS* mutant. However, while the incorporation of CM from the *luxS* mutant was reported to have no effect (although the respective figure

shows that it increased the halo size of the complemented mutant), addition of CM from the wild-type resulted in a large halo of a similar size for both wild-type and mutant and promoted only a very small halo with the complemented mutant. Sperandio *et al.* (2001) suggested that AI-2 present in wild-type CM, together with the overproduction of AI-2 by the complemented mutant, might have reduced growth by this strain even further. In agreement with transcriptional data and the motility plate assays, the *luxS* mutant produced less flagellin and fewer flagella and also showed a reduced swimming speed, probably because it tumbles more often. Similar data were obtained in a later study for an EPEC *luxS* mutant, which was unable to swim and produce flagella when grown in DMEM medium (Giron *et al.*, 2002). However, motility was restored in the presence of CM derived from the wild-type grown in DMEM. Furthermore, the *luxS* mutant was motile when grown in LB medium.

A two-component system termed QseBC (originally termed YgiYX), which is present in both EHEC and *E. coli* K12, appears to mediate the *luxS*-dependent regulation of motility (Sperandio, 2002b). *qseB* encodes a putative response regulator and *qseC* the corresponding sensor kinase. A *qseB–lacZ* fusion was downregulated in the *luxS* mutant in comparison to the EHEC 86–24 parent and the complemented strain, but only 2.5-fold in contrast to the 17-fold reduction observed for the transcripts in the gene array study. This was attributed to the presence of *qseB–lacZ* on a multicopy plasmid. Again, CM from the wild-type stimulated the expression of this fusion but less than 2-fold, whereas CM from the *luxS* mutant had no effect. CM from the complemented mutant, reported to contain more AI-2 (2-fold), stimulated the fusion 7-fold. Sperandio *et al.* (2002b) concluded that the reporter was activated by AI-2. A *qseC* mutant expressed considerably less flagellin, formed smaller halos on motility plates, and was drastically downregulated in *flhD*-, *fliA*-, *fliC*-, and *motA-lacZ* expression. Thus, a EHEC *qseC* mutant has similar characteristics with regard to flagellar gene expression and motility as the EHEC *luxS* mutant. However, the motility defect of the *luxS* mutant could be restored by the addition of CM from the parent strain, which was not the case with a *qseC* mutant and it was therefore concluded that the latter cannot respond to AI-2 (Sperandio *et al.*, 2002b). Further experiments demonstrated that QseBC also activates the flagella regulon in the *E. coli* K12 strain MC1000, and that this regulation, at least for *fliA*, is dependent on the presence of functional FlhDC. However, in a study by Oshima *et al.* (2002a) where all two-component systems of *E. coli* K12 were individually inactivated and the resulting mutants analyzed for changes in their transcript profiles,

a deletion of this system was not reported to alter expression of motility-related genes significantly (see also supplementary data at http://ecoli.aist-nara.ac.jp/xp_analysis/2_components).

Since the EHEC *qseC* mutation had no effect on the secretion of EspA and EspB via the type III secretion system encoded by the LEE pathogenicity island, and *qseBC* overexpression did not affect the transcription of the LEE operons, it was concluded that either QseC is one of several AI-2 sensors and dedicated to the regulation of flagellar genes only, or, alternatively, that the *qseBC* system itself is regulated by a master system in response to AI-2. Both scenarios are possible. However, from the data presented so far, it can only be concluded that a factor which is present in CM of the wild-type, but missing (or reduced) in the CM of a *luxS* mutant, induces the *qseBC* system. Whether this factor turns out to be AI-2 remains to be established. Alternatively, an inhibitory factor could be present in CM from *luxS* mutants. These considerations also apply to all other results obtained with CM. Indeed, a recent study by Sperandio *et al.* (2003) demonstrates that *in vitro* synthesized AI-2 does not activate motility and type III secretion; they also speculate that an unknown autoinducer, AI-3, the production of which also depends on *luxS*, is responsible for the activation seen in previous studies.

b. E. coli *K12.* DeLisa *et al.* (2001c) used a different approach to identify AI-2–regulated genes in *E. coli* K12. In their study, a *luxS* mutant was grown to an OD_{600} of 1, the culture split evenly, and the cells resuspended in CM prepared from either wild-type or *luxS* mutant and thus either containing or lacking AI-2 activity. Differences in the transcript profile after 20 min of incubation were then analyzed using DNA microarrays. In total, 242 genes (5.6%) of the genome were at least 2.3-fold upregulated (154 genes) or downregulated (88 genes) in the presence of wild-type-derived CM. Twenty-three genes were upregulated more than 5-fold, notably *frwC* (33-fold; similar to enzyme II component of the fructose PTS) and two other genes of unknown function, *yeiK* (25.4-fold) and *yidS* (21.3-fold). On the other hand, 25 genes were more than 5-fold repressed, including *thiH* (19.2-fold; involved in thiamin synthesis), *b2650* (27.8-fold), and *b2247* (15.2-fold). Many genes involved in cell division, DNA processing, and morphological processes were differentially expressed. Other changes included genes known or suggested to be involved in regulatory processes. For instance, *ygeV*, encoding a putative NtrC-like protein with similarity to LuxO, was upregulated in the presence of wild-type CM (3.6-fold), as well as *yhbH*, encoding a putative σ^{54} modulating protein (2.5-fold).

DeLisa *et al.* (2001c) therefore postulated that *E. coli* may employ σ^{54} during quorum sensing in a fashion analogous to that of *V. harveyi*.

In a previous study, DeLisa *et al.* (2001a) had suggested that a LuxR-type regulator, SdiA, activated the p2 promoter of the *ftsQAZ* cell division gene cluster in response to AI-2 contained in CM from the wild-type. However, in the gene array study, *sdiA*, *ftsQ*, *ftsA*, and *ftsZ* were relatively unchanged. The latter results are in agreement with data for *E. coli* and *S. typhimurium* provided by Surette and Bassler (1999), who had demonstrated that under the conditions used, AI-2 was not involved in the modulation of SdiA activity in these organisms. DeLisa *et al.* (2001a, 2001c) suggested that these contradictions may be explained by the fact that, under physiological conditions, *ftsQAZ* expression is influenced by the overlapping regulation from the RpoS-dependent p1 promoter.

Surprisingly, the *b1513* operon, which encodes the *E. coli* equivalent of the AI-2 uptake system Lsr in *S. typhimurium* (Taga *et al.*, 2001), was not induced in the presence of AI-2 (see supplementary data for DeLisa *et al.* (2001c) provided at http://www.umbi.umd. edu/∼cab/bentley/AI-2_array.html). This could indicate that either the incubation with AI-2 had not been long enough to activate transcription significantly or that the cells, which had been grown in the presence of glucose, were not in the required physiological state to permit induction of this system. Furthermore, many of the most drastic changes observed for EHEC *luxS* mutants, notably effects on *sdiA*, *minE*, *sulA*, and genes encoding ribosomal proteins, were not observed by DeLisa *et al.* (2001c; supplementary data). Similarly, the vast majority of motility-related genes in *E. coli* K12 were not significantly affected or showed the opposite trend to that previously reported. Whether these differences can be attributed to differences in the quorum-sensing system of both strains, the growth conditions (DMEM versus LB glucose) and thus the physiological state of the cells, or the metabolic effects of *luxS* inactivation remains to be seen. In common with the analysis presented by Sperandio *et al.* (2001), this study only analyzed a single timepoint during growth in a single set of growth conditions. Addition of CM to a *luxS* mutant, in contrast with the direct comparison of parent strain and mutant, reduced but not completely eliminated the possibility that some of the observed changes were caused by the metabolic consequences of *luxS* inactivation rather than AI-2 signaling. The CM of wildtype and *luxS* mutant may well differ in more than just the presence or absence of AI-2, given that the expression of several hundred genes, including many with metabolic functions, was apparently modulated in both studies.

c. S. typhimurium. AI-2 production by *S. typhimurium* has already been described in detail in Section VIII. It has been suggested (Surette and Bassler, 1999) that AI-2–based quorum sensing may be critical when cells undergo the transition from the free-living to the host-associated form, because certain conditions which are encountered by the bacterium upon first interaction with host (high osmolarity, low pH, certain nutrients) are known to favor the production of AI-2. However, in a comprehensive screening study which involved 11,000 mutants, the only genes differentially regulated in *luxS* mutant and wild-type were found to be a putative AI-2 uptake system (described in Section IX. C) and *metE* (Taga *et al.*, 2001). This is puzzling, given the fact that in both *E. coli* K12 and EHEC so many different phenotypes have been attributed to the inactivation of *luxS* or the lack of AI-2. As explained in more detail in Section VI, growth conditions are likely to influence the phenotype of *luxS* mutants. It is possible that more *luxS*-dependent phenotypes will be identified in a system that mimics the *in vivo* situation of animal and human hosts more closely.

A role for AI-2–based quorum sensing in biofilm formation has been suggested by Prouty *et al.* (2002) who reported that a *S. typhimurium luxS*::MudJ mutant was unable to form biofilms on gallstones within 14 days. Whether this was a result of metabolic or signaling defects was not established. Furthermore, in *S. typhimurium* LuxS protein levels were reduced in a *slyA* mutant in comparison with a *slyA* overexpressing strain (Spory *et al.*, 2002). The *slyA* gene is required for full virulence of *S. typhimurium*, but in common with *luxS*, its inactivation influences the level of several other metabolic enzymes and stress-related proteins (Spory *et al.*, 2002). It is therefore not clear whether *slyA* is directly or indirectly involved in the regulation of AI-2–based quorum sensing or whether it also plays a role in the coordination of metabolism. Furthermore, a stationary phase culture of an *S. typhimurium luxS* mutant was not affected in its ability to suppress the growth of wild-type cells introduced after 24 h (Rychlik *et al.*, 2002), indicating that AI-2-dependent signaling is not involved in the inhibition of growth usually seen in stationary phase cultures of this organism. However, earlier timepoints need to be analyzed before final conclusions can be drawn, because the experiment was undertaken in LB medium without glucose where no AI-2 activity remains after 24 h (Beeston and Surette, 2002).

d. Shigella flexneri. *Shi. flexneri* efficiently invades host cells, and after its escape from the phagolysosome, flourishes in the cytosol. Day and Maurelli (2001) demonstrated that inactivation of *luxS*

modulated expression of the virulence gene regulator *virB* but did not attenuate virulence in the Sereny test, which assesses the ability of a strain to induce keratoconjunctivitis in the guinea pig eye. VirB controls the expression of the type III secretion operons *mxi* and *spa* as well as the *ipaA* and *ipaB* genes, which encode proteins delivered by the type III secretion system to the host cell surface. Expression of *spa47*- and *mxiA-lacZ* fusions were not changed in the presence of CM, but an *ipaB* fusion was about 2-fold upregulated in the presence of CM derived from either *Shi. flexneri* or *E. coli* MC4100 stationary phase cultures. A *virB-lacZ* fusion was also upregulated but only in the presence of CM derived from late exponential cultures of *Shi. flexneri* or *E. coli*. This was attributed to the presence of an extracellular factor believed to be AI-2, because *virB-lacZ* activation was not observed with AI-2-free CM from either a *Shi. flexneri luxS* mutant or *E. coli* DH5α. Furthermore, the expression of *virB-lacZ* in wild-type and *luxS* mutant was essentially identical during early exponential and stationary phase, but a peak in expression, observed for the wild-type during the transition from late exponential to stationary phase, was not observed in the mutant. Again, it remains to be seen whether these changes can be attributed to AI-2. Day and Maurelli (2001) suggested that AI-2–based quorum sensing may not be used to control virulence gene expression in bacterial pathogens that colonize host tissues not occupied by normal flora and therefore not containing AI-2.

e. Proteus vulgaris. In *P. vulgaris*, the accumulation of extracellular AI-2 appears to be correlated with swarming (Schneider *et al.*, 2002). AI-2 activity at the edge of a swarming colony was close to the background level during the entire length of the experiment, apart from a short window 3 h after inoculation where the activity increased dramatically. This peak in activity corresponded to the time at which the cells started their swarming migration and may be growth-related, since cell numbers increased more than 100-fold between inoculation and the beginning of swarming migration. The subsequent fall in activity, however, was not correlated with a reduction in cell density and may have been caused by AI-2 degradation. After 5 h, when the cells had completed one cycle of differentiation, migration, and consolidation, there was also a spatial difference in the low remaining AI-2 activity on the swarm plate, which decreased from the center toward the edge of the colony.

However, inactivation of *luxS* did not affect swimming motility, chemotaxis, swarming motility, or the swarming colony pattern. Furthermore, phenotypes such as growth rate or the production of urease,

protease, and hemolysin remained unchanged *in vitro*. Consequently, the authors studied mutant and wild-type under conditions that challenged the survival of the cells by comparing their pathogenicity in a mouse model of urinary tract infection. The *luxS* mutant was not attenuated and the authors concluded that AI-2–dependent signaling was not required for urinary tract infection.

It has been argued in an earlier section that disruption of *luxS* is expected to cause methionine deficiency *in vivo* under sulfur-restricted conditions. If this is true for *P. mirabilis*, should it not have resulted in attenuation in the *in vivo* model? Organic sulfur sources are known to be present in urine (Lentner, 1981) and cysteine-dependent strains of *E. coli* and *Klebsiella* sp. have been isolated from this environment (McIver and Tapsall, 1990), suggesting that organic sulfur compounds would not have been severely limited in the mouse model used.

2. H. pylori *and* C. jejuni

Although it has been speculated that AI-2 may play a role in the regulation of *H. pylori* gene expression, no phenotypes could be attributed to *luxS* inactivation in the two independent studies by Forsyth and Cover (2000) and Joyce *et al.* (2000). Both studies demonstrated that *H. pylori luxS* mutants did not exhibit any growth defects under the conditions tested. Furthermore, inactivation of *luxS*, which completely abolished AI-2 production, did not affect the expression of known virulence factors, such as *vacA* (encoding a vacuolating cytotoxin) which peaks at the onset of stationary phase (Forsyth and Cover, 2000). There was also no difference in urease activity, motility, Cag-induced IL-8 production by Hep-2 cells, or the ability of concentrated supernatants to induce vacuoles in Hep-2 cells (Joyce *et al.*, 2000). Furthermore, no differences in the protein profiles of wild-type and mutant were detectable by 2-D gel analysis. It was suggested that *H. pylori* may produce AI-2 solely to interfere with the communication of other bacteria (Forsyth and Cover, 2000), or that, at least *in vitro*, the presence of other converging signaling pathways similar to those of *V. harveyi* may have masked any obvious phenotypes in a *luxS* mutant.

In *Campylobacter jejuni*, inactivation of *luxS* did not significantly alter growth, the resistance to oxidative stress, or adherence and invasion of Caco-2 cells (Elvers and Park, 2002). However, a *luxS* mutant consistently produced smaller halos in semi-solid agar, suggesting that motility was affected. However, no attempt was made to restore the parental phenotype through the addition of CM or *in vitro*–synthesized AI-2 (Elvers and Park, 2002).

3. *Periodontal Pathogens*

Communication between oral bacteria has been considered essential for initial colonization and the subsequent formation of mixed biofilms on the tooth surface (see Kolenbrander *et al.* [2002] for a review). It is therefore intriguing that AI-2 production has been demonstrated for the periodontal pathogens *Fusobacterium nucleatum* and *Prevotella intermedia* and for some but not all strains of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* (Chung *et al.*, 2001; Fong *et al.*, 2001; Frias *et al.*, 2001; Burgess *et al.*, 2002). Furthermore, certain members of the genus *Streptococcus* which are predominant early colonizers on the tooth surface are either known (*Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus mutans*) or likely (e.g., *Streptococcus oralis* and *Streptococcus sanguis*) to possess *luxS* homologues in their genome. For these reasons, it has been suggested that AI-2 may mediate the communication between various members of mixed oral biofims (Fong *et al.*, 2001; Kolenbrander *et al.*, 2002; McNab *et al.*, 2003). However, there is presently only limited evidence to support this idea. The strains of *S. mitis*, *S. mutans*, *S. oralis*, and *S. sanguis* tested by Frias *et al.* (2001) did not produce detectable amounts of AI-2, and the extracellular AI-2 levels produced by various *F. nucleatum* strains differed dramatically (although this may have been a consequence of the growth conditions, which were obviously quite different from those encountered *in vivo*). Inactivation of *luxS* did not significantly impair mono-species biofilm formation by *S. mutans* on a plastic surface (Wen and Burne, 2002) but resulted in more granular biofims on a glass surface (Merritt *et al.*, 2003). Inactivation of luxS had no effect on mono-species biofilms of *S. gordonii* (McNab *et al.*, 2003) and the formation of a mixed biofilm (in conjunction with *S. gordonii*) was not affected in a *P. gingivalis luxS* mutant (Chung *et al.*, 2001; McNab *et al.*, 2003). However, Blehart *et al.* (2003) recently reported that a *luxS* mutant exhibited altered microcolony architecture within *S. gordonii* biofilms formed in saliva. Interestingly, however, a *S. gordonii luxS* mutant was unable to form a mixed-species biofilm together with a *P. gingivalis luxS* mutant (McNab *et al.*, 2003). Since the presence of a functional *luxS* gene in either one of the two species resulted in a normal mixed-species biofilm, it was suggested that ''LuxS-based signaling'' is essential for the development of a mixed-species biofilm containing these two species (McNab *et al.*, 2003). This is indeed a possibility, but alternative functions of LuxS, or even AI-2, have to be taken into account before final conclusions can be drawn. In *S. gordonii*, inactivation of *luxS* does not affect the production of SspA and

SspB adhesins (McNab *et al.*, 2003), which are known to mediate adhesion between *S. gordonii* and bacteria such as *P. gingivalis* and *Actinomyces naeslundii*.

The function of *luxS* in *P. gingivalis* has been analyzed in more detail in two independent studies by Chung *et al.* (2001) and Burgess *et al.* (2002). Differential display analysis by Chung *et al.* (2001) indicated that *hemR* (encoding a putative TonB-dependent outer membrane receptor which is also known to be regulated by hemin) as well as the genes encoding putative homologues of TonB and excinuclease ABC (*uvrB*; Fong *et al.*, 2001) were downregulated in a *P. gingivalis luxS* mutant. This study also suggested the upregulation of *rgpA*, encoding an arginine specific protease, and *hasF* (Fong *et al.*, 2001), encoding a putative outer membrane hemin acquisition protein. With the exception of *rgpA*, the differential regulation of these genes was confirmed by RT-PCR. In an independent study by Burgess *et al.* (2002), however, the upregulation of *rgpA* was not confirmed at the level of enzymatic activity. Instead, a *P. ginigivalis luxS* mutant was found to produce around 45% less Rgp protease and also 30% less Kgp protease than the parent strain. The *luxS* mutant also exhibited a 4-fold reduced hemaglutination titer. Nevertheless, wild-type and mutant were equally virulent in a murine soft tissue model (Burgess *et al.*, 2002). In addition, *luxS* inactivation had no effect on the efficiency of gingival epithelial cell invasion (Chung *et al.*, 2001).

AI-2–dependent signaling has also been studied in *A. actinomycetemcomitans* (Fong *et al.*, 2001). Early exponential phase cells of this organism were incubated with either sterile medium or CM of the same strain obtained at mid- to late exponential phase. In the presence of CM, leukotoxicity of the cells was increased two- to three-fold, indicating the presence of an inducing factor. A similar increase was observed using CM derived from *E. coli* DH5α expressing the *A. actinomycetemcomitans luxS* gene when compared to CM derived from *E. coli* DH5α containing an inactivated copy of the same gene. For the latter experiment, the observed differences were also confirmed using anti-leukotoxin antibodies. Furthermore, the transcript level of *afuA*, encoding a periplasmic iron transport protein, was 8-fold increased in the presence of CM from *E. coli* DH5α containing the *luxS* gene, but no change was observed for *cdtB*, encoding the cytolethal distending toxin protein B. The authors attributed the observed changes in gene expression to the presence of AI-2 in CM of *E. coli* DH5α expressing *luxS*.

Examination of an *A. actinomycetemcomitans luxS* mutant (Fong *et al.*, 2003) revealed a growth defect in BHI medium, under iron-limiting

conditions. Inactivation of *luxS* also influenced the expression of several iron acquisition genes, including *afuA*, under iron-replete conditions. *AfuA* and genes encoding putative homologues for ferritin (*ftnAB*), ferric citrate transport as well as transferrin-, hemoglobin-, and hemophore-binding proteins were downregulated, whereas two genes for putative siderophore transport proteins were upregulated. Where tested, these changes could be reversed through the introduction of a plasmid-borne copy of *luxS*. The authors suggested that ''LuxS-dependent signaling'' regulated the acquisition of iron by this organism, which may be important for adaptation and subsequent growth under iron limitation.

Fong *et al*. (2001) also examined the expression of *uvrB* and *hasF* in a *P. gingivalis luxS* mutant after the addition of CM from *E. coli* DH5α expressing the *A. actinomycetemcomitans luxS* gene in comparison with sterile medium. In the presence of CM, and thus AI-2, *uvrB* expression was increased and *hasF* expression was turned off, whereas expression of a control gene, *fimA*, remained unchanged. Again, these effects were explained by the presence of ''*A. actinomycetemcomitans* AI-2'' in the CM and it was speculated that the strong effect seen with *hasF* may have arisen from a higher AI-2 concentration in *E. coli*–derived CM. Fong *et al*. (2001) interpreted their results as evidence for interspecies communication between *A. actinomycetemcomitans* and *P. gingivalis* and possibly other bacteria in the oral cavity. In support of this, Chung *et al*. (2001) and Fong *et al*. (2001) both reported the presence of putative LuxO and LuxQ homologues in *P. gingivalis* and *A. actinomycetemcomitans*, and discussed their possible involvement in AI-2 signal transduction. However, a closer look reveals that these so-called ''LuxO'' and ''LuxQ homologues'' are, in fact, more closely related to other known σ54-dependent regulators and to tripartite sensor kinases. For example, the LuxQ homologue of *P. gingivalis* is more similar to the styrene sensor kinase StyS in *Pseudomonas sp*, strain Y2, whereas the respective homologue of *A. actinomycetemcomitans*, although sharing significant similarity with LuxQ in the C-terminal region, is even more similar to the aerobic respiration sensor-response protein ArcB of *E. coli* or *Haemophilus influenzae*. Interestingly, in addition to LuxQ, several ArcB-like proteins can be found in the *V. fulnificus* and *V. cholerae* genomes. A mutant of the putative *arcB* homologue in *A. actinomycetemcomitans*, in common with the $luxS_{Aa}$ mutant, has a growth defect under iron-limiting conditions and shows reduced expression of *afuA* and *ftnAB* (Fong *et al.*, 2003). The authors suggested that ArcB may function as a sensor for AI-2 and control the acquisition and storage of iron in *A. actinomycetemcomitans*. In other organisms, the ArcA/

ArcB two-component signal transduction system is an important regulator of central metabolic functions, which are regulated in response to oxygen availability (Lynch and Lin, 1996). Although a role of ArcB in iron acquisition has not yet been described, iron transport is probably regulated in response to oxygen concentration (Kammler *et al.*, 1993), which could explain the observed phenotype of the *A. actinomycetemcomitans arcB* mutant. Presently, a role of this protein in AI-2 signal transduction can neither be confirmed nor excluded.

4. Neisseria meningitidis

In *Neisseria meningitidis*, AI-2 accumulates during growth and reaches maximal level during entry into stationary phase (Winzer *et al.*, 2002d). The *luxS* gene of two *N. meningitidis* serogroup B strains, MC58 and B16B6, was deleted and the resulting mutants were analyzed for their ability to cause bacteremic infection in infant rats after intraperitoneal injection together with the wild-type in a 1:1 ratio. For both strains, the inactivation of *luxS* resulted in attenuation. Wild-type levels of virulence were restored after the introduction of an intact *luxS* gene in single copy on a different part of the chromosome, indicating that attenuation was not caused by second-site mutation. However, it is not clear from this study whether the reduction in virulence is caused by a disruption of AI-2–based signaling, a reduction in general fitness due to a metabolic defect, or a combination of the two. It can be argued that AI-2 produced by the wild-type could have cross-fed the *luxS* mutant in the mixed-inoculum experiments and that the impact of AI-2–based signaling on meningococcal virulence may have been underestimated in this study. However, recent unpublished data from the authors' laboratory point toward a reduction in fitness due to a metabolic defect. While parent strains and mutants showed comparable growth in rich medium (Mueller-Hinton), a growth defect was observed in defined medium which lacked methionine and was restricted for cysteine. Wild-type levels of growth could be restored through the addition of cysteine but not *in vitro* synthesized AI-2, which is consistent with the idea that inactivation of *luxS* increases the need for methionine biosynthesis, which in turn depends on the availability of suitable sulfur sources (see Section VI.B). Indeed, access of *N. meningitidis* to organic sulfur sources appears to be limited at some stages during infection, as mutants containing defects in methionine biosynthesis genes have been shown to be attenuated in the infant rat model (Sun *et al.*, 2000) and genes involved in the uptake of sulfate and *de novo* synthesis of cysteine are upregulated upon interaction with human epithelial cells (Grifantini *et al.*, 2002). These results

also demonstrate the importance of choosing the appropriate growth conditions when analyzing the phenoytypes of *luxS* mutants *in vitro*.

A role of AI-2 in meningococcal cell-to-cell communication cannot, however, be completely ruled out on the basis of these experiments. Indeed, a study by Levin *et al.* (2002) suggested that AI-2 may regulate a number of different target genes in *N. menigitidis* MC58. A *luxS* mutant was grown in Muller-Hinton broth and its protein profile compared with that of the parent strain by 2-D gel analysis. The levels of at least 11 proteins were changed significantly, including BfrA (bacterioferritin A) and PdxA (an NAD-dependent dehydrogenase involved in pyridoxal phosphate synthesis) which were both upregulated, and as a putative peptidyl-formyl cis-trans isomerase (NMB0281), which was downregulated. These changes were also confirmed at a transcriptional level by RT-PCR and gene array analysis, showing 3.8- and 2.9-fold upregulation for *bfrA* and *pdxA*, respectively, and 2.5-fold downregulation of NMB0281. In most cases, the observed changes could be reversed by the presence of 50 μg/ml (0.44 mM) ''synthetic AI-2'' (MHF; Levin, personal communication). As discussed in more detail in Section IV, MHF is a compound which exhibits AI-2 activity and is formed *in vitro* in the LuxS reaction (Winzer *et al.*, 2002a). It can fully activate bioluminescence in the *V. harveyi* BB170 assay at a concentration of 2 mM (see Winzer *et al.*, 2002a) but also possesses toxic properties (Hiramoto *et al.*, 1996) and inhibits the AI-2 bioassay at high concentrations. The fact that MHF could reverse the effects of *luxS* inactivation on a number of proteins is consistent with a role in signaling, but the unusually high concentration as well as its toxic properties may also affect gene expression in signaling-independent manner. In an independent gene array study, no significant differences between a *N. meningitidis luxS* mutant and its parent strain could be identified (Dove *et al.*, 2003).

5. *Other Gram-negative Bacteria*

a. Borrelia burgdorferi. The spirochete *Borrelia burgdorferi* is the causative agent of Lyme disease in humans. This Gram-negative bacterium lives in the midgut of ticks but can be transmitted when the latter feed on mammals.

AI-2 activity was not present in detectable amounts in the culture supernatants of different *B. burgdorferi* strains, even when grown at different pH values and temperatures, although the *B. burgdorferi luxS* gene ($luxS_{Bg}$) was functional in *E. coli* DH5α (Stevenson and Babb, 2002; Hübner et al., 2003). It is possible that AI-2, like other substances synthesized by this bacterium, is only produced under specific conditions encountered *in vivo* but not *in vitro*. Nevertheless, addition of

CM from *E. coli* DH5α expressing *luxS*$_{Bg}$, strongly affected the level of several proteins in *B. burgdorferi*. At 34°C, at least 18 proteins were upregulated and 7 proteins downregulated and most of these were different from the proteins found to be altered at 23°C. Furthermore, at 34°C, the levels of the complement factor H-binding proteins ErpA/I/N were two-fold increased in the presence of CM from *E. coli* DH5α expressing *luxS*$_{Bg}$, whereas the surface protein OspC (a transmission-associated protein) remained unchanged. Stevenson and Babb (2002) concluded that *B. burgdorferi* responds to AI-2 in a temperature-dependent manner by modulating the production of specific proteins.

Apart from the problems associated with the use of CM, this interpretation is further complicated by the fact that after the addition of CM, cells were grown for a further 2 (34°C.) or 3 days (23°C), which was equivalent to 2 to 3 generations and no data were made available to demonstrate the stability of AI-2 activity under these conditions. If the response was indeed AI-2–dependent, the signal reception must be fairly sensitive, as the CM from *E. coli* was diluted 1:100 in the *B. burgdorferi* culture fluid.

b. Photorhabdus luminescens. The luminescent bacterium *Photorhabdus luminescens* inhabits the intestine of a soil nematode from where it is expelled when the nematode enters a susceptible insect. Once they are released, the bacteria kill the insect and enhance the conditions for nematode reproduction. It is believed that the production of several antibiotics prevents contamination of the insect carcass with other bacterial species, which is important for the successful completion of the nematode life cycle.

Interestingly, the inactivation of *luxS* in *P. luminescens* upregulated the production of a carbapenem-like antibiotic, which is effective against several gram-negative bacteria (Derzelle *et al.*, 2002). This was indicated by the antibiosis zones produced by the *luxS* mutant, which were consistently larger than those of the wild-type, suggesting that it produced more antibiotic. Furthermore, the expression pattern of the *cpm* gene cluster, which is required for the biosynthesis of the carbapenem–like antibiotic, was altered. In the parent strain, *cpm* transcript level peaked during midexponential phase and then declined rapidly before entry into stationary phase. In the mutant, however, this decline was less pronounced and significant amounts of *cpm* mRNA were still detectable during the early stationary phase. Pigment production and the growth rate in Schneider medium was not affected, although the growth of the mutant resumed with a short

delay after inoculation into fresh medium. No attempts were made to restore the wildtype pattern.

Derzelle *et al.* (2002) suggested that repression of antibiotic production by quorum sensing may function to limit carbapenem biosynthesis to logarithmic growth, which could help to prevent intoxication of *P. luminescens* by its own antibiotic in the confined space of the insect carcass. This pattern of antibiotic production is strikingly different from that of *E. carotovora*, which employs AHL-based quorum sensing to induce carbapenem production only at high cell densities although both species contain homologous mechanisms of self-protection against the antibiotic (Whitehead *et al.*, 2002).

c. Pasteurellaceae. AI-2 production by *A. actinomycetemcomitans* has already been described in Section IX.B.3. Other members of this family, e.g., *Manheimia hemolytica, Pasteurella multocida, Pasteurella trehalosi, Actinobacillus suis*, and *Actinobacillus pleuropneumoniae*, also produce AI-2 but sometimes only at a very low level (Malott and Lo, 2002). It is not clear whether the low activities are due to limited export or the production of less active AI-2 derivatives. Interestingly, the genome sequence of *P. multocida* (May *et al.*, 2001; GenBank accession number AE004439) suggests the presence of a putative ABC transporter system, which appears to be homologous to the AI-2 transporter identified in *S. typhimurium*.

6. *Gram-positive Bacteria*

a. Streptococci. In *S. pyogenes* HSC5, AI-2 activity is undetectable early during growth but then increases dramatically and reaches maximal levels after entry into stationary phase. AI-2 activity then falls rapidly (Lyon *et al.*, 2001). Interestingly, a *luxS* mutant of this strain was shown not to produce any AI-2 up to the point were the activity produced by the wild-type started to decline. Then, however, a small increase in activity was observed, although maximal activities were still 100-fold lower than those produced by the wild-type. Because the sequenced genomes of various *S. pyogenes* strains contain only one *luxS* homologue, the reason for this AI-2 activity is not clear. Work from the author's laboratory has identified an alternative route of AI-2 formation via the spontaneous convertion of sugar phosphates, in particular ribulose-5-phosphate. It is also possible that other compounds produced by the mutant had stimulatory effects, e.g., on growth and thus bioluminescence, in the *V. harveyi* BB170 bioassay.

Inactivation of *luxS* in HSC5 had a strong effect on the production of the virulence factor streptolysin S (SLS, a hemolysin), which in the

wild-type is produced during the transition to stationary phase and then rapidly decays. Although the pattern of temporary production and decay was not altered, the *luxS* mutant produced highly elevated SLS level (Lyon *et al.*, 2001). In agreement with this observation, the *sagA* gene (streptolysin-associated gene A) which is required for SLS production, was more than 10-fold upregulated. Production of a second hemolysin, streptolysin O, was not altered in the *luxS* mutant.

Lyon *et al.* (2001) also demonstrated that the *luxS* mutant was defective for secreted proteolytic activity. Activity of the secreted cysteine protease SpeB appears abruptly during the late exponential phase and, like SLS, declines several hours into stationary phase. However, the *luxS* mutant produced only a 20-fold lower activity at the time where SpeB production by the wild-type was maximal. This defect occurred posttranslationally, possibly at the level of secretion and processing of the protease precursor. The mutant secreted approximately 8-fold less protease polypeptide than the wild-type, whereas *speB* transcript level were unaffected. All detectable protease secreted by the mutant was present as the inactive precursor.

The *luxS* mutant also exhibited a medium-dependent growth defect. In peptide-rich and carbohydrate-poor C medium, no difference in growth was observed, whereas in Todd-Hewitt yeast extract medium, the mutant showed a 30% reduction in the rate of growth, which was not caused by the observed reduction in proteolytic activity.

Interestingly, an insertion element, *IS1239*, was identified immediately upstream of *luxS* in a strain characterized by low production of SpeB activity. When this region was exchanged against the IS*1239*-free but otherwise identical region of strain HSC5, SpeE activity increased significantly and reached the level observed for *S. pyogenes* HSC5. The molecular basis for the variable production of proteolytic activity in many clinical isolates of *S. pyogenes* is not well understood, but differential expression through the integration of an insertion element next to *luxS* may represent at least one mechanism that can modulate virulence during infection.

In contrast to many other species, inactivation of *luxS* in *S. pyogenes* strongly influenced specific phenotypes. However, since the phenotypes could not be restored by the addition of CM from wildtype cultures (Lyon *et al.*, 2001), the observed changes could not be attributed to an AI-2 signaling defect.

In *S. mutans*, expression of *luxS* was detectable in early exponential phase cells and was optimal in the mid-exponential phase of growth (Wen and Burne, 2002). Maximal AI-2 levels were reported for mid- to late logarithmic growth (Merritt *et al.*, 2003). Inactivation of *luxS*

did not affect growth in BHI or semi-defined BH medium and did not significantly impair biofilm formation on plastic surfaces (Wen and Burne, 2002). However, on glass slides biofilms were reported to be more granular (Merritt *et al.*, 2003).

The importance of *luxS* for mixed-species biofilms formed by *S. gordonii* has already been discussed (see Section IX.B.3). Genes differentially regulated in the $luxS_{Sg}$ mutant and wild-type include those with putative functions in metabolism (notably sugar metabolism) and transport (McNab *et al.*, 2003). For *Strep. pneumoniae*, mutation of *luxS* reduced virulence in a mouse model but did not affect colonization of the mouse nasopharynx (Stroeher *et al.*, 2003).

b. Clostridium perfringens. The anaerobic pathogen *C. perfringens* is known to produce extracellular virulence factors, several of which are regulated by the VirR/VirS two-component system in response to an unknown signal (Lyristis *et al.*, 1994; Shimizu *et al.*, 1994, Ba-Thein *et al.*, 1996). These include the alpha-, theta-, and kappa-toxins, which are encoded by the *plc*, *pfoA*, and *colA* genes, respectively. Inactivation of *luxS* resulted in an approximately two-fold reduction of alpha-, theta-, and kappa-toxin levels in the culture supernatant after 5 h, although growth characteristics in Gifu anaerobic medium was not affected (Ohtani *et al.*, 2002). Northern blot analysis indicated that *phoA* transcript levels were also significantly reduced during mid-exponential growth in the *luxS* mutant, whereas the *plc* and *colA* transcripts levels remained largely unchanged.

The influence of CM from wild-type and mutant on toxin production was analyzed to obtain evidence for their regulation by AI-2. *luxS* mutants produced more alpha, theta-, and kappa-toxins when incubated with CM from the wild-type when compared with incubation with AI-2-free CM from the mutant itself. Under the same conditions, *phoA* transcript levels increased four-fold, whereas *colA* or *plc* transcription remained unchanged. A similar effect on *phoA* transcription was observed in the presence of CM from *E. coli* DH5α expressing either the *C. perfringens ycgJ-metB-cysK-luxS* gene cluster, or *luxS* alone, when compared to CM from the same strain containing a control plasmid. Ohtani *et al.* (2002) concluded that extracellular AI-2 positively regulates *pfoA* transcription and may have an effect on post-transcriptional regulation of alpha- and kappa-toxins.

In *C. perfringens*, *luxS* is organized in an operon together with a gene of unknown function, *ycgJ*, and the metabolic genes *metB* and *cysK* (gene order: *ycgJ-metB-cysK-luxS*; Banu *et al.*, 2000; see Section VII). The importance of *metB* and *cysK* for cell-to-cell signaling was analyzed

through the construction of a *metB* mutant, which, probably due to a polar effect on *luxS* expression, was also impaired for AI-2 production (Ohtani *et al.*, 2002). This mutant also showed drastically reduced *phoA* transcript levels at the mid-exponential growth phase. The introduction of plasmid-borne copies of either *luxS*, *ycg-luxS*, or the complete operon increased *phoA* levels 3.5-fold, whereas constructs containing other genes of the operon but lacking *luxS* had no effect. However, even in the presence of *luxS, phoA* levels were still drastically lower than those of the wild-type. The reasons for the reduced *phoA* levels in the presence of *luxS*-containing plasmids was not further investigated, but the supernatants of these strains also contained only 19 to 31% of the AI-2 activity found for the wild-type. The authors concluded that only *luxS* (but not *metB* or *cysK*) is important for the increase in *phoA* expression.

The relationship between VirR/VirS and the potential AI-2 signaling system was also examined. No *phoA* transcripts, and only very little *plc* and *colA* mRNA, was detected in a *virR* mutant, and this pattern, which is far more dramatic than that observed after *luxS* inactivation, did not alter in the presence of plasmid-borne copy of *luxS*. By contrast, when the *virR* gene was overexpressed in a *luxS* mutant, *col*, *plc*, and particularly *pfoA* transcript level were enhanced to approximately wild-type levels. These data suggested that VirR/VirS is not only required for the ''*luxS*-mediated'' regulation of toxins but, more importantly, that it clearly has the ability to regulate toxins in the absence of AI-2 production. This is also in agreement with the earlier finding by Banu *et al.* (2000) that VirR/VirS negatively regulates the *ycgJ-metB-cysK-luxS* operon.

In contrast to many other bacteria, where toxin production occurs mainly during stationary phase, *C. perfingens* is unique in that it secretes toxins at low cell density but switches production off at later stages. Ohtani *et al.* (2002) suggested that AI-2, which peaks during the mid-exponential phase, may be responsible for *pfoA* activation at this stage of growth whereas other mechanisms may be involved at earlier stages. The question remains, however, whether it was really the disruption of AI-2–based signaling which was responsible for the observed changes in toxin production in the *luxS* mutant. The stimulatory effects of AI-2 containing CM from both *C. perfringens* and *E. coli* support the author's interpretation, as well as the observation that a reduction in *phoA* transcript levels after the introduction of a polar mutation in *metB* could be partially restored by the introduction of *luxS* but not *metB* together with *cysK*. Reduced toxin production in response to the disruption of the methionine and cysteine biosynthesis pathways should have been reversed in the presence *metB* and *cysK*.

However, the small increase of *phoA* transcript levels and extracellular AI-2 activity in the presence of a plasmid borne copy of *luxS* or even the complete *ycgJ-metB-cysK-luxS* operon suggests that the metabolic functions in the complemented strains had not been fully restored.

C. Conclusions

Several gram-negative and gram-positive bacteria contain *luxS* genes and accumulate, often temporarily, AI-2 activity in their culture supernatants. Inactivation of *luxS* is known to affect multiple phenotypes, including growth, protein production and secretion, motility, iron acquisition, or virulence in an animal model, depending on the species and conditions tested. These changes are caused by either a lack of AI-2 signaling activity or the disruption of the activated methyl cycle. *V. harveyi* and *V. cholerae* are presently the only species shown to respond to extracellular AI-2 by regulating specific target genes not related to reuptake and degradation of the molecule. Other bacteria such as *E. coli* may also respond to AI-2, but final proof requires the use of purified AI-2 and the identification of a signal transduction pathway.

X. The Role of AI-2 in Interspecies Communication and Alternative Functions

Given that AI-2 is produced by so many different bacteria, it is perhaps not surprising that the molecule has given rise to much speculation about its function (Bassler, 1999; Fuqua and Greenberg, 1998; Coulthurst *et al.*, 2002; Winans, 2002; Winzer *et al.*, 2002a,b). Building on suggestions first unveiled by Greenberg *et al.* (1979) regarding the involvement of *V. harveyi* in bacterial cross-talk, the idea that AI-2 may serve as a signal in interspecies communication has been put forward by Bonnie Bassler's group and has certainly been influential, inspiring much of the research presented in this chapter (Bassler *et al.*, 1999; Schauder and Bassler, 2001; Miller and Bassler, 2001; Bassler, 2002; see also Section X.A.4).

A. A Role in Interspecies Communication?

The production of AI-2 by so many different bacteria remains enigmatic. True AI-2–based cross-species communication in natural habitats, while an intriguing theoretical exercise, still awaits experimental confirmation. Although certain scenarios can be envisaged where AI-2 is involved in bacterial crosstalk in nature, e.g., the communication

between marine *Vibrio* species, it is not clear why so many different bacteria in general need to communicate their metabolic situation or other information to separate and probably competing species. The exchange of information could make sense in symbiotic relationships, but, in that case, a specific signal which cannot be distorted or ''bugged'' by other bacteria would appear to be more reliable. It is now clear that AI-2 production profiles can differ dramatically among species, and some bacteria may degrade the molecule at the same time others continue to produce it. Therefore, decoding the message contained in the AI-2 level of a mixed population appears to be a rather complex task. Thus, interspecies communication could occur predominantly in situations where the number of different AI-2 producers is restricted and where other signal molecules or excreted metabolites reveal additional information about the presence or absence of individual AI-2 producers or consumers and their physiological state.

B. A Signal for Metabolic Activity?

It has been suggested that in some species AI-2 is used to signal metabolic activity (DeLisa *et al*, 2001b; Beeston and Surette, 2002), the potential of a given environment for growth (Surette and Bassler, 1999), or the growth conditions of a population (Sperandio *et al.*, 2001) rather than simply cell density. These possibilities cannot be excluded, although many bacteria have developed highly sophisticated mechanisms for sensing a multitude of environmental parameters directly. Nevertheless, it could be useful for bacteria to coordinately downregulate growth and prepare for starvation before nutrients become severely limited (Withers and Nordström, 1998). In this situation, information on both size and metabolic state of a population will become important. Other species-specific metabolites could be used for this purpose, but in naturally occurring mixed populations, AI-2 has the advantage that it may be produced by more than one species and that its formation is directly dependent on central metabolic processes.

C. AI-2 as a Secreted Metabolite

In many cases, AI-2 may simply represent a metabolic end product or temporarily secreted metabolite which some bacteria, notably of the genus *Vibrio*, have adopted for signaling purposes (Winans, 2002; Winzer *et al.*, 2002b). Nevertheless, it is still possible that some bacteria, e.g., *V. cholerae* or EHEC, when infecting the gut, ''listen in'' and try to

make sense of the information conveyed by ''unsuspecting'' noncommunicating AI-2 producers. For EHEC, it has been suggested that AI-2 produced by the normal flora of the large intestine could activate the *LEE* genes and allow intimin-mediated colonization to proceed (Sperandio *et al.*, 1999). Another possibility is that some bacteria produce AI-2 solely for the purpose of interference with the communication of other species (Forsyth and Cover, 2000). It has also been suggested that AI-2 is excluded from the cell because it has toxic properties (Winzer *et al.*, 2002b). This suggestion was based on the properties of MHF (Hiramoto *et al.*, 1996), another derivative formed from the reactive DPD *in vitro* (Winzer *et al.*, 2002a). With the discovery of the relatively stable furanosyl borate diester as the principal ligand of LuxP, this interpretation has to be reexamined. Presently, it is not known whether additional toxic DPD derivatives like MHF are formed *in vivo*. If toxic AI-2 precursors do exist, their export under certain growth conditions could explain the temporal and varying AI-2 activity observed in bacterial culture supernatants. It is also possible that DPD serves as a precursor in an (as yet unidentified) anabolic pathway and that AI-2 is a minor side product.

D. A Role in Boron Acquisition or Boric Acid Sensing?

Coulthurst *et al.* (2002) suggested that AI-2 might act as a boron transporter, employed for the uptake of boron under certain growth and environmental conditions. This is an interesting idea worth pursuing. Boron is essential for plants and diatoms and (at least) some animals (for a review, see Brown *et al.*, 2002). The element has been shown to stimulate yeast growth (Bennett *et al.*, 1999) and boron deficiency under certain growth conditions affects nitrogen fixation in some heterocyste-forming cyanobacteria, possibly because it is required for the structural integrity of the heterocyst envelope, the main barrier against oxygen diffusion (reviewed by Cakmak and Römheld, 1997). In plant cell walls, rhamnogalacturonan-II is present predominantly as a dimeric molecule, where two monomers are cross-linked through the formation of a tetrahedral borate–diol diester with two apiosyl residues (Power and Woods, 1997). Boron also plays a role in plant metabolism, although the mechanisms remain unclear, and is likely to be important for membrane structure and function in plants and animals (Brown *et al.*, 2002). On the other hand, high concentrations of boric acid have been shown to be toxic.

Is it possible that bacteria obtain boron by excreting pro-AI-2 (Fig. 3), which then specifically reacts with boric acid and is taken up again in the form of AI-2 (acting as a ''borophore,'' in analogy to iron uptake by

siderophores)? In plants, where boron acquisition has been studied most intensively, boron is absorbed mainly as undissociated boric acid (H_3BO_3). Boric acid is a very weak acid with a pK of 9.1 and at a physiological or acidic pH, very little is present in the form of the borate anion ($H_4BO_4^-$) (Power and Woods, 1997). In plants, boric acid can enter the cells via passive diffusion but there is also evidence for active transport (at a concentration below 1 μM passive permeation is considered inadequate to satisfy the boron requirements of plants) (Dordas and Brown, 2000; Brown *et al.*, 2002). Recently, an efflux-type boron transporter (Bor1) which is required for xylem loading has been discovered in *Arabidopsis thaliana*, and genes encoding similar proteins are present in animals and in *Saccharomyces cerevisiae* (Takano *et al.*, 2002).

Apart from cyanobacteria, the boron requirements of other prokaryotes have not been studied in detail and therefore it is not known whether bacteria in general require boron for growth. Close homologues of the *A. thaliana* Bor1 transporter are not present in the currently sequenced bacterial genomes, but alternative systems may exist. Boric acid is known to react spontaneously with a variety of organic mono-, di-, or polyhydroxy compounds, but the most stable (cyclic) borate esters are formed with cis-diols on a furanoid ring, such as apiose and ribose (Mazurek and Perlin, 1963). AI-2, which is derived from a ribosyl moiety, may represent another example for a comparatively stable borate diester (Chen *et al.*, 2002). Whether AI-2 plays a role in boron acquisition as suggested by Coulthurst *et al.* (2002) remains to be seen, but the uptake of a stable borate ester rather than boric acid itself is a possibility. AI-2 is not the only known boron compound formed by bacteria. Boron-containing polyketide antibiotics have been isolated from the culture fluids of *Streptomyces antibioticus* (boromycin; Hütter *et al.*, 1967; Dunitz *et al.*, 1971), *Streptomyces griseus* SS120 (aplasmomycin; Nakamura *et al.*, 1977), the myxobacterium *Sorangium cellulosum* (tartrolon B; Irschik *et al.*, 1995; Schummer *et al.*, 1996), and the cyanobacteria *Nostoc linckia* and *Nostoc spongiaeforme* (borophycin; Hemscheidt *et al.*, 1994; Banker and Carmeli, 1998). In fact, boromycin was the first defined biological boron compound to be described (Hütter *et al.*, 1967). Interestingly, desboroplasmomycin (the polyketide structure of aplasmomycin without the central boron atom) was easily converted to aplasmomycin in the presence of boric acid at pH 6 or pH 8 (Chen *et al.*, 1980). Furthermore, tartrolon A (tartolon B polyketide lacking boron) was by far the predominant product when *S. cellulosum* cultures were grown in a stainless steel bioreactor, whereas tartolon B was the major product in glass flasks (Irschik *et al.*, 1995). This suggests

that tartrolon A was converted to tartolon B in the presence of contaminating boron compounds originating from the glassware. As discussed in Section IV. C, it is possible that AI-2 is formed from pro-AI-2 nonenzymatically, depending on the presence and concentration of boric acid. In this case, AI-2 may be looked upon as a molecule which modulates the AI-1– and CAI-1–driven quorum-sensing systems of *V. harveyi* and *V. cholerae*, respectively, in response to the extracellular concentration of boric acid. Thus, the AI-2 systems in these organisms may act as boric acid sensors rather than cell density sensing systems.

However, AI-2–induced bioluminescence in conjunction with boric acid may fulfill an important function in the ecology of *V. harveyi* and other marine bacteria. *V. harveyi*, as well as other luminous bacteria, have been isolated in the free-living planktonic form from seawater but also in large numbers from the gut of fish (Reichelt and Baumann, 1973; Hastings and Nealson, 1977; Ruby and Morin, 1979; Ramesh *et al.*, 1986; Ramesh and Venugopalan, 1988). *V. harveyi* is also a pathogen of certain crustaceans. The enteric contents of animals are one of the largest pools of organic matter available to marine bacteria, but it appears that after their uptake, luminous bacteria inhabit the gut of fish only transiently (Ruby and Morin, 1979; O'Brien and Sizemore, 1979). After ingestion, the bacteria move down the intestinal tract together with digested food material and are finally expelled together with the fecal pellet. These fecal pellets start to bioluminesce once they have left the anaerobic environment of gut (Ruby and Morin, 1979). As well as oxygen, bioluminescence requires the appropriate autoinducer concentration for the induction of luciferase. In some cases, the required autoinducer concentration may already be reached in the gut, but feces-associated bacteria have also been shown to proliferate extensively after excretion with a concomitant increase in bioluminescence intensity (Ruby and Morin, 1979).

After the release of the fecal pellet, the high concentration of boric acid (400 mM) present in seawater may particularly stimulate AI-2–dependent induction of bioluminescence, by enhancing the conversion of pro-AI-2 to AI-2 (see Section IV.C). This mechanism could ensure that a high level of bioluminescence is maintained even at low autoinducer concentrations, e.g., on the surface of fecal pellets, due to the very tight binding of AI-2 to LuxP. It has been suggested that the bioluminescence of bacteria associated with fecal pellets might serve to attract fish or other marine organisms to ensure the reuptake of bacteria through consumption of these particles (Hastings and Nealson, 1977; Ruby and Morin, 1979). Hastings and Nealson (1977) have pointed out that this may also be beneficial for the fish, as *V. harveyi* and other

luminous bacteria are capable of producing chitinases which may facilitate the degradation of the exoskeletons of marine crustaceans. AI-2–dependent activation of bioluminescence in the presence of high boric acid concentrations may therefore represent an example for a concerted multispecies effort, where a number of different feces-associated bacteria act together to ensure reuptake into the nutrient-rich resorvoir of the fish gut.

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References

Alexeev, D., Alexeeva, M., Baxter, R. L., Campopiano, D. J., Webster, S. P., and Sawyer, L. (1998). The crystal structure of 8-amino-7-oxononanoate synthase: A bacterial PLP-dependent, acyl-CoA-condensing enzyme. *J Mol. Biol.* **284,** 401–419.

Arnold, R. J., and Reilly, J. P. (1999). Observation of *Escherichia coli* ribosomal proteins and their posttranslational modifications by mass spectrometry. *Anal. Biochem.* **269,** 105–112.

Atkinson, S., Throup, J. P., Stewart, G. S., and Williams, P. (1999). A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. *Mol. Microbiol.* **33,** 1267–1277.

Baldwin, J. E., and Krebs, H. (1981). The evolution of metabolic cycles. *Nature* **291,** 381–382.

Banker, R., and Carmeli, S. (1998). Tenuecyclamides A–D, cyclic hexapeptides from the cyanobacterium *Nostoc spongiaeforme* var. tenue. *J. Nat. Prod.* **61,** 1248–1251.

Banu, S., Ohtani, K., Yaguchi, H., Swe, T., Cole, S. T., Hayashi, H., and Shimizu, T. (2000). Identification of novel VirR/VirS-regulated genes in *Clostridium perfringens*. *Mol. Microbiol.* **35,** 854–864.

Bassler, B. L. (1999). How bacteria talk to each other: Regulation of gene expression by quorum sensing. *Cur. Opin. Microbiol.* **2,** 582–587.

Bassler, B. L. (2002). Small talk: Cell–cell communication in bacteria. *Cell* **109,** 421–424.

Bassler, B. L., Wright, M., Showalter, R. E., and Silverman, M. R. (1993). Intercellular signaling in *Vibrio harveyi*: Sequence and function of genes regulating expression of luminescence. *Mol. Microbiol.* **9.4,** 773–786.

Bassler, B. L., Wright, M., and Silverman, M. R. (1994). Multiple signaling systems controlling expression of luminescence in *Vibrio harveyi*: Sequence and function of genes encoding a second sensory pathway. *Mol. Microbiol.* **13,** 273–286.

Bassler, B. L., Greenberg, E. P., and Stevens, A. M. (1997). Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J. Bacteriol.* **179,** 4043–4045.

Ba-Thein, W., Lyristis, M., Ohtani, K., Nisbet, I. T., Hayashi, H., Rood, J. I., and Shimizu, T. (1996). The virR/virS locus regulates the transcription of genes encoding extracellular toxin production in *Clostridium perfringens*. *J. Bacteriol.* **178,** 2514–2520.

Bechthold, A., and Floss, H. G. (1994). Overexpression of the thiostrepton-resistance gene from *Streptomyces azureus* in *Escherichia coli* and characterization of recognition sites of the 23S rRNA A1067 2′-methyltransferase in the guanosine triphosphatase center of 23S ribosomal RNA. *Eur. J. Biochem.* **224,** 431–437.

Beeston, A. L., and Surette, M. G. (2002). *pfs*-dependent regulation of autoinducer 2 production in *Salmonella enterica* serovar typhimurium. *J. Bacteriol.* **184,** 3450–3456.

Bennett, A., Rowe, R. I., Soch, N., and Eckhert, C. D. (1999). Boron stimulates yeast (*Saccharomyces cerevisiae*) growth. *J. Nutr.* **129,** 2236–2238.

Biran, D., Gur, E., Gollan, L., and Ron, E. Z. (2000). Control of methionine biosynthesis in *Escherichia coli* by proteolysis. *Mol. Microbiol.* **37,** 1436–1443.

Björk, G. R. (1996). Stable RNA modification. *In* ''Escherichia coli and Salmonella: Cellular and Molecular Biology'' (F. C. Neidhardt, R. Curtiss, III, C. A. Gross, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger, eds.), 2nd ed., Vol. 1, pp. 861–886 ASM Press, Washington, DC.

Blehert, D. S., Palmer, R. J. Jr., Xavier, J. B., Almeida, J. S., and Kolenbrander, P. E. (2003). Autoinducer 2 production by *Streptococcus gordonii* DL1 and the biofilm phenotype of a *luxS* mutant are influenced by nutritional conditions. *J. Bacteriol.* **185,** 4851–4860.

Brown, P. H., Bellaloui, N., Wimmer, M. A., Bassil, E. S., Ruiz, J., Hu, H., Pfeffer, H., Dannel, F., and Römheld, V. (2002). Boron in plant biology. *Plant Biol.* **4,** 205–223.

Bugaut, M. (1987). Occurrence, absorption, and metabolism of short chain fatty acids in the digestive tract of mammals. *Comp. Biochem. Physiol. B.* **86,** 439–472.

Burgess, N. A., Kirke, D., Williams, P., Winzer, K., Hardie, K. R., Meyer, N. L., Aduse-Opoku, J., Curtis, M. A., and Cámara, M. (2002). LuxS-dependent quorum sensing in *Porphyromonas gingivalis* modulates protease and hemagglutinin activities but is not essential for virulence. *Microbiology* **148,** 763–772.

Cadieux, N., Bradbeer, C., Reeger-Schneider, E., Koster, W., Mohanty, A. K., Wiener, M. C., and Kadner, R. J. (2002). Identification of the periplasmic cobalamin-binding protein BtuF of *Escherichia coli*. *J. Bacteriol.* **184,** 706–717.

Cai, X. Y., Redfield, B., Maxon, M., Weissbach, H., and Brot, N. (1989). The effect of homocysteine on MetR regulation of *metE*, *metR*, and *metH* expression *in vitro*. *Biochem. Biophys. Res. Commun.* **163,** 79–83.

Cakmak, I., and Römheld, V. (1997). Boron deficiency-induced impairments of cellular functions in plants. *Plant and Soil* **193,** 71–83.

Caldas, T., Binet, E., Bouloc, P., and Richarme, G. (2000). Translational defects of *Escherichia coli* mutants deficient in the Um(2552) 23S ribosomal RNA methyltransferase RrmJ/FTSJ. *Biochem. Biophys. Res. Commun.* **271.3,** 714–718.

Cao, J. G., and Meighen, E. A. (1989). Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *J. Biol. Chem.* **264,** 21670–21676.

Cao, J. G., and Meighen, E. A. (1993). Biosynthesis and stereochemistry of the autoinducer controlling luminescence in *Vibrio harveyi*. *J. Bacteriol.* **175,** 3856–3862.

Carmel-Harel, O., and Storz, G. (2000). Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu. Rev. Microbiol.* **54,** 439–461.

Chatterjee, J., Miyamoto, C. M., and Meighen, E. A. (1996). Autoregulation of *luxR*: The *Vibrio harveyi lux*-operon activator functions as a repressor. *Mol. Microbiol.* **20,** 415–425.

Chatterjee, J., Miyamoto, C. M., Zouzoulas, A., Lang, B. F., Skouris, N., and Meighen, E. A. (2002). MetR and CRP bind to the *Vibrio harveyi lux* promoters and regulate luminescence. *Mol. Microbiol.* **46,** 101–111.

Chen, T. S., Chang, C. J., and Floss, H. G. (1980). Biosynthesis of the boron-containing antibiotic aplasmomycin. Nuclear magnetic resonance analysis of aplasmomycin and desboroaplasmomycin. *J. Antibiot.* **33,** 1316–1322.

Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B. L., and Hughson, F. M. (2002). Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415,** 545–549.

Chung, W. O., Park, Y., Lamont, R. J., McNab, R., Barbieri, B., and Demuth, D. R. (2001). Signaling system in *Porphyromonas gingivalis* based on a LuxS protein. *J. Bacteriol.* **183,** 3903–3909.

Cloak, O. M., Solow, B. T., Briggs, C. E., Chen, C. Y., and Fratamico, P. M. (2002). Quorum sensing and production of autoinducer-2 in *Campylobacter spp.*, *Escherichia coli* O157:H7, and *Salmonella enterica* serovar Typhimurium in foods. *Appl. Environ. Microbiol.* **68,** 4666–4671.

Collier, G. B., Mattson, T. L., Connaughton, J. F., and Chirikjian, J. G. (1994). A novel Tn10 tetracycline regulon system controlling expression of the bacteriophage T3 gene encoding S-adenosyl-L-methionine hydrolase. *Gene* **148.1,** 75–80.

Cotter, P. A., and DiRita, V. J. (2000). Bacterial virulence gene regulation: An evolutionary perspective. *Annu. Rev. Microbiol.* **54,** 519–565.

Coulthurst, S. J., Whitehead, N. A., Welch, M., and Salmond, G. P. C. (2002). Can boron get bacteria talking? *Trends Biochem. Sci.* **27,** 217–219.

Cronan, Jr., J. E., Reed, R., Taylor, F., and Jackson, M. B. (1979). Properties and biosynthesis of cyclopropane fatty acids in *Escherichia coli*. *J. Bacteriol.* **138,** 118–121.

Crooks, P. A., Tribe, M. J., and Pinney, R. J. (1984). Inhibition of bacterial DNA cytosine-5-methyltransferase by S-adenosyl-L-homocysteine and some related compounds. *J. Pharm. Pharmacol.* **36,** 85–89.

Croxatto, A., Chalker, V. J., Lauritz, J., Jass, J., Hardman, A., Williams, P., Camara, M., and Milton, D. L. (2002). VanT, a homologue of *Vibrio harveyi* LuxR, regulates serine, metalloprotease, pigment, and biofilm production in *Vibrio anguillarum*. *J. Bacteriol.* **184,** 1617–1629.

Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P., and Macfarlane, G. T. (1987). Short-chain fatty acids in human large intestine, portal, hepatic, and venous blood. *Gut* **28,** 1221–1227.

Das, S. K., Sedelnikova, S. E., Baker, P. J., Ruzheinikov, S. N., Foster, S., Hartley, A., Horsburgh, M. J., and Rice, D. W. (2001). Cloning, purification, crystallization, and preliminary crystallographic analysis of *Bacillus subtilis* LuxS. *Acta Crystallogr. D. Biol. Crystallogr.* **57,** 1324–1325.

Day, W. A., and Maurelli, A. T. (2001). *Shigella flexneri* LuxS quorum sensing system modulates *virB* expression but is not essential for virulence. *Infect. Immun.* **69,** 15–23.

Deguchi, T., and Barchas, J. (1971). Inhibition of transmethylations of biogenic amines by S-adenosylhomocysteine. Enhancement of transmethylation by adenosylhomocysteinase. *J. Biol. Chem.* **246,** 3175–3181.

DeLisa, M. P., Valdes, J. J., and Bentley, W. E. (2001a). Mapping stress-induced changes in autoinducer AI-2 production in chemostat-cultivated *Escherichia coli* K-12. *J. Bacteriol.* **183,** 2918–2928.

DeLisa, M. P., Valdes, J. J., and Bentley, W. E. (2001b). Quorum signaling via AI-2 communicates the ''metabolic burden'' associated with heterologous protein production in *Escherichia coli Biotechnol. Bioeng.* **75,** 439–450.

DeLisa, M. P., Wu, C. F., Wang, L., Valdes, J. J., and Bentley, W. E. (2001). DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli. J. Bacteriol.* **183,** 5239–5247.

de Rudder, K. E., Thomas-Oates, J. E., and Geiger, O. (1997). *Rhizobium meliloti* mutants deficient in phospholipid N-methyltransferase still contain phosphatidylcholine. *J. Bacteriol.* **179,** 6921–6928.

Derzelle, S., Duchaud, E., Kunst, F., Danchin, A., and Bertin, P. (2002). Identification, characterization, and regulation of a cluster of genes involved in carbapenem biosynthesis in *Photorhabdus luminescens*. *Appl. Environ. Microbiol.* **68,** 3780–3789.

Diggle, S. P., Winzer, K., Lazdunski, A., Williams, P., and Camara, M. (2002). Advancing the quorum in *Pseudomonas aeruginosa*: MvaT and the regulation of *N*-acylhomoserine lactone production and virulence gene expression. *J. Bacteriol.* **184,** 2576–2586.

Doherty, N. C., Winzer, K., Holden, M., Williams, P. (2002). Characterization and mutagenesis of the *luxS* locus in *Staphylococcus aureus.* 150th ordinary meeting of the Society for General Microbiology, University of Warwick, April. Abstract MI09, booklet p. 58.

Dordas, C., and Brown, P. H. (2000). Permeability of boric acid across lipid bilayers and factors affecting it. *J. Membrane Biol.* **175,** 95–105.

Dove, J. E., Yasukawa, K., Tinsley, C. R., and Nassif, X. (2003). Productin of the signalling molecule, autoinducer-2, by *Neisseria meningitidis*: Lack of evidence for a concerted transcriptional response. *Microbiology* **149,** 1859–1869.

Duerre, J. A., Baker, D. J., and Salisbury, L. (1971). Structure elucidation of a carbohydrate derived from *S*-ribosylhomocysteine by enzymatic cleavage. *Fed. Proc.* **30,** 88.

Duerre, J. A., and Bowden, P. M. (1964). Utilization of ribosylhomocysteine by various microorganisms. *Biochem. Biophys. Res. Commun.* **16.2,** 150–155.

Duerre, J. A., and Miller, C. H. (1966). Cleavage of *S*-ribosyl-L-homocysteine by extracts from *Escherichia coli. J. Bacteriol.* **91,** 1210–1217.

Duerre, J. A., and Walker, R. D. (1977). Metabolism of adenosylhomocysteine. *In* ''The Biochemistry of Adenosylmethionine'' (F. Salvatore, E. Borek, V. Zappia, H. G.

Williams-Ashman, and F. Schlenk, eds.), pp. 43–57, Columbia University Press, New York.

Dunitz, J. D., Hawley, D. M., Miklos, D., White, D. N., Berlin, Y., Marusic, R., and Prelog, V. (1971). Structure of boromycin. *Helv. Chim. Acta.* **54,** 1709–1713.

Elvers, K. T., and Park, S. F. (2002). Quorum sensing in *Campylobacter jejuni*: Detection of a *luxS* encoded signaling molecule. *Microbiology* **148.5,** 1475–1481.

Fahey, R. C., Brown, W. C., Adams, W. B., and Worsham, M. B. (1978). Occurrence of glutathione in bacteria. *J. Bacteriol.* **133,** 1126–1129.

Fallingborg, J. (1999). Intraluminal pH of the human gastrointestinal tract. *Dan. Med. Bull.* **46,** 183–196.

Feather, M. S. (1981). Amine-assisted sugar dehydration reactions. *Prog. Food Nutr. Sci.* **5,** 37–45.

Fidopiastis, P. M., Miyamoto, C. M., Jobling, M. G., Meighen, E. A., and Ruby, E. G. (2002). LitR, a new transcriptional activator in *Vibrio fischeri*, regulates luminescence and symbiotic light organ colonization. *Mol. Microbiol.* **45,** 131–143.

Fong, K. P., Chung, W. O., Lamont, R. J., and Demuth, D. R. (2001). Intra- and interspecies regulation of gene expression by *Actinobacillus actinomycetemcomitans* LuxS. *Infect. Immun.* **69,** 7625–7634.

Fong, K. P., Gao, L., and Demuth, D. R. (2003). *luxS* and *arcB* control aerobic growth of *Actinobacillus actinomycetemcomitans* under iron limitation. *Infect. Immun.* **71.1,** 298–308.

Forsyth, M. H., and Cover, T. L. (2000). Intercellular communication in *Helicobacter pylori*: *luxS* is essential for the production of an extracellular signaling molecule. *Infect. Immun.* **68,** 3193–3199.

Freeman, J. A., and Bassler, B. L. (1999a). Sequence and function of LuxU: A two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. *J. Bacteriol.* **181.3,** 899–906.

Freeman, J. A., and Bassler, B. L. (1999b). A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. *Mol. Microbiol.* **31.2,** 665–677.

Freeman, J. A., Lilley, B. N., and Bassler, B. L. (2000). A genetic analysis of the functions of LuxN: A two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. *Mol. Microbiol.* **35.1,** 139–149.

Frey, P. A. (2001). Radical mechanisms of enzymatic catalysis. *Annu. Rev. Biochem.* **70,** 121–148.

Frias, J., Olle, E., and Alsina, M. (2001). Periodontal pathogens produce quorum sensing signal molecules. *Infect. Immun.* **69.5,** 3431–3434.

Fuqua, C., and Greenberg, E. P. (1998). Cell-to-cell communication in *Escherichia coli* and *Salmonella typhimurium*: They may be talking, but who's listening? *Proc. Natl. Acad. Sci. USA* **95,** 6571–6572.

Fuqua, C., and Greenberg, E. P. (2002). Listening in on bacteria: Acyl-homoserine lactone signaling. *Nat. Rev. Mol. Cell. Biol.* **3,** 685–695.

Fuqua, W. C., Winans, S. C., and Greenberg, E. P. (1994). Quorum sensing in bacteria: The LuxR/LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **176,** 269–275.

Gefter, M., Hausmann, R., Gold, M., and Hurwitz, J. (1966). The enzymatic methylation of ribonucleic acid and deoxyribonucleic acid. X. Bacteriophage T3-induced S-adenosylmethionine cleavage. *J. Biol. Chem.* **241.9,** 1995–2006.

Giron, J. A., Torres, A. G., Freer, E., and Kaper, J. B. (2002). The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Mol. Microbiol.* **44,** 361–379.

Goldfine, H. (1972). Comparative aspects of bacterial lipids. *Adv. Microb. Physiol.* **8,** 1–58.

Goldfine, H. (1982). Lipids of prokaryotes—Structure and distribution. *Curr. Top. Membr. Transp.* **17,** 1–43.

Goldfine, H., and Ellis, M. E. (1964). N-methyl groups in bacterial lipids. *J. Bacteriol.* **87,** 8–15.

Greenberg, P., Hastings, J. W., and Ulitzur, S. (1979). Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. *Arch. in Microbiol.* **120,** 87–91.

Greene, R. C. (1996). Biosynthesis of methionine. *In ''Escherichia coli* and *Salmonella*: Cellular and Molecular Biology'' (F. C. Neidhardt, R. Curtiss, III, C. A. Gross, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger, eds.), 2nd ed., Vol. 1, pp. 861–886. ASM Press, Washington DC.

Grifantini, R., Bartolini, E., Muzzi, A., Draghi, M., Frigimelica, E., Berger, J., Ratti, G., Petracca, R., Galli, G., Agnusdei, M., Giuliani, M. M., Santini, L., Brunelli, B., Tettelin, H., Rappuoli, R., Randazzo, F., and Grandi, G. (2002). Previously unrecognized vaccine candidates against group B meningococcus identified by DNA microarrays. *Nature Biotechnol.* **20,** 914–921.

Gur, E., Biran, D., Gazit, E., and Ron, E. Z. (2002). *In vivo* aggregation of a single enzyme limits growth of *Escherichia coli* at elevated temperatures. *Mol. Microbiol.* **46.5,** 1391–1397.

Han, K., Hong, J., and Lim, H. C. (1993). Relieving effects of glycine and methionine from acetic acid inhibition in *Escherichia coli* fermentation. *Biotechnol. Bioeng.* **41,** 316–324.

Hanzelka, B. L., and Greenberg, E. P. (1996). Quorum sensing in *Vibrio fischeri*: Evidence that *S*-adenosylmethionine is the amino acid substrate for autoinducer synthesis. *J. Bacteriol.* **178.17,** 5291–5294.

Hanzelka, B. L., Parsek, M. R., Val, D. L., Dunlap, P. V., Cronan, Jr., J. E., and Greenberg, E. P. (1999). Acylhomoserine lactone synthase activity of the *Vibrio fischeri* AinS protein. *J. Bacteriol.* **181,** 5766–5770.

Hardie, K. R., Cooksley, C., Green, A. D., and Winzer, K. (2003). Autoinducer 2 activity in *Escherichia coli* culture supernatants can be actively reduced despite maintenance of an active synthase, LuxS. *Microbiology* **149,** 715–728.

Hastings, J. W., and Nealson, K. H. (1977). Bacterial bioluminescence. *Annu. Rev. Microbiol.* **31,** 549–595.

Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Umayam, L., Gill, S. R., Nelson, K. E., Read, T. D., Tettelin, H., Richardson, D., Ermolaeva, M. D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R. D., Nierman, W. C., and White, O. (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406,** 477–483.

Hemscheidt, T., Puglisi, M. P., Larsen, L. K., Patterson, G. M. L., Moore, R. E., Rios, J. L., and Clardy, J. (1994). Structure and biosynthesis of borophycin, a new boeseken complex of boric-acid from a marine strain of the blue-green-alga *Nostoc linckia*. *J. Org. Chem.* **59,** 3467–3471.

Hildesheim, J., Goguillon, J. F., and Lederer, E. (1973). Selective inhibitions of tRNA methyltransferases by S-adenosylhomocysteine and two of its analogues. *FEBS Lett.* **30,** 177–180.

Hilgers, M. T., and Ludwig, M. L. (2001). Crystal structure of the quorum-sensing protein LuxS reveals a catalytic metal site. *Proc. Nat. Acad. Sci. USA* **98.20,** 11169–11174.

Hiramoto, K., Sekiguchi, K., Ayuha, K., Aso-o, R., Morya, N., Kato, T., and Kikugawa, K. (1996). DNA breaking activity and mutagenicity of soy sauce: Characterization of the active components and identification of 4-hydroxy-5-methyl-3(*2H*)-furanone. *Mutation Res.* **359,** 119–132.

Holmes, W. M., Andraos-Selim, C., and Redlak, M. (1995). tRNA-m1G methyltransferase interactions: Touching bases with structure. *Biochimie.* **77,** 62–65.

Holmgren, A. (1989). Thioredoxin and glutaredoxin systems. *J. Biol. Chem.* **264,** 13963–13966.

Horinouchi, S. (2002). A microbial hormone, A-factor, as a master switch for morphological differentiation and secondary metabolism in *Streptomyces griseus*. *Front. Biosci.* **7,** 2045–2057.

Hsing, W., and Silhavy, T. J. (1997). Function of conserved histidine-243 in phosphatase activity of EnvZ, the sensor for porin osmoregulation in *Escherichia coli*. *J. Bacteriol.* **179,** 3729–3735.

Hübner, A., Revel, A. T., Nolen, D. M., Hagman, K. E., and Norgard, M. V. (2003). Expression of a *luxS* gene is not required for *Borrelia burgdorferi* infection of mice via needle inoculation. *Infect. Immun.* **71,** 2892–2896.

Hughes, J. A., Brown, L. R., and Ferro, A. J. (1987). Expression of the cloned coliphage T3 S-adenosylmethionine hydrolase gene inhibits DNA methylation and polyamine biosynthesis in *Escherichia coli*. *J. Bacteriol.* **169,** 3625–3632.

Hütter, R., Keller-Schierlein, W., Knüsel, F., Prelog, V., Rodgers, Jr., G. C., Suter, P., Vogel, G., Voser, W., and Zähner, H. (1967). The metabolic products of microorganisms. Boromycin. *Helv. Chim. Acta* **50,** 1533–1539.

Igarashi, K., and Kashiwagi, K. (2000). Polyamines: Mysterious modulators of cellular functions. *Biochem. Biophys. Res. Commun.* **271.3,** 559–564.

Irschik, H., Schummer, D., Gerth, K., Höfle, G., and Reichenbach, H. (1995). The tartrolons, new boron-containing antibiotics from a myxobacterium, *Sorangium cellulosum*. *J. Antibiot.* **48,** 26–30.

Jelsbak, L., and Søgaard-Andersen, L. (2000). Pattern formation: Fruiting body morphogenesis in *Myxococcus xanthus*. *Curr. Opin. Microbiol.* 637–642.

Jiang, Y., Camara, M., Chhabra, S. R., Hardie, K. R., Bycroft, B. W., Lazdunski, A., Salmond, G. P., Stewart, G. S., and Williams, P. (1998). *In vitro* biosynthesis of the *Pseudomonas aeruginosa* quorum-sensing signal molecule *N*-butanoyl-L-homoserine lactone. *Mol. Microbiol.* **28,** 193–203.

Jobling, M. G., and Holmes, R. K. (1997). Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. *Mol. Microbiol.* **26,** 1023–1034.

Joyce, E. A., Bassler, B. L., and Wright, A. (2000). Evidence for a signaling system in *Helicobacter pylori*: Detection of a *luxS*-encoded autoinducer. *J. Bacteriol.* **182,** 3638–3643.

Kammler, M., Schon, C., and Hantke, K. (1993). Characterization of the ferrous iron uptake system of *Escherichia coli*. *J. Bacteriol.* **175.19,** 6212–6219.

Kaneshiro, T., and Law, J. H. (1964). Phosphatidylcholine synthesis in *Agrobacterium tumefaciens*. I. Purification and properties of a phosphatidylethanolamine *N*-methyltransferase. *J. Biol. Chem.* **239,** 1705–1713.

Karlin, S., and Zhu, Z. Y. (1997). Classification of mononuclear zinc metal sites in protein structures. *Proc. Natl. Acad. Sci. USA* **94,** 14231–14236.

Kim, S. Y., Lee, S. E., Kim, Y. R., Kim, C. M., Ryu, P. Y., Choy, H. E., Chung, S. S., and Rhee, J. H. (2003). Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. *Mol. Microbiol.* **48,** 1647–1664.

Kirkpatrick, C., Maurer, L. M., Oyelakin, N. E., Yoncheva, Y. N., Maurer, R., and Slonczewski, J. L. (2001). Acetate and formate stress: Opposite responses in the proteome of *Escherichia coli*. *J. Bacteriol.* **183,** 6466–6477.

Kolenbrander, P. E., Andersen, R. N., Blehert, D. S., Egland, P. G., Foster, J. S., and Palmer, Jr., R. J. (2002). Communication among oral bacteria. *Microbiol. Mol. Biol. Rev.* **66,** 486–505.

Kovacikova, G., and Skorupski, K. (2002). Regulation of virulence gene expression in *Vibrio cholerae* by quorum sensing: HapR functions at the *aphA* promoter. *Mol. Microbiol.* **46,** 1135–1147.

Kovacikova, G., and Skorupski, K. (2001). Overlapping binding sites for the virulence gene regulators AphA, AphB, and cAMP-CRP at the *Vibrio cholerae tcpPH* promoter. *Mol. Microbiol.* **41,** 393–407.

Kredich, N. (1996). Biosynthesis of Cysteine. *In* ''*Escherichia coli* and *Salmonella*: Cellular and Molecular Biology'' (F. C. Neidhardt, R. Curtiss, III, C. A. Gross, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger, eds.), 2nd ed., Vol. 1, pp. 514–527. ASM Press, Washington, DC.

Latifi, A., Foglino, M., Tanaka, K., Williams, P., and Lazdunski, A. (1996). A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol. Microbiol.* **21,** 1137–1146.

Lazazzera, B. A., and Grossman, A. D. (1998). The ins and outs of peptide signaling. *Trends Microbiol.* **6,** 288–294.

Lentner, C. (1981). *Geigy scientific tables,* Vol. 1, 8th ed., Ciba-Geigy, Basel, Switzerland.

Levin, J. C., Grant, C. C. R., Surette, M. G., and Dammel, C. S. (2002). Poster B798. 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, California. See also http://www.quorex.com/QuorexHTM/InThePress/F798.pdf.

Lewis, H. A., Furlong, E. B., Laubert, B., Eroshkina, G. A., Batiyenko, Y., Adams, J. M., Bergseid, M. G., Marsh, C. D., Peat, T. S., Sanderson, W. E., Sauder, J. M., and Buchanan, S. G. (2001). A structural genomics approach to study of quorum sensing: Crystal structures of three LuxS orthologs. *Structure* **9,** 527–537.

Lilley, B. N., and Bassler, B. L. (2000). Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. *Mol. Microbiol.* **36.4,** 940–954.

Lopez-Lara, I. M., and Geiger, O. (2001). Novel pathway for phosphatidylcholine biosynthesis in bacteria associated with eukaryotes. *J. Biotechnol.* **91,** 211–221.

Low, D. A., Weyand, N. J., and Mahan, M. J. (2001). Roles of DNA adenine methylation in regulating bacterial gene expression and virulence. *Infect. Immun.* **69.12,** 7197–7204.

Lynch, A. S., and Lin, E. C. C. (1996). Responses to molecular oxygen. *In* ''*Escherichia coli* and *Salmonella*: Cellular and Molecular Biology'' (F. C. Neidhardt, R. Curtiss, III, C. A. Gross, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger, eds.), 2nd ed., Vol. 1, pp. 1526–1538. ASM Press, Washington, DC.

Lyon, W. R., Madden, J. C., Levin, J. C., Stein, J. L., and Caparon, M. G. (2001). Mutation of *luxS* affects growth and virulence factor expression *in Streptococcus pyogenes*. *Mol. Microbiol.* **42,** 145–157.

Lyristis, M., Bryant, A. E., Sloan, J., Awad, M. M., Nisbet, I. T., Stevens, D. L., and Rood, J. I. (1994). Identification and molecular analysis of a locus that regulates extracellular toxin production in *Clostridium perfringens*. *Mol. Microbiol.* **12,** 761–777.

Macintyre, G., Atwood, C. V., and Cupples, C. G. (2001). Lowering *S*-adenosylmethionine levels in *Escherichia coli* modulates C-to-T transition mutations. *J. Bacteriol.* **183.3,** 921–927.

Malott, R. J., and Lo, R. Y. (2002). Studies on the production of quorum-sensing signal molecules in *Mannheimia haemolytica* A1 and other *Pasteurellaceae* species. *FEMS Microbiol. Lett.* **206,** 25–30.

Matthews, R. G. (1996). One-carbon metabolism. *In* ''*Escherichia coli* and *Salmonella*: Cellular and Molecular Biology'' (F. C. Neidhardt, R. Curtiss, III, C. A. Gross, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger, eds.), 2nd ed., Vol. 1, pp. 861–886. ASM Press, Washington, DC.

Mazurek, M., and Perlin, A. S. (1963). Borate complexing by five-membered-ring viv-diols. Vapor pressure equilibrium and N.M.R. spectral observations. *Can. J. Chem.* **41,** 2403–2411.

May, B. J., Zhang, Q., Li, L. L., Paustian, M. L., Whittam, T. S., and Kapur, V. (2001). Complete genomic sequence of *Pasteurella multocida*, Pm70. *Proc. Nat. Acad. Sci. USA* **98,** 3460–3465.

McDougald, D., Rice, S. A., and Kjelleberg, S. (2000). The marine pathogen *Vibrio vulnificus* encodes a putative homologue of the *Vibrio harveyi* regulatory gene, *luxR*: A genetic and phylogenetic comparison. *Gene* **248,** 213–221.

McDougald, D., Rice, S. A., and Kjelleberg, S. (2001). SmcR-dependent regulation of adaptive phenotypes in *Vibrio vulnificus*. *J. Bacteriol.* **183,** 758–762.

McIver, C. J., and Tapsall, J. W. (1990). Assessment of conventional and commercial methods for identification of clinical isolates of cysteine-requiring strains of *Escherichia coli* and *Klebsiella* species. *J. Clin. Microbiol.* **28,** 1947–1951.

McLaggan, D., Logan, T. M., Lynn, D. G., and Epstein, W. (1990). Involvement of gamma-glutamyl peptides in osmoadaptation of *Escherichia coli*. *J. Bacteriol.* **172,** 3631–3636.

McNab, R., Ford, S. K., El-Sabaeny, A., Barbieri, B., Cook, G. S., and Lamont, R. J. (2003). LuxS-based signaling in *Streptococcus gordonii*: Autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. *J. Bacteriol.* **185.1,** 274–284.

Meighen, E. A. (1991). Molecular biology of bacterial bioluminescence. *Microbiol. Rev.* **55,** 123–142.

Mellies, J. L., Elliott, S. J., Sperandio, V., Donnenberg, M. S., and Kaper, J. B. (1999). The Per regulon of enteropathogenic *Escherichia coli*: Identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Mol. Microbiol.* **33,** 296–306.

Merritt, J., Qi, F., Goodman, S. D., Anderson, M. H., and Shi, W. (2003). Mutation of *luxS* affects biofilm formation in *Streptococcus mutans*. *Infect. Immun.* **71,** 1972–1979.

Messerschmidt, A., Worbs, M., Steegborn, C., Wahl, M., Huber, R., Laber, B., and Clausen, T. (2003). Determinants of enzymatic specificity in the Cys-Met-metabolism PLP-dependent enzymes family: Crystal structure of cystathionine γ-lyase from yeast and intrafamiliar structure comparison. *Biol. Chem.* **384,** 373–386.

Miller, C. H., and Duerre, J. A. (1968). *S*-ribosylhomocysteine cleavage enzyme from *Escherichia coli*. *J. Biol. Chem.* **243,** 92–97.

Miller, M. B., and Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55,** 165–199.

Miller, M. B., Skorupski, K., Lenz, D. H., Taylor, R. K., and Bassler, B. L. (2002). Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* **110,** 303–314.

Milton, D. L., Chalker, V. J., Kirke, D., Hardman, A., Camara, M., and Williams, P. (2001). The LuxM homologue VanM from *Vibrio anguillarum* directs the synthesis of *N*-(3-hydroxyhexanoyl)homoserine lactone and *N*-hexanoylhomoserine lactone. *J. Bacteriol.* **183,** 3537–3547.

Miyamoto, C. M., Smith, E. E., Swartzman, E., Cao, J. G., Graham, A. F., and Meighen, E. A. (1994). Proximal and distal sites bind LuxR independently and activate expression of the *Vibrio harveyi lux* operon. *Mol. Microbiol.* **14,** 255–262.

Miyamoto, C. M., Chatterjee, J., Swartzman, E., Szittner, R., and Meighen, E. A. (1996). The role of *lux* autoinducer in regulating luminescence in *Vibrio harveyi*: Control of *luxR* expression. *Mol. Microbiol.* **19,** 767–775.

Miyamoto, C. M., Dunlap, P. V., Ruby, E. G., and Meighen, E. A. (2003). LuxO controls *luxR* expression in *Vibrio harveyi*: Evidence for a common regulatory mechanism in Vibrio. *Mol. Microbiol.* **48,** 537–548.

Mok, K. C., Wingreen, N. S., and Bassler, B. L. (2003). Vibrio harveyi quorum sensing: A coincidence detector for the two autoinducers controls gene expression. *EMBO J.* **22,** 870–881.

More, M. I., Finger, L. D., Stryker, J. L., Fuqua, C., Eberhard, A., and Winans, S. C. (1996). Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. *Science* **272,** 1655–1658.

Nakamura, H., Iitaka, Y., Kitahara, T., Okazaki, T., and Okami, Y. (1977). Structure of aplasmomycin. *J. Antibiot.* **30,** 714–719.

Narasimhan, S., Santiago, F., Koski, R. A., Brei, B., Anderson, J. F., Fish, D., and Fikrig, E. (2002). Examination of the *Borrelia burgdorferi* transcriptome in *Ixodes scapularis* during feeding. *J. Bacteriol.* **184.11,** 3122–3125.

Nealson, K. H., and Hastings, J. W. (1979). Bacterial bioluminescence: Its control and ecological significance. *Microbiol. Rev.* **43,** 496–518.

Nedvidek, W., Ledl, F., and Fischer, P. (1992). Detection of 5-hydroxymethyl1-2-methyl-3(2H)-furanone and of alpha-dicarbonyl compounds in reaction mixtures of hexoses and pentoses with different amines. *Z. Lebensm. Unters. Forsch.* **194,** 222–228.

Newman, E. B., Budman, L. I., Chan, E. C., Greene, R. C., Lin, R. T., Woldringh, C. L., and D'Ari, R. (1998). Lack of S-adenosylmethionine results in a cell division defect in *Escherichia coli*. *J. Bacteriol.* **180.14,** 3614–3619.

Novick, R. P., and Muir, W. M. (1999). Virulence gene regulation by peptides in *Staphylococci* and other Gram-positive bacteria. *Curr. Opin. Microbiol.* **2,** 40–45.

O'Brien, C. H., and Sizemore, R. K. (1979). Distribution of the luminous bacterium *Beneckea harveyi* in a semi-tropical estuarine environment. *Appl. Environ. Microbiol.* **38,** 928–933.

Ohtani, K., Hayashi, H., and Shimizu, T. (2002). The *luxS* gene is involved in cell–cell signaling for toxin production in *Clostridium perfringens*. *Mol. Microbiol.* **44.1,** 171–179.

Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Sugiura, M., Wanner, B. L., Mori, H., and Mizuno, T. (2002a). Transcriptone analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Mol. Microbiol.* **46.1,** 281–291.

Oshima, T., Wada, C., Kawagoe, Y., Ara, T., Maeda, M., Masuda, Y., Hiraga, S., and Mori, H. (2002b). Genome-wide analysis of deoxyadenosine methyltransferase-mediated control of gene expression in *Escherichia coli*. *Mol. Microbiol.* **45,** 673–695.

Palmer, L. M., and Colwell, R. R. (1991). Detection of luciferase gene sequence in nonluminescent *Vibrio cholerae* by colony hybridization and polymerase chain reaction. *Appl. Environ. Microbiol.* **57,** 1286–1293.

Pajula, R. L., and Raina, A. (1979). Methylthioadenosine, a potent inhibitor of spermine synthase from bovine brain. *FEBS Lett.* **99,** 343–345.

Pesci, E. C., Pearson, J. P., Seed, P. C., and Iglewski, B. H. (1997). Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J. Bacteriol.* **179,** 3127–3132.

Posnick, L. M., and Samson, L. D. (1999). Influence of *S*-adenosylmethionine pool size on spontaneous mutation, *dam* methylation, and cell growth of *Escherichia coli*. *J. Bacteriol.* **181.21,** 6756–6762.

Power, P. P., and Woods, W. G. (1997). The chemistry of boron and its speciation in plants. *Plant and Soil* **193,** 1–13.

Prouty, A. M., Schwesinger, W. H., and Gunn, J. S. (2002). Biofilm formation and interaction with the surfaces of gallstones by *Salmonella spp*. *Infect. Immun.* **70,** 2640–2649.

Raina, A., Tuomi, K., and Pajula, R. L. (1982). Inhibition of the synthesis of polyamines and macromolecules by 5′-methylthioadenosine and 5′-alkylthiotubercidins in BHK21 cells. *Biochem. J.* **204,** 697–703.

Ramesh, A., Nandakumar, R., and Venugopalan, V. K. (1986). Enteric microflora of the pond-cultures milk fish *Chanos chanos* (Forskal). *Microb. Ecol.* **12,** 231–235.

Ramesh, A., and Venugopalan, V. K. (1988). Lumimous microflora associated with the fishes *Mugil cephalus* and *Tachysurus arius*. *FEMS Microbiol. Ecol.* **53,** 27–34.

Redfield, R. J. (2002). Is quorum sensing a side effect of diffusion sensing? *Trends Microbiol.* **10,** 365–370.

Reichelt, J. L., and Baumann, P. (1973). Taxonomy of the marine, luminous bacteria. *Arch. Microbiol.* **94,** 283–330.

Reisenauer, A., Kahng, L. S., McCollum, S., and Shapiro, L. (1999). Bacterial DNA methylation: A cell cycle regulator? *J. Bacteriol.* **181.17,** 5135–5139.

Riscoe, M. K., Tower, P. A., and Ferro, A. J. (1984). Mechanism of action of 5′-methylthioadenosine in S49 cells. *Biochem. Pharmacol.* **33,** 3639–3643.

Roe, A. J., O'Byrne, C., McLaggan, D., and Booth, I. R. (2002). Inhibition of *Escherichia coli* growth by acetic acid: A problem with methionine biosynthesis and homocysteine toxicity. *Microbiology* **148,** 2215–2222.

Ron, E. Z. (1975). Growth rate of *Enterobacteriaceae* at elevated temperatures: Limitation by methionine. *J. Bacteriol.* **124,** 243–246.

Ron, E. Z., and Davis, B. D. (1971). Growth rate of *Escherichia coli* at elevated temperatures: Limitation by methionine. *J. Bacteriol.* **107,** 391–396.

Ron, E. Z., and Shani, M. (1971). Growth rate of *Escherichia coli* at elevated temperatures: Reversible inhibition of homoserine trans-succinylase. *J. Bacteriol.* **107,** 397–400.

Roth, J. R., Lawrence, J. G., and Bobik, T. A. (1996). Cobalamin (coenzyme B12): Synthesis and biological significance. *Annu. Rev. Microbiol.* **50,** 137–181.

Ruby, E. G., and Morin, J. G. (1979). Luminous enteric bacteria of marine fishes: A study of their distribution, densities, and dispersion. *Appl. Environ. Microbiol.* **38,** 406–411.

Russell, J. B., and Diez-Gonzalez, F. (1998). The effects of fermentation acids on bacterial growth. *Adv. Microb. Physiol.* **39,** 205–234.

Ruzheinikov, S. N., Das, S. K., Sedelnikova, S. E., Hartley, A., Foster, S. J., Horsburgh, M. J., Cox, A. G., McCleod, C. W., Mekhalfia, A., Blackburn, G. M., Rice, D. W., and Baker, P. J. (2001). The 1.2 A structure of a novel quorum-sensing protein, *Bacillus subtilis* LuxS. *J. Mol. Biol.* **313.1,** 111–122.

Rychlik, I., Martin, G., Methner, U., Lovell, M., Cardova, L., Sebkova, A., Sevcik, M., Damborsky, J., and Barrow, P. A. (2002). Identification of *Salmonella entericaserovar* Typhimurium genes associated with growth suppression in stationary-phase nutrient broth cultures and in the chicken intestine. *Arch. Microbiol.* **178,** 411–420.

Schaefer, A. L., Val, D. L., Hanzelka, B. L., Cronan, Jr., J. E., and Greenberg, E. P. (1996). Generation of cell-to-cell signals in quorum sensing: Acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. *Proc. Natl. Acad. Sci. USA* **93,** 9505–9509.

Schauder, S., and Bassler, B. L. (2001). The languages of bacteria. *Genes Dev.* **15.12,** 1468–1480.

Schauder, S., Shokat, K., Surette, M. G., and Bassler, B. L. (2001). The LuxS family of bacterial autoinducers: Biosynthesis of a novel quorum sensing signal molecule. *Mol. Microbiol.* **41,** 463–476.

Schneider, R., Lockatell, C. V., Johnson, D., and Belas, R. (2002). Detection and mutation of a *luxS*-encoded autoinducer in *Proteus mirabilis*. *Microbiology* **148.3,** 773–782.

Schummer, D., Schomburg, D., Irschik, H., Reichenbach, H., and Höfle, G. (1996). Antibiotics from gliding bacteria. Absolute configuration and biosynthesis of tartalon B, a boron-containing macrolide from *Sorangium cellulosum*. *Liebigs Ann.* **6,** 965–969.

Seiflein, T. A., and Lawrence, J. G. (2001). Methionine-to-cysteine recycling in *Klebsiella aerogenes*. *J. Bacteriol.* **183,** 336–346.

Sekowska, A., Kung, H. F., and Danchin, A. (2000). Sulfur metabolism in *Escherichia coli* and related bacteria: Facts and fiction. *J. Mol. Microbiol. Biotechnol.* **2,** 145–177.

Shaw, N. M., Birch, O. M., Tinschert, A., Venetz, V., Dietrich, R., and Savoy, L. A. (1998). Biotin synthase from *Escherichia coli*: Isolation of an enzyme-generated intermediate and stoichiometry of *S*-adenosylmethionine use. *Biochem. J.* **330,** 1079–1085.

Shimizu, S., Shiozaki, S., Ohshiro, T., and Yamada, H. (1984). Occurrence of S-adenosylhomocysteine hydrolase in prokaryote cells. Characterization of the enzyme from *Alcaligenes faecalis* and role of the enzyme in the activated methyl cycle. *Eur. J. Biochem.* **141,** 385–392.

Shimizu, T., Ba-Thein, W., Tamaki, M., and Hayashi, H. (1994). The *virR* gene, a member of a class of two-component response regulators, regulates the production of perfringolysin O, collagenase, and hemagglutinin in *Clostridium perfringens*. *J. Bacteriol.* **176,** 1616–1623.

Showalter, R. E., Martin, M. O., and Silverman, M. R. (1990). Cloning and nucleotide sequence of *luxR*, a regulatory gene controlling bioluminescence in *Vibrio harveyi*. *J. Bacteriol.* **172.6,** 2946–2954.

Skorupski, K., and Taylor, R. K. (1997). Cyclic AMP and its receptor protein negatively regulate the coordinate expression of cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* **94.1,** 265–270.

Slaughter, J. C. (1999). The naturally occurring furanones: Formation and function from pheromone to food. *Biol. Rev. Camb. Phil. Soc.* **74,** 259–276.

Sperandio, V., Mellies, J. L., Nguyen, W., Shin, S., and Kaper, J. B. (1999). Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc. Nat. Acad. Sci. USA* **96,** 15196–15201.

Sperandio, V., Torres, A. G., Giron, J. A., and Kaper, J. B. (2001). Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. *J. Bacteriol.* **183,** 5187–5197.

Sperandio, V., Li, C. C., and Kaper, J. B. (2002a). Quorum-sensing *Escherichia coli* regulator A: A regulator of the LysR family involved in the regulation of the locus of enterocyte effacement pathogenicity island in enterohemorrhagic *E. coli*. *Infect. Immun.* **70.6,** 3085–3093.

Sperandio, V., Torres, A. G., and Kaper, J. B. (2002b). Quorum sensing *Escherichia coli* regulators B and C (QseBC): A novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. *Mol. Microbiol.* **43.3,** 809–821.

Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P., and Kaper, J. B. (2003). Bacteria-host communication: The language of hormones. *Proc. Natl. Acad. Sci. USA.* **100,** 8951–8956.

Spory, A., Bosserhoff, A., von Rhein, C., Goebel, W., and Ludwig, A. (2002). Differential regulation of multiple proteins of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium by the transcriptional regulator SlyA. *J. Bacteriol.* **184,** 3549–3559.

Stancik, L. M., Stancik, D. M., Schmidt, B., Barnhart, D. M., Yoncheva, Y. N., and Slonczewski, J. L. (2002). pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. *J. Bacteriol.* **184,** 4246–4258.

Steiner, K., and Malke, H. (2001). *relA*-Independent amino acid starvation response network of *Streptococcus pyogenes*. *J. Bacteriol.* **183.24,** 7354–7364.

Stevenson, B., and Babb, K. (2002). LuxS-mediated quorum sensing in *Borrelia burgdorferi*, the Lyme disease spirochete. *Infect. Immun.* **70,** 4099–4105.

Stroeher, U. H., Paton, A. W., Ogunniyi, A. D., and Paton, J. C. (2003). Mutation of *luxS* of *Streptococcus pneumoniae* affects virulence in a mouse model. *Infect. Immun.* **71,** 3206–3212.

Sun, Y. H., Bakshi, S., Chalmers, R., and Tang, C. M. (2000). Functional genomics of *Neisseria meningitidis* pathogenesis. *Nat. Med.* **6,** 1269–1273.

Surette, M. G., and Bassler, B. L. (1998). Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proc. Nat. Acad. Sci. USA* **95,** 7046–7050.

Surette, M. G., and Bassler, B. L. (1999). Regulation of autoinducer production in *Salmonella typhimurium*. *Mol. Microbiol.* **31,** 585–595.

Surette, M. G., Miller, M. B., and Bassler, B. L. (1999). Quorum sensing in *Escherichia coli, Salmonella typhimurium*, and *Vibrio harveyi*: A new family of genes responsible for autoinducer production. *Proc. Nat. Acad. Sci. USA* **96,** 1639–1644.

Swartzman, E., Silverman, M., and Meighen, E. A. (1992). The *luxR* gene product of *Vibrio harveyi* is a transcriptional activator of the *lux* promoter. *J. Bacteriol.* **174,** 7490–7493.

Swift, S., Throup, J. P., Williams, P., Salmond, G. P., and Stewart, G. S. A. B. (1996). Quorum sensing: A population-density component in the determination of bacterial phenotype. *Trends Biochem. Sci.* **21,** 214–219.

Swift, S., Downie, J. A., Whitehead, N. A., Barnard, A. M., Salmond, G. P., and Williams, P. (2001). Quorum sensing as a population-density-dependent determinant of bacterial physiology. *Adv. Microb. Physiol.* **45,** 199–270.

Taga, M. E., Semmelhack, J. L., and Bassler, B. L. (2001). The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*. *Mol. Microbiol.* **42.3,** 777–793.

Takano, J., Noguchi, K., Yasumori, M., Kobayashi, M., Gajdos, Z., Miwa, K., Hayashi, H., Yoneyama, T., and Fujiwara, T. (2002). *Arabidopsis* boron transporter for xylem loading. *Nature* **420,** 337–340.

Taylor, F., and Cronan, Jr., J. E. (1976). Selection and properties of *Escherichia coli* mutants defective in the synthesis of cyclopropane fatty acids. *J. Bacteriol.* **125,** 518–523.

Urbanowski, M. L., Stauffer, L. T., Plamann, L. S., and Stauffer, G. V. (1987). A new methionine locus, *metR*, that encodes a trans-acting protein required for activation of *metE* and *metH* in *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* **169,** 1391–1397.

Urbanowski, M. L., and Stauffer, G. V. (1989). Role of homocysteine in *metR*-mediated activation of the *metE* and *metH* genes in *Salmonella typhimurium* and *Escherichia coli*. *J. Bacteriol.* **171,** 3277–3281.

Val, D. L., and Cronan, Jr., J. E. (1998). *In vivo* evidence that S-adenosylmethionine and fatty acid synthesis intermediates are the substrates for the LuxI family of autoinducer synthases. *J. Bacteriol.* **180.10,** 2644–2651.

Van Bibber, M., Bradbeer, C., Clark, N., and Roth, J. R. (1999). A new class of cobalamin transport mutants (*btuF*) provides genetic evidence for a periplasmic binding protein in *Salmonella typhimurium*. *J. Bacteriol.* **181,** 5539–5541.

Walker, R. D., and Duerre, J. A. (1975). *S*-adenosylmethionine metabolism in various species. *Can. J. Biochem.* **53,** 312–319.

Wang, A.-Y., and Cronan, Jr., J. E. (1994). The growth phase-dependent synthesis of cyclopropane fatty acids in *Escherichia coli* is the result of an RpoS(KatF)-dependent promoter plus enzyme instability. *Mol. Microbiol.* **11,** 1009–1017.

Weissbach, H., and Brot, N. (1991). Regulation of methionine synthesis in *Escherichia coli*. *Mol. Microbiol.* **5,** 1593–1597.

Wen, Z. T., and Burne, R. A. (2002). Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*. *Appl. Environ. Microbiol.* **68,** 1196–1203.

Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J., and Salmond, G. P. (2001). Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol. Rev.* **25,** 365–404.

Whitehead, N. A., Byers, J. T., Commander, P., Corbett, M. J., Coulthurst, S. J., Everson, L., Harris, A. K., Pemberton, C. L., Simpson, N. J., Slater, H., Smith, D. S., Welch, M., Williamson, N., and Salmond, G. P. (2002). The regulation of virulence in phytopathogenic *Erwinia* species: Quorum sensing, antibiotics, and ecological considerations. *Antonie Van Leeuwenhoek* **81,** 223–231.

Winans, S. C. (2002). Bacterial esperanto. *Nat. Struct. Biol.* **9,** 83–84.

Winzer, K., Hardie, K. R., Burgess, N., Dhoherty, N., Kirke, D., Holden, M. T. G., Linforth, R., Cornell, K. A., Taylor, A. J., Hill, P. J., and Williams, P. (2002a). LuxS: Its role in central metabolism and the *in vitro* synthesis of 4-hydroxy-5-methyl-3(*2H*)-furanone. *Microbiology* **148,** 909–922.

Winzer, K.., Hardie, K. R., and Williams, P. (2002b). Bacterial cell-to-cell communication: Sorry, can't talk now—gone to lunch! *Curr. Opin. Microbiol.* **5.2**, 216–222.

Winzer, K., Hardie, K. R., Williams, P. (2002c). LuxS: Its role in central metabolism and the *in vitro* synthesis of 4-hydroxy-5-methyl-3(*2H*)-furanone. Presented at the annual meeting of the Vereinigung für Allgemeine und Angewandte Mikrobiologie, Göttingen, Germany (oral presentation KC011, abstract booklet p44).

Winzer, K., Sun, Y-H., Green, A., Delory, M., Blackley, D., Hardie, K. R., Baldwin, T. J., and Tang, C. M. (2002d). The role of *Neisseria meningitidis luxS* in cell-to-cell signaling and bacteraemic infection. *Infect. Immun.* **70.4,** 2245–2248.

Wisniewski-Dye, F., and Downie, J. A. (2002). Quorum-sensing in *Rhizobium*. *Antonie Van Leeuwenhoek* **81,** 397–407.

Withers, H. L., and Nordström, K. (1998). Quorum-sensing acts at initiation of chromosomal replication in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **95,** 15694–15699.

Withers, H., Swift, S., and Williams, P. (2001). Quorum sensing as an integral component of gene regulatory networks in Gram-negative bacteria. *Curr. Opin. Microbiol.* **4,** 186–193.

Wohrl, B. M., Wehmeier, U. F., and Lengeler, J. W. (1990). Positive and negative regulation of expression of the L-sorbose (*sor*) operon by SorC in *Klebsiella pneumoniae*. *Mol. Gen. Genet.* **224,** 193–200.

Wu, W. F., Urbanowski, M. L., and Stauffer, G. V. (1992). Role of the MetR regulatory system in vitamin B_{12}-mediated repression of the *Salmonella typhimurium metE* gene. *J. Bacteriol.* **174,** 4833–4837.

Yocum, R. R., Perkins, J. B., Howitt, C. L., and Pero, J. (1996). Cloning and characterization of the *metE* gene encoding *S*-adenosylmethionine synthetase from *Bacillus subtilis*. *J. Bacteriol.* **178,** 4604–4610.

Zhu, J., Dizin, E., Hu, X., Wavreille, A. S., Park, J., and Pei, D. (2003). S-Ribosylhomocysteinase (*LuxS*) is a mononuclear iron protein. *Biochemistry* **42,** 4717–4726.

Zhu, J., Miller, M. B., Vance, R. E., Dziejman, M., Bassler, B. L., and Mekalanos, J. J. (2002). Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* **99,** 3129–3314.

Microbiological Contributions to the Search for Extraterrestrial Life

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I. Preface and Introduction

''NASA's mission [is] to understand and protect our home planet; to explore the Universe and search for life; and to inspire the next generation of explorers . . . as only NASA can.''

—U.S. President's Management Agenda, 2002

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A. The Search for Life in the Universe

This chapter will describe the current microbiological knowledge base that supports the quest for life beyond Earth. The interests of researchers funded by the National Aeronautics and Space Administration (NASA) and other Western sponsors—specifically, the European and Russian space agencies—have converged on the search for life beyond earth (Chyba, in press). The characteristics of current ''terrestrial'' targets, which include the planets Mars and Venus as well as the Jovian satellites Io, Ganymede, Europa, and possibly the Saturnian satellites Titan and Enceladus, are described at the websites listed in Table I. Additional relevant information is available elsewhere (Bennett *et al.*, 2003; National Research Council, 2002b). The agencies' effort differs substantially from the older, more popularly familiar ''search for extraterrestrial intelligence'' (SETI) in that the goal is the recognition, characterization, and identification of life forms *in situ*—in the absence of hard evidence for the existence of extraterrestrial life. Indeed, one of NASA's current goals is the demonstration that such life exists. The agency's strategy may be explained via a systems engineering approach (Table II).

This author has a background in general microbiology as well as the physical and engineering sciences and has hands-on experience in the development of biological processes for management of contaminated—i.e., ''extreme''—environments. Involvement in both discovery-based and hypothesis-driven experimentation, as well as in student education, has coincided with an interest in astronomy and science fiction. All of these threads will be examined here and will be applied to the NASA mission ''to improve life here, to extend life to there, and to find life beyond'' (http://www.nasa.gov). Activities beyond the agency initiative will be described in brief.

The exploration of the cosmos by NASA encompasses the field of astrobiology (http://astrobiology.arc.nasa.gov/). ''Astrobiology'' is defined as ''the study of the origin, distribution, and destiny of life in the universe.'' Indeed, there is now a NASA Astrobiology Institute (NAI) encompassing several universities and research centers, and charged with responsibility for research on the origin, distribution, and future of life in the universe (http://nai.arc.nasa.gov/). NASA also has an Exobiology Program, whose mission is slightly more goal-oriented, and which currently focuses on the planet Mars:

TABLE I

WEB-BASED INFORMATION SOURCES DESCRIBING CURRENT EXOBIOLOGICAL EXPLORATION TARGETS

Planetary body	Satellite	Location of information on physical characteristics
Solar system		http://nssdc.gsfc.nasa.gov/planetary/planetfact.html
		http://solarsystem.nasa.gov/features/planets/planet_profiles.html
Venus		http://astrobiology.arc.nasa.gov/topics/venus.html
		http://nssdc.gsfc.nasa.gov/planetary /factsheet/venusfact.html
Earth		http://nssdc.gsfc.nasa.gov/planetary/factsheet/earthfact.html
		http://nssdc.gsfc.nasa.gov/planetary /factsheet/planet_table_ratio.html (planetary comparisons to Earth)
	Moon	http://nssdc.gsfc.nasa.gov/planetary/factsheet/moonfact.html
Mars		http://cmex-www.arc.nasa.gov /SiteCat/sitecat2/mars.htm
Jupiter		http://nssdc.gsfc.nasa.gov/planetary/factsheet/galileanfact_table.html (Galilean satellites—Callisto, Io, Europa, Ganymede)
		http://nssdc.gsfc.nasa.gov/planetary/factsheet/jupiterfact.html
		http://solarsystem.nasa.gov/features/planets/jupiter/jupiter/html
	Callisto	http://galileo.jpl.nasa.gov/moons/callisto.html
	Io	http://solarsystem.nasa.gov/features/planets/jupiter/io.html
	Europa	http://galileo.jpl.nasa.gov/moons/europa.html
	Ganymede	http://galileo.jpl.nasa.gov/moons/ganymede.html
Saturn		http://nssdc.gsfc.nasa.gov/planetary/factsheet/saturnfact.html
	Enceladus	http://nssdc.gsfc.nasa.gov/planetary/factsheet/saturniansatfact.html
		http://solarsystem.nasa.gov/features /planets/saturn/enceladus.html
		http://solarsystem.nasa.gov/features/planets/saturn/titan.html
Extrasolar bodies		http://exoplanets.org /planet_table.shtml
		http://jwst.gsfc.nasa.gov/science/exoplanets.htm
		http://obswww.unige.ch/~udry/planet/planet.html
		http://www.obspm.fr/encycl/catalog.html
		http://www.esa.int.export/esaHS/ESA6FBPZ9NC_future.0.html
		http://www.ciw.edu/boss/IAU/div3/wgesp/planets.shtml

TABLE II

SYSTEMS ENGINEERING AS APPLIED TO THE SEARCH FOR EXTRATERRESTRIAL LIFE[a]

1. State problem clearly (e.g., ''How may we design a system that will detect and identify life beyond Earth?'')
   a. Define operating environment(s)
   b. Identify relevant constraints
      i. Physical/chemical laws
      ii. Mathematical probability
      iii. Available resources
2. Identify useful components (i.e., contributing areas of expertise)
   a. Life sciences including, but not limited to, microbiology
      i. Microbiology (prokaryotic)
      ii. Molecular biology (prokaryotic and eukaryotic)
      iii. Cell biology (eukaryotic)
   b. Planetary sciences
      i. Hydrology
      ii. Mineralogy
      iii. Atmospheric sciences
   c. Physical sciences
      i. Chemistry
      ii. Physics
      iii. Astronomy
      iv. Cosmology
   d. Engineering science
   e. Mathematics
3. Reuse existing knowledge, using Terran life as a model
   a. Define relevant physical/chemical laws
   b. Correlate laws with environment(s) under study
4. Design exploratory system
   a. Establish logical basis for experimentation
   b. Determine operational requirements for primary system
   c. Develop interfaces with other necessary operating systems (Probes? Humans?)
   d. Devise communications tools and/or sensors
   e. Recognize necessary tradeoffs via modeling, simulation, and analysis
      i. Conceptual approach
      ii. System development
      iii. Risk assessment and management protocols (outgoing/incoming)

> . . .to understand the origin, evolution, and distribution of life in the universe. Research is focused on tracing the pathways taken by the biogenic elements, leading from the origin of the universe through the major epochs in the evolution of living systems and their precursors. These epochs are 1) The cosmic evolution of the biogenic compounds, 2) prebiotic evolution, 3) the early evolution of life, and 4) the evolution of advanced life. (http://astrobiology.arc.nasa.gov/workshops/1996/palebluedot/abstracts/meyer01.html; http://cmex-www.arc.nasa.gov/CMEX/data/SiteCat/sitecat2/exobiolo.htm; http://research.hq.nasa.gov/code s/nra/current/NRA-02-OSS-01/appenA2 1 0.doc)

Within NASA, astrobiology includes studies of life in the universe, including exobiology as well as issues surrounding the transport of terrestrial life to other planetary bodies. In contrast, exobiology (studies of life beyond Earth) is synonymous with astrobiology outside the United States.

The role of microbiology in exobiology research is paramount, in that microorganisms essentially dominate the only planet now known to harbor life (i.e., Earth). Indeed, it is estimated that the majority of biomass (''life'') on Earth is bacterial and that this amount can be estimated as a global microbial load of 10^{30} (Sherratt, 2001). This author is not confident of the estimate, in that the mass of funguslike organisms is soil is thought to outweigh that of bacteria; and that some of these estimates predate our recognition of two prokaryotic domains, *Bacteria* and *Archaea* (Woese *et al.*, 1990). (After all, the archaea were first considered to be unusual bacteria.) Perhaps more important, if the domain *Eukarya* is considered by the NASA Exobiology Program to be ''advanced life'' (including humans), the Theory of Serial Endosymbiosis dictates that prokaryotes coevolved to generate eukaryotes (Margulis, 1993). Thus, prokaryotes are important to the evolution of advanced life and are perhaps the product of ''early evolution'' as described previously. Molecular biology has an equally important role in the study of exobiology. Prokaryotic organisms have been postulated to be the earliest life forms, viz. the biological maxims that individual cells are the smallest units of life and that cells may differ greatly from those of humans. This and other important advances in the microbiological sciences—including molecular biology, a field that was organized in the mid-1970s—are described and attributed in Table III. This

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[a] Systems engineering focuses on problem-solving methodology rather than on the actual solution of those problems. The field encourages both basic and applied research—i.e., development of mathematical models and algorithms, to be employed for the design and operation of novel tools and methods—for solving multiple problems within complex and/or unfamiliar environments. Adapted from a presentation by Harley McAdams, Stanford University. 2002. US-DOE Bioinformatics Contractors' Meeting. Washington, DC. 6–7 September 2000. http://devbio1.stanford.edu/usr/hm/.

TABLE III

CORRELATED TIMELINE: THE HISTORY OF SCIENCE AND TECHNOLOGY IN SUPPORT OF THE SEARCH FOR EXTRATERRESTRIAL LIFE, 1546–2001.

Event	Microbiological Sciences	Space Exploration
1546	Fracastoro, an Italian physician, associates human disease with ''invisible organisms,'' and suggests that humans are affected by as yet unknown life forms	Copernicus, a Polish astronomer, publishes treatise describing heliocentric solar system and suggests that Earth is not the center of the universe
~1600	The brothers Janssen (professions unknown; Netherlands) invent first compound light microscope	
1610		Galileo (astronomer and physicist; Italy) improves telescope sufficiently to gather data supporting Copernicus' theory
1632		Galileo publishes treatise describing a heliocentric theory of Earth's solar system but is forced to recant the following year
1664	Hooke (astronomer and physicist; Great Britain) describes fungal structure with aid of low-power light microscope of his own design	
1665	Hooke first describes biological compartments as ''cells''	
1668		Newton (physicist; Great Britain) designs and builds the first reflecting telescope, as first envisioned by Gregory in 1663
1676	Van Leeuwenhoek (custodian and microscopist; Netherlands) improves light microscope, and discovers ''animalcules'' (motile microorganisms—eukaryotic protists)	
1680		Hooke and Newton independently calculate gravitational attractions and suggest elliptical orbits for planetary bodies
1683	Van Leeuwenhoek discovers prokaryotes	
1687		Newton publishes volume detailing methods for launch of an artificial satellite and establishes law of universal gravitation

(*Continued*)

TABLE III (*Continued*)

Event	Microbiological Sciences	Space Exploration
1729	Bourget (profession unknown) publishes letters making the distinction between organic and inorganic growth	
1742	Baker (profession unknown; Great Britain) publishes manuscript introducing the construction and use of the light microscope to the layman	Robins (mathematician; Great Britain) founds science of ballistics
1778	Volta (physicist; Italy) discovers ''flammable air'' in marshes, a first indication that atmospheric composition can be affected by microbial activity	
1781		Herschel (astronomer; Germany) discovers Uranus, the first previously unknown planet
1793	Young (physicist and physician; Great Britain) is first to apply engineering concepts to a biological problem (human vision)	
1794		Chladni (physicist; Germany) proves that meteors are extraterrestrial
1799–1804		Cayley (aerodynamicist; Great Britain) designs first flying machine with fixed wings, control surfaces on the tail, and a means of propulsion; determines basic principles of aerodynamics; becomes first person to fly in a machine heavier than air; and states essential principles required for flight
1802	Lamarck and Trevierons (naturalists; France) independently coin the term ''biology''	
1825		Cuvier (paleontologist; France) introduces ''catastrophe theory,'' suggesting that major chemical and/or physical events have caused the extinction of large groups of species and have altered Earth

(*Continued*)

TABLE III (*Continued*)

Event	Microbiological Sciences	Space Exploration
1827	Brown (botanist; Great Britain), using a microscope, discovers ''Brownian motion,'' the first indication that molecules really exist and interact with cells	
1831	Brown discovers the nucleus of eukaryotic cells	
1838	Schwann (physiologist; Germany) and Scheiden (botanist; Germany) publish work establishing cell as smallest unit of life	Bessel (astronomer; Germany) is first to measure distance to another star, other than the Sun, by measuring parallax of 61 Cygni
1851	Stokes (mathematician; Ireland) describes motion of very small particles through fluids, in quantitative fashion	
1855	Fick (physicist and physician; Germany) describes laws of sedimentation within fluids	
1856	Mendel (monk and botanist; Austria) initiates genetic experiments	
1857	Pasteur (chemist; France) demonstrates life in the absence of oxygen, i.e., fermentation	
1858	Virchow (pathologist; Germany) declares that cells are only produced by other cells—the theory of biogenesis	
1859	Darwin (naturalist; Great Britain) publishes a theory of natural selection, *The Origin of Species*	Arrhenius (physical chemist; Sweden) suggests that microbial life is distributed throughout the universe: the ''panspermia'' theory [a concept revisited by Oparin (biochemist; Russia) in 1936]
1860	Mendel discovers the laws of heredity; Pasteur disproves theory of spontaneous generation (i.e., that life arises wholly from nonliving material)	
1862	Pasteur announces germ theory of disease	
1863		Huggins (astronomer; Great Britain) uses spectra of stars to show that the same elements exist in the stars as on Earth

(*Continued*)

TABLE III (*Continued*)

Event	Microbiological Sciences	Space Exploration
1869	Miescher (biologist; Switzerland) discovers nucleic acids (DNA, RNA); Mendelye'ev (chemist; Russia) states periodic law of the chemical elements	
1871–1874	Lister (microbiologist and pathologist; Great Britain) defines aseptic techniques and establishes concept of pure (axenic) culture	
1876	Tyndall (microbiologist; Great Britain) proves that microorganisms are carried on dust	
1878	Warrington (profession unknown; Great Britain) shows that microorganisms are basis of a global nitrogen cycle and establishes microorganisms as key players in planetary chemistry	
1881		Ganswindt (inventor, Germany) calculates velocity needed to escape Earth's gravitational force, i.e., the ''escape velocity''
1883		The brothers Tissandier (profession unknown; France) launch first airship powered with electric engine
1884	Koch (physician; Germany) publishes ''postulates'' for infectious disease, providing a model linking microorganisms and humans; LeChâtelier (chemical engineer; France) clarifies principles underlying chemical equlibrium	
1887	Winogradsky (microbiologist; France) initiates study of oxygenated respiratory substrates (used in the absence of oxygen) and establishes concept of anaerobic growth	
1890	Winogradsky demonstrates autotrophic growth (use of CO_2 as carbon source), first attack on the ''viable-but-nonculturable'' problem	
1892	Ivanovski (botanist; Russia) isolates plant virus: noncellular ''life''	

(*Continued*)

TABLE III (*Continued*)

Event	Microbiological Sciences	Space Exploration
1897	Thomson (physicist; Great Britain) discovers the electron	Schwarz (profession unknown; Germany) builds first rigid-hulled airship, using an aluminum frame and sheeting
1899	Beijerinck (microbiologist; Netherlands) postulates that viruses reproduce within (parasitize) cells	
1903		Tsiolkovsky (aeronautical and astronomical scientist; Russia) proposes that liquid oxygen be used for powering rocket engines for space travel and use of multistage rockets
1909	Garrod (physician; Great Britain) posits that specific proteins correspond to specific individual genes	
1910	Rous (physician; United States) discovers that viruses can cause cancer in ''higher'' organisms	
1912	Loeb (physiologist; Germany) publishes *The Mechanistic Conception of Life*, using chemistry and physics to explain the origin of life	Hess (profession unknown; Austria and United States) discovers cosmic rays during a balloon ascent
1913–1914	Bohrs (physicist; Netherlands) writes trilogy on the constitution of atoms and molecules; Rutherford (physicist; Great Britain) discovers the proton	Goddard (physicist; United States) develops experimental rockets
1915–1917	D'Herelle (microbiologist; France) and Twort (bacteriologist; Great Britain) independently publish description of (bacterial) DNA viruses, extending ecological concepts to the microbial world	Einstein (physicist; Germany and United States) completes his theory of gravitation, known as the general theory of relativity
1918		Shapley (astronomer; United States) makes first accurate estimate of the size of the Milky Way galaxy and locates the solar system near its outer edge
1924		Oberth (profession unknown; Germany) gives first truly scientific account of techniques for space research

(*Continued*)

TABLE III (*Continued*)

Event	Microbiological Sciences	Space Exploration
1932		Piccard (physicist; Belgium) becomes first human to enter the stratosphere, via a balloon
1933	Ruska and Ruedenberg (physicists; Germany) independently build first electron microscopes	
1934		Von Braun (physicist; Germany and United States) successfully launches first rockets powered by alcohol and liquid oxygen
1936	Bawden and Pirie (physical chemists; Great Britain) describe an RNA virus (tobacco mosaic)	
1937	Chatton (microbiologist; France) discriminates between prokaryotes and eukaryotes; Krebs (biologist; Germany and Great Britain) describes citric acid (respiratory) cycle	
1941	Beadle and Tatum (biochemists; United States) describe transmission of hereditary characteristics, from nucleic acids through proteins, thus supporting Garrod's hypothesis	
1944	Avery, MacLeod, and McCarty (biochemists; Canada and United States) demonstrate that DNA carries information in most organisms	Schrödinger (physicist; Austria) publishes agenda to define biology in mechanistic terms: *What is Life?*
1951	McClintock (cytogeneticist; United States) presents discovery of transposable genetic elements in plants; concept was extended to prokaryotes by Jacob and Hedges in 1974	
1952	Gale (chemical microbiologist; Great Britain) posits ''Theory of Microbial Infallibility''	
1953	Crick (molecular biologist; Great Britain), Franklin (physical chemist; Great Britain), Gosling (physical chemist; Great Britain), Stokes (mathematical physicist; Great Britain), Watson (biochemist; United States), Wilkins (biophysicist; Great Britain), and Wilson (biophysicist; Great Britain) describe structure of	Miller (chemist; United States) and Urey (astronomer and physical chemist; United States) create biomolecules under conditions simulating what was then postulated of early Earth

(*Continued*)

TABLE III (*Continued*)

Event	Microbiological Sciences	Space Exploration
	deoxyribonucleic acid (DNA), a deduction based on their three papers that appeared simultaneously within the same issue of *Nature*	
1956	Anderson (microbiologist; United States) discovers *Deinococcus radiodurans*, an extraordinarily radiation-resistant microorganism, in can of irradiated meat	
1957		Soviet Union launches first artificial satellite, with German technology; ''space age'' begins
1958	United States launches first artificial satellite, using German technology; ''space race'' begins; Lederberg (molecular biologist; United States) encourages U.S. National Academy of Sciences to have NASA incorporate planetary protection, i.e., microbial management measures, into U.S. space missions; International Council of Scientific Unions, now the International Council for Science, establishes Committee on Space Research (COSPAR) during an international meeting in London, U.K.	
1960	Drake (astronomer; United States) develops algorithm to calculate the probability of ''complex'' life in the universe	
1961		Gagarin (cosmonaut; Soviet Union) makes first orbital flight in spacecraft; Shepard (astronaut; United States) makes first American spaceflight
1962		United States launches first object (space probe Mariner 2) to voyage to another planet, Venus
1963	Sagan (astronomer; United States) detects adenosine triphosphate (ATP) in a mixture of chemicals thought to be present early in Earth's history	
1967	Caskey, Marshall, and Nirenberg (biochemists; United States) suggest that the genetic code is a universal system used by all life forms	
1969		Armstrong (astronaut; United States) first walks on moon
1971		First space station, Salyut I, established by Soviet Union
1974	Jacob and Hedges (molecular biologists; United States) discover transposons, movable genetic elements within bacteria	Soviet Union lands space probe on Mars
1975		First cooperative space mission (United States and Soviet Union) launched

(*Continued*)

TABLE III (*Continued*)

Event	Microbiological Sciences	Space Exploration
1976		First space probes, from United States, soft-land on Mars and send back direct pictures and other data
1977	Woese (molecular biologist; United States) establishes *Archaea* as distinct from *Bacteria*	
1979	Ballard (oceanographer; United States) and Grassie (biologist; United States) discover submarine ecosystem supported by microbial chemical energy transformations	
1981	Margulis (microbiologist; United States) proposes Serial Endosymbiosis Theory of the origin of eukaryotic cells	
1982	Prusiner (physician; United States) proposes existence of prions (protein-based ''life'' forms)	
1986		Soviet Union establishes first space station
1987	Gantí (microbiologist; Hungary) describes organization of living systems in engineering terms	Campbell, Walker, and Yang (astronomers; United States) announce discovery of extrasolar planets
1991–1992		Wolszczan and Frail (astrophyscists; Switzerland) report discovery of first extrasolar planets (PSR 1257+12 A,B,C,D)
1995	NASA establishes Astrobiology Institute in United States	
1996	McKay (planetary scientist; United States) posits that meteorite ALH 84001 from Mars contains evidence of microbial life	
1998	NASA names Nealson (microbiologist; United States) as head of domestic Exobiology Branch	
1999	Gold (astronomer; United States) suggests subsurface microbial systems as key target for exobiological exploration (*The Hot Deep Biosphere*)	
2000	Ward (geologist; United States) and Brownlee (astonomer; United States) define ''habitable'' planetary bodies and suggest that microbial life is common in the universe (*Rare Earth*)	
2001	Horneck (biologist; Germany) and colleagues demonstrate that microbial cells are unlikely to survive interstellar transport unless associated with planetary ejecta and advance concept of ''pan*litho*spermia''	

Sources: Barnes-Svarney, P. (1995); Brock, T. D. (1999); Bunch, B., and Hellemans, A. (1993); Hellemans, A., and Bunch, B. (1991); Stevenson, D. J. (2000).

information is correlated with advances in flight and space exploration. The table begins with the interesting coincidence that 1546 ushered in the twin realizations that (1) life on Earth is not unique to humans (and that invisible life forms may affect humans) and (2) humans, and their lives, are not necessarily the focus of the universe.

What are the minimal criteria for life, where might it be found, and how might it be recognized? This chapter analyzes the roles of microbiology and (to a lesser extent) molecular biology in answering the first two questions by charting the contributions that microbiological research has made to the search for life in nonterrestrial environments. Prokaryotic microbiology encompasses the study of the simplest, but possibly the most complex, cellular life forms—bacteria and archaeons. Environmental prokaryotic microbiology has explored some of the extreme (for humans) environments where these organisms live and has measured their environmental impact thereon. The field has historically included study of not-quite-alive forms: viruses (of varying degrees of complexity, including the presence or absence of a membranous enclosure), viroids (naked RNA), and prions (apparently naked protein). But these acellular almost-life forms—as well as naked DNA, the key informational molecule for ''advanced life''—may well fall within the province of molecular biology. Microbiology and molecular biology are thus critical to understanding early events in prebiotic and biotic evolution. This chapter also postulates a working definition for life, to aid in recognizing its signature.

B. Predictions Concerning Life Beyond Earth

Human exploration of apparently empty space, for purposes of discovery or exploitation, has required critical technological advances (Table III). Before invention of the microscope and telescope, humans could only philosophize about what they could not see; in fact, a form of solipsism—''what I can't see isn't there or at least it won't hurt me''—pervaded. After introduction and popular usage of these two instruments, two different self-referential thoughts arose. The first anthropocentric thought was that ''All life forms require what I require,'' where those requirements were oxygen, moderate temperature and pressure, and avoidance of hazardous and/or radioactive materials. The second idea was that life forms would be macroscopic animals. These conceptions still prevail among some nonmicrobiologists.

But this anthropocentrism regarding the search for extraterrestrial life may be waning. In the first days of the space programs, the concern

about humanoid ''invaders'' (little green men with advanced weapons) has changed: first, to fears of bellicose, weird-looking animals with equally dangerous weapons; then, to alarms about microbial pathogens able to cause disease or to destroy materièl; and finally, to an appreciation that the off-world creatures may simply be different and have no specific feeling toward, or interest in, humans at all.

In the scientific domain, humans had first thought to search deliberately for ''intelligent'' [animal?] life. The Search for Extraterrestrial Intelligence (SETI) operated on the assumption that nonterrestrial life forms would resemble humans intellectually and technologically, and demanded the use of advanced technologies for communication (Clark, 2000). If one accepts a conservative calculation, there should be a minimum of 2400 ''communicating civilizations'' in the universe. However, in the nearly 50 years since the initiation of space flight and SETI, there has been no good evidence for the existence of advanced life forms, according to human criteria, elsewhere in the universe. It is possible that humans are alone in the universe—if life is defined solely as being humanoid with respect to scientific and technological advances.

But if one accepts the following principles (Clark, 2000), it seems statistically improbable that there is no life whatsoever beyond Earth:

- Principle of Uniformity of Nature: that the laws of nature are the same throughout the universe
- Copernican Principle: that the Earth is not the center of the Universe
- Principle of Plenitude: that anything that *can* happen *will* happen.

And astrobiology researchers have described Earth as merely being in ''the right [cosmic] place at the right [cosmological] time'' for the development of ''advanced life'', ''intelligence'', and ''communicating civilizations'' (Ward and Brownlee, 2000). [The use of quotation marks is deliberate; the biophysicochemical definition of each of these terms is open to debate.] The authors maintain instead that there is life on other planetary bodies but that this life is more likely than not to be microbial; hence, the importance of microbiology to NASA. [Note: the interplanetary exchange of solid material, possibly including microorganisms, has been described (Gladman *et al.*, 1996). The current controversy over Martian meteorite ALH84001 hinges on that argument.] The reader should note that such predictions are couched in probabilistic and not absolute terms.

C. Goal Statement

This chapter builds upon the conceptual framework outlined above—that there probably is extraterrestrial life and it is probably microbial in nature—to describe the basis for a search for extraterrestrial life. It is possible that this extraterrestrial life will be more advanced, i.e., nucleated, macroscopic, and/or multicellular. A focus on prokaryotic life would be valid because (1) prokaryotes are thought to be subunits of advanced life forms, probably as former endosymbionts that have become organelles and (2) advanced life forms live in necessary symbiosis with microorganisms, which support metabolic function. This chapter does not address symbiotic issues in any detail, except to note microbes' role in establishing functional ecosystems. The focus here will be on prokaryotes—*Bacteria* and *Archaea*—and portions thereof. Endosymbiotic possibilities, and hence *Eukarya*, are beyond our scope except for mention of unicellular fungi (yeasts). Molecular biological issues will be entertained only for purposes of defining life.

II. How Has Microbiology Contributed to a Definition of Extracellular Life?

A. Clarification of the Basic Unit of Life on Earth

1. *Life's Spatiotemporal Characteristics*

Life, as exhibited by any organism, is a dynamic process. The basic attributes of living things—growth, feeding (including excretion and elimination), enlargement, reproduction (including limited change over generations), and death—are easily visualized in a manageable timeframe by study of prokaryotes. Moreover, these qualities are readily measurable; life forms can be readily distinguished from nonliving things, such as crystals or bubbles. The dynamic state is evident, in that the rates at which changes are made are greater than those associated with nonlife: in other words, the kinetics of biotic activities are more rapid than are those of abiotic events. The living thing is in a state of disequilibrium and can respond to changes and perturbations in its environment. In some cases the response is death—the attainment of thermodynamic equilibrium. Microbiological studies have documented these phenomena in real time and allowed their characterization.

2. *Development of a Cell-based Model for ''Natural'' Phenomena*

Humans are known to ascribe significant events and conditions to a vaguely defined ''Mother Nature'' or to a higher power. Table III chronicles microbiological advances that clarified the basis of certain of these

phenomena. This timetable necessarily correlates microbiological progress with advances in imaging technologies.

Van Leeuwenhoek's experiments had demonstrated the presence of ubiquitous, tiny life forms. But the simplest assay, before sophisticated technical equipment was available, for microbial activity is pathogenesis—a term that encompasses the creation of disease in higher organisms (humans as well as macroscopic plants and animals). Indeed, it was this activity that brought microorganisms to prominence as a subject of study, in part, through establishment of Koch's postulates. At the beginning of the twentieth century, it was shown that cellular organisms were themselves susceptible to pathogenesis by acellular microbial forms. These final ecological observations closely preceded the distinction between prokaryotes and eukaryotes (Chatton) and the identification of the genetic, or informational, materials in cellular and acellular life forms (various twentieth-century workers). A current ecological thesis holds that microorganisms are responsible for what are not yet recognized as infectious chronic diseases (Ewald, 1996). In the context of this chapter, it is ironic that a microbiological theory on the source of human disease first coincided with an astronomical theory regarding man's place in the universe.

In studies reaching as far back as the eighteenth century, it was shown that microorganisms could alter the gaseous (Volta's observations) or mineral (Winogradsky's work) composition of a given niche. This work, as well as a growing realization that microorganisms are responsible for the cycling of key elements in Earth's environment, led to identification of energetic metabolism that is not related to oxygen. [''Oxygen,'' as the etymological root of *oxidation*, causes an unfortunate confusion of the latter term with *oxygenation*. The critical concept in element cycling is ''redox,'' or *oxidation-reduction*.] Microorganisms, by oxidizing and reducing elements to which they were exposed, could change the chemistry of their environment. Cumulative microbial activities over time apparently contributed to today's atmosphere and geochemistry.

Investigations like those described here have encouraged careful microbial analysis. Various researchers, whose identities are lost to time, slowly revealed that microbial biomass is based on primarily carbon, plus hydrogen, as scaffolding elements, and that hydrogen, oxygen, nitrogen, sulfur, and phosphorus are also important biomass components. It is generally accepted that elemental analysis of microbial biomass, normalized to nitrogen content, is $C_4H_7O_{1.5}N$ (bacteria) and $C_9H_{15}O_4N$ (fungi). These values may be compared to that for humans: $C_{7.2}H_{72}O_{21}N$. The composition of soil organic matter, which

includes residues of microorganisms, plants, animals, and other macroorganisms, is $C_{10}H_{13}O_5N$. The concentrations of sulfur and phosphorus in biomass are negligible. There are available methods and data for calculating the elemental compositions of microorganisms (http://www.chemeng.drexel.edu/web_books/EngBio/Hidden/react/Ch2/2_1.htm) and humans (http://fig.cox.miami.edu/~cmallery/150/life/elements.htm)

3. *Prokaryotes as a Model for Life: A Reductionist Argument*

Ever since Schwann and Schleiden (Hellemans and Bunch, 1991) established the concept of the cell as basic unit of life, organisms that consist of one cell have been considered, in a way, a basic form of life. Cells (with notable exceptions such as certain animal eggs) tend to be smaller than the human eye can resolve—a value traditionally taken as 0.2 mm. Prokaryotes tend to be approximately one micron (1 μm or 0.001 mm) in their longest dimension, although visible bacteria (*Epiviscularium* and *Thiomargarita* spp.) are known (Angert, Clements, and Pace, 1993; Clements and Bullivant, 1991; Shulz *et al.*, 1999). However, current controversies whirl around the question, ''what is the smallest detectable living thing?'' (National Research Council, 1999). The answer necessarily depends on the limits of current technologies.

A symbiotic archaean has been identified that is just 400 nm (0.4 μm or 0.0004 mm) in diameter and lives on the surface of another slightly larger archaeum (Huber *et al.*, 2002). This recent description appears to set a lower boundary for dependent prokaryotic life, at least in conceptual terms. That report is moreover consistent with understanding of mollicutes (e.g., mycoplasmas, eukaryotic symbionts), which can be as little as 100 nm in length. Smaller nanometer-sized structures called nan(n)obacteria, or ''nanobes,'' have been described but have not yet been demonstrated to be living (Uwins *et al.*, 1998).

Acellular symbiotic entities—where the symbiosis involves a parasitic, sometimes pathogenic, lifestyle—are well known. Viruses, consisting of DNA or RNA contained within a protein coat and/or lipid envelope, are submicroscopic and range from 20 to 200 nm in size. Even smaller lifelike forms—prions, viroids, or various poorly defined viruslike oddities (''virinos'')—are the size of a macromolecule. But acellular forms are not capable of independent propagation. Similarly, macromolecular biomass such as plasmids, cosmids, transposons, and ribozymes, whose manipulation and assay require cells, are considered neither living nor nonliving. The cell is the basic unit of life as understood by humans.

Cells are defined as being truly autopoietic (i.e., autonomous and self-maintaining) and to be separated from their environment by some sort of envelope. That environment must also include macromolecular assemblages necessary for acquisition of mass, conversion of energy, and processing of information—the three integral and integrative capabilities of life on Earth, as described by microbiologists. But underlying these capabilities are principles delineated within the fields of mathematics (topology, geometry, and probability); chemistry, whether physical, inorganic, or organic; and physics.

Current biological, chemical, and physical understanding of life demands that the cell envelope include a semipermeable membrane that allows constant communication with, and exploitation of, the environment (Fox, 1973; Morowitz *et al.*, 1988). An elastic, but relatively rigid, external wall with many of the same characteristics would also be needed to protect the membrane contents from the effects of turgor (hydrostatic pressure) (Koch and Silver, 2003). Activities essential to life must occur within or adjacent to the space defined by the envelope. Specific approaches to matter and energy acquisition can be combined independently. Cell shape may be spherical or cylindrical, to allow for an optimal ratio of volume to surface area even during growth (extension). The reaction matrix for biological activities is water; indeed, this solvent accounts for up to 90% of cell biomass (and hence cell volume). Cells live in hydric or humid microenvironments which may consist of only a monolayer of liquid water. Biochemical reactions, whether intracellular or extracellular, occur in the aqueous phase; the laws of solution chemistry insofar as they concern kinetics and thermodynamics apply. Concepts underlying equilibrium and hydrodynamics are also relevant. Life appears to be powered by electromagnetic conversions related to irradiation or redox but may acknowledge gravitational forces. Physicochemical discontinuities (e.g., interfaces) and other inhomogeneities may stimulate specific biological behaviors. Life responds to gradients.

This understanding is based on all current data from the one planetary system known to harbor life: Earth. This document will characterize unicellular Earth-based life but will extrapolate from current understanding to enable humans to recognize life—even ''xeno-life'' elsewhere. The definition of cellular terrestrial life provided in the preceding paragraphs relies heavily on physical and chemical laws. This definition further suggests that life can someday be expressed in the form of a universal mathematical model that integrates biological tenets with their cosmological realities. Such a model would accommodate the cosmic abundance of elements (www.humboldt.edu/~gdg1/

cosmicab.html), ambient conditions on specific planetary bodies, and proven biological factors [evolution, including randomness, biochemical (genetic) blueprinting, and natural selection; and ecology (Leadbetter and Poindexter, 1985; Madigan *et al.*, 1997). But the immediate challenge is to identify *life as we know it* beyond Earth.

Earthlike life can be detected by its biosignatures (http://nai.arc.nasa.gov/institute/lead_teams_details.cfm?ID=6; Cady, 2001; Crawford *et al.*, 2001; Des Marais *et al.*, 2002; Gorbushina *et al.*, 2002). A systems engineering approach to the search for extraterrestrial life (Table II) may prove to be a good model for a systems approach to biology, as suggested by Gantí and Schrödinger in the recent past and that is currently under development (Drossel, 1999; Ideker *et al.*, 2001). Still, an interim generic model for defining life, on the level of a single microorganism, is provided in Table IV. A primer on microbial structure and function is provided next.

4. *Biochemical and Biophysical Attributes of Microorganisms*

Prokaryotes are bounded by a semipermeable cytoplasmic membrane consisting of a lipid bilayer structure that is impregnated with functional proteins. The lipids define the cell margin, and the proteins aid in import and excretion of material. Water and certain dissolved ions, however, pass freely through the membrane. Membrane lipids are phosphorylated (i.e., are phospholipids) and thus polar; however, the membrane interior is hydrophobic. Archaeal lipids contain fatty alcohols; bacterial lipids, like those of eukaryotes, contain fatty acids. The cell envelope may extend beyond the cytoplasmic membrane to include a peptidoglycan cell wall that may itself be coated with lipids. Peptidoglycan is composed of specific amino acids and aminomonosaccharides. Certain archaeans have a pseudopeptidoglycan cell wall instead; others have walls composed of polysaccharide, glycoprotein, or protein. [The prebiotic composition of the cell wall is currently under study (Dworkin *et al.*, 2001; Martin and Russell, 2002).]

The ''gram-positive'' prokaryotes, so called because of their staining characteristics in the laboratory, have acidic phosphopolysaccharides (teichoic or lipoteichoic acids) attached to their cell wall. These organisms have a negative surface charge. ''Gram-negative'' prokaryotes possess a second, outer lipopolysaccharide membrane. There is some evidence that gram-positive organisms have outer membranes as well. The periplasmic space, between the two membranes, contains lipoproteins and enzymes. Bacteria and archaea can be distinguished on a basis of their distinctive membrane lipids, as can different groups

within these domains. Although most amino acids within microbial proteins possess the L configuration upon optical analysis, cell surface proteins may contain D-amino acid residues.

The cytoplasm of prokaryotes is an organized, rather viscous gel that contains most of the metabolic machinery that supports life. This machinery includes various polysaccharides and proteins, the latter of which are enzymes that carry out necessary matter interconversions and energy harvesting. Information, however, seems to be stored primarily in polymers that contain nucleic acids: DNA and RNA. The relatively recent identification of prions as pathogenic agents, when coupled with traditional understanding of the conformation of enzyme active sites, suggests that significant information is stored within proteins as well. In both cases (nucleic acid and protein), the critical aspect of information storage appears to be not the two-dimensional sequence of monomers, but instead the three-dimensional structure. It is this structure that confers information-managing activity. Some degeneracy in biological systems has been detected with respect to catalysis (by ribozymes, or RNA sequences) as well (Edelman and Gally, 2001; Ideker *et al.*, 2001). This phenomenon, of using completely different tools to accomplish the same task, differs clearly from redundancy, and possibly can best be explored through study of manmade information systems. Nucleic acid analysis is routinely used to distinguish among different microorganisms; protein profiles are somewhat less revealing—suggesting a basis for nucleic acids as primary informational molecules. However, those seeking to identify extraterrestrial life must appreciate that DNA/RNA are not the only keys to information storage.

B. Bridge between the Physical, Life, and Social Sciences

1. *Microbiology as a Manifestation of Physical Laws*

The study of microbial systems, from the individual through the community levels, has illustrated aspects of cosmology that can actually be expressed in mathematical terms. Cosmological analysis indicates that hydrogen is quite plentiful in the universe. Physical understanding explains that hydrogen is the building block of matter and that the abundance of most elements decreases exponentially with atomic number. Conversely, chemical experimentation has shown that the electromagnetic reactivity of elements decreases with atomic number. The three principles stated earlier (Section 1.2), when applied to biology, are consistent with life existing in the universe *as*

TABLE IV

LIFE—A GENERALIZED WORKING DEFINITION[a]

Life is a transient condition that treads close to the bounds of the second law of thermodynamics. In kinetic terms, life is a state of dynamic disequilibrium that obeys the laws of physics and chemistry, reflects the tenets of cosmology and the planetary sciences, and makes manifest the concepts of ecology and evolution. The behavior of individual life forms may ultimately be described in mathematical terms that integrate these subject fields but recognize the existence of chaotic and/or stochastic processes. The behavior of the aggregate, however, might be made more predictable through understanding of ecological relations and evolutionary processes. But life of individual units, as we know it, cannot be sustained in the absence of other life forms and their collective action. Nor can life be sustained in the absence of an external source of energy, via chemical physics, which is well known; irradiation via photosynthesis, which is also well understood, or cosmic rays (Bernstein *et al.*, 2001; Blankenship, manuscript in preparation); or possibly radiolysis (Fredrickson and Onstott, 1997; Onstott *et al.*, manuscript in preparation).

Biomass may currently be described as a complex, locally ordered assemblage of macromolecules contained within one or more semipermeable envelopes. These biomolecules are based on scaffolding elements that have a minimal number of shells (i.e, are on the top row of their groups) and are near the middle of their periods. In an oxidizing Earth environment, these elements are typically slightly electronegative. Biomolecules consist of a primary structure stabilized by covalent bonding. Associated secondary to quaternary structures may be ascribed to ionic- or hydrogen-bonding, Van der Waals forces, and/or weak interactions with other macromolecules and solvents. The topology (three-dimensional conformation) accomplished by these associations contributes to the molecules' activity. The biomolecules are dissolved or suspended in protonated liquid solvents capable of forming ionic and hydrogen bonds, with relevant transformational activities occurring within gradients or at interfaces. The rules of fluid mechanics, which are affected in turn by gravitational forces, dominate the physical aspects of biomolecular activities. Electron transfer reactions characterize the activities' chemical aspects.

The selection of elements for scaffolding or energy capture is based on the elements' localized availability. Cosmology and physics dictate that low atomic number elements will predominate in the universe and may predict their locations, but does not define their suitability. Planetary science then determines ambient oxidation–reduction potential, temperature, and pressure—all of which will affect the elements' physicochemical state.

Life changes its environment through manipulation of elemental energy states; current understanding of this manipulation is that life on Earth exploits the electromagnetic forces (Deamer, 1997). Biological work, such as osmosis, metabolism, and movement, is made possible by capturing energy from an external source. The captive energy is contained within multiply-bonded functionalities involving electronegative elements at or near the top row of their groups.

(*Continued*)

TABLE IV (*Continued*)

Transformations of mass and energy within organisms are mutually dependent, with growth and reproduction differing significantly from survival. For a given organism, the choice of substrate(s) for energy capture, electron transfer, and acquisition of the scaffolding element(s) is determined stochastically. On this planet, energy capture is described by phototrophy versus chemotrophy, electron donors and acceptors by lithotrophy versus organotrophy, and scaffolding element by autotrophy versus heterotrophy. This nomenclature implies a binary code for execution of life's processes on Earth; however, these geocentric semantic distinctions—like those of oxidation versus oxygenation—may cloud an underlying truth.
Life is autopoietic though not autocatalytic. The instructions that guide transformation of biomass and energy, as well as reproduction, are contained in informational molecules. The necessary information is encoded in specialized biomolecules as described, and replicated through succeeding generations. However, random (physicochemical) and nonrandom (biological) events, such as mutation, genetic recombination, excision-repair, and natural selection ensure that succeeding generations are not identical to the parent(s). The fitness of these latter generations may be determined by their interrelationships with the environment and other life forms. Evolution and ecology are interdependent.
A given planetary body, and any life forms that live therein, may appear to be a closed system. But local increases in thermodynamic order—within, among, and caused by life forms whose population increases exponentially—contribute to localized transient decreases in entropy. These decreases may raise the overall entropy of a planetary environment and possibly the universe beyond.

[a] This table attempts to provide a working definition that is linear and positive rather than negative or circular. This definition dwells on neither the hallmarks of life (see text) nor the qualities that describe what life is *not* and extrapolates from the only data now available—those describing life on Earth. Sources: Haldane, 1947; Margulis, 1998; Orgel, 1973; Wolstencroft and Raven, 2002.

long as that life is considered in the context of the planetary system under study. This requirement would tailor life to ambient temperature, pH, redox potential (E_h), and pressure. Table IV provides a generalized working definition of such life by describing its physicochemical influences. Each of those influences can be described by formulae such as Fick's laws—which require definition of locally specific constant values—and/or scientific axioms such as LeChâtelier's principle. The relationships described by these formulations are also assumed here to be universal. These relationships are perhaps best appreciated by study of life as an engineering problem requiring some understanding of mass and heat (energy) transfer within a fluid medium. On Earth, that medium is water. However, current biological understanding that cellular functioning is inextricably linked to the proton-motive force, and macromolecular assemblages *in vivo* are stabilized by hydrogen

bonding, suggests that other protonated solvents capable of hydrogen-bond formation (such as neat ammonia?) would support life.

2. *Microbiology as Illustration of Sociobiological Principles*

There are perhaps only two main impulses underlying life: survival and reproduction. Successful resource management would be achieved only if the organisms would disperse. Microorganisms were traditionally studied in axenic (pure) culture, with initially unlimited resources. These studies have led to a synthesis of the ''hallmarks of [uni]cellular life'' (Madigan *et al.*, 1997): self-feeding, or nutrition; self-replication, or growth; differentiation, which includes both morphological and biochemical development, in response to environmental change; chemical signaling to other organisms; and evolution, or production of offspring that differ from the parent. These principles may be extrapolated to ''higher'' life forms, including eukaryotes and other multicellular communities. The multicellular communities noted here include prokaryotic communities within axenic and mixed cultures, including colonies, biofilms, and flocs.

Prior ecological studies have delineated six principles of symbiosis, or communal life (Boddy and Wimpenny, 1992). The same relationships—amensalism, antagonism, commensalism, mutualism, neutralism, and parasitism/predation—have been demonstrated within microbial communities. These sociological aspects of microbiology appear to reflect an adaptation to the immediate surroundings, as demonstrated in terrestrial and laboratory environments. Adaptation over long periods is reflected in evolutionary trends. Microbial metabolism meanwhile can change the surrounding pH, Eh, and water activity. These concomitant changes reflect a coevolution of environment and life. Studies of life in so-called extreme environments on Earth may provide clues to detecting life, or its signatures, in extreme nonterrestrial environments. But the following sections examine the characteristics of these environments individually.

III. Microbial Life in Extreme Environments

A. Microbial Exploitation of Water

1. *Water Availability*

''Extremophiles'' usually live within environments where more than one characteristic—of pressure, temperature, etc.—differs from those familiar to humans (Pace, 1991; Colwell *et al.*, 1997; Chang, 1988;

Rothschild, 2002). Indeed, such organisms are polyextremophiles, and their categorization and description are difficult. But life on Earth depends on liquid water as reaction matrix. This document proceeds from that starting point. It is relevant, though, that a prokaryote that existed in a desiccated state for 250 million years in the form of spores was recently revived (Vreeland *et al.*, 2000). This latter finding suggests that water is necessary not for life but for normal metabolism.

The apparent requirement for liquid water (but see the following text) was thought to limit life to a particular range of temperatures and pressures. However, pure water is known to remain liquid at temperatures and pressures beyond its cardinal points for freezing and boiling, as does water containing solutes. Microorganisms exploit both these physical and colligative properties of water. Microbial communities have been detected in superheated and supercooled water, at low and high pressures. These microbial types will be described. This section, however, describes organisms capable of life in environments where quantities of water is limited, i.e., those that are xerotolerant. This classification includes those organisms that live in environments of low water activity (a_w), i.e., the osmotolerant and halotolerant.

True xerotolerance has been demonstrated among microorganisms that have cell walls; indeed, the purpose of the cell wall may be to protect the cytoplasmic membrane during times of osmotic stress. Among the prokaryotes, the thick-walled gram-positive organisms are most likely to survive water limitation. Fungi, however, are also xerotolerant. In either case, the absolute lower limit for xerotolerance occurs at $a_w = 0.55$, at which point DNA becomes disordered (Brown, 1995). At the other end of the water activity spectrum, organisms capable of growth in nearly pure water (a_w near 1.0) are also known. In heterogeneous settings, microorganisms have been reported to migrate toward water (Garcia-Pichel, unpublished). The ionic basis for microbial xerotolerance—discrimination among different cations or anions or between salts and organic solutes—has not been rigorously studied. However, organisms are known to pump ions out of the cell or to produce compatible solutes in order to maintain physiological equilibrium.

2. *Microbial Relations to pH*

Although most known organisms live at circumneutral pH, certain prokaryotes have been shown to grow at pH extremes from 0.7 (Schleper *et al.*, 1995) to 10 (Tindall *et al.*, 1984). The physiological basis for their acidophily or alkaliphily is under investigation (Pace, 1991).

B. Microorganisms and Pressure Extremes

Organisms that survive under high pressure can be described either as barophiles (''weight-loving'') or, more recently, piezophiles (''pressure-loving''). Metabolically active organisms have been isolated from a pressurized, deep-sea habitat at 10,987 m (Kobayashi *et al.*, 1998). Elsewhere, microorganisms have been shown to be active at a pressure of 1680 megaPascals (Sharma *et al.*, 2002). These phenomena are new and are being studied, as is that of a terrestrial prokaryote that was inadvertently exposed to the vacuum of space for 3 years, but survived (http://nai.arc.nasa.gov/astrobio/astrobio_detail.cfm?ID=170).

C. Life's Apparent Temperature Limits

Hyperthermophilic prokaryotes have been isolated from terrestrial and marine environments. The current known limit for life at high temperatures is 113°C, as demonstrated in a deep-sea vent (Blöchl *et al.*, 1997). Psychrophilic organisms that could be cultured at temperatures of 0.3 to 4°C have also been found in subglacial sediments (Skidmore *et al.*, 2000).

D. Microbes and Irradiation

The electromagnetic spectrum spans a variety of wavelengths; those described here are in the range of 10^{-4} to 10^{-16} meters—infrared light through gamma rays. It is useful to note that irradiation doses of 1.5 to 3 kilo Grays (where one Gray is equivalent to one joule per kilogram) are routinely used for industrial sterilization, but that ultrasound exposure has also recently been shown to be effective for microbial killing (Hoover *et al.*, 2002). Microbial susceptibility to the killing effects of irradiation in space has been studied in detail (Petras and Bisa, 1968).

Phototropic microorganisms are capable of metabolism through conversion of light energy to chemical energy. In these and chemotrophic microbes, blue light serves a trigger for morphological and/or biochemical differentiation. Radiotolerance has been demonstrated in still others. The organism that still holds the record for radiotolerance (15 kGy) was first discovered in 1956. The isolation of microorganisms from the stratosphere (~40 km above sea level) has been reported but not well documented.

E. Microbial Redox Relations

Terrestrial life appears to depend on electromagnetism in the form of chemical energy. Macroscopic life forms, including humans, depend on oxygen as terminal electron acceptor. Both macro- and microorganisms have been found to conduct some metabolic tasks via redox of organic compounds (fermentation, and/or organotrophy). But only prokaryotes have been shown definitively to live lithotrophically—via the transfer of electrons to inorganic elements such as iron, sulfur, or chlorine (as perchlorate). Indeed, molecular oxygen and its partially reduced forms are toxic to most organisms). It is possible that SETI has not been productive simply because it focuses on identification of organisms capable of intelligent action, where that action required executive function with high energy demands. It is also possible that such intelligence could be based on reduction of nitrogen, fluorine, or hydrogen. Still, life—the focus of the current search—can perhaps be found through study of electron cycling in nonterrestrial environments.

Microorganisms that are carbon-based, but carry out energy transformations at the expense of other elements, do so via anaerobic respiration (Madigan *et al.*, 1997). The oxidant substrate for such transformations may be water-soluble, such as certain partially oxidized organic compounds, nitrates, hydrogen gas, or metal ions, or it may be insoluble. The electron acceptor (reductant) may be organic or it may be some insoluble form of ferric ion, manganese, or possibly other elements. [Reduction of soluble nitrate or sulfate is well known and may result in a phase change for the electron acceptor.] Clearly, microorganisms are capable of affecting the gaseous, dissolved, and mineral phases of their environments. Molecular oxygen is not required for microbial life. Moreover, any organism that can carry out energy-yielding, biological redox at rates greater than the corresponding abiotic activities is viable. In other words, ''As long as the biology [biochemical reaction kinetics] beats the chemistry [chemical reaction kinetics], you've got it made.'' (K. Nealson, personal communication).

F. Microbial Proliferation—The Scaffolding Elements

Organisms described in this document, and life as we know it, are carbon-based (Section II.A.2). The metabolic product of carbon metabolism is CO_2. Life's basis on carbon may simultaneously reflect

carbon's reactivity, relative to elements in its group, and its lack of reactivity relative to other elements in its period. Carbon's energy content and chemical stability to oxidation or reduction are probably more important to the definition of life than its reasonable abundance in the universe. Similarly, with regard to periodic law, life is hydrogen-dependent, but this dependence possibly refers to protons rather than to the element itself (Section 2.2.1). This requirement for hydrogen is usually met via water solvation.

Microorganisms can live under conditions of either low (oligotrophic) or high (copiotrophic) carbon availability, where that carbon is at least partially reduced. Organisms that utilize inorganic carbon (i.e., CO_2, CO, cyanide, and methane) are also known. The key issue regarding oligotrophy may the organism's Monod constant (K_s), i.e, its substrate saturation concentration in g/L. But microorganisms are able to live in distilled water—although that matrix is admittedly in contact with air, an atmosphere that contains 4% CO_2. Others are quite versatile regarding carbon substrates and can shift to alternate sources when a preferred carbon source becomes limiting.

Successful culture of new environmental isolates in the laboratory requires formulation of the proper growth medium. However, microbiologists have long been plagued by the ''viable but non culturable'' (VBNC) phenomenon, in which environmental isolates simply will not grow in the laboratory, in axenic culture. It is true that slow growth may not be easily detected or that growth in the absence of other organisms capable of completing an element or electron cycle may be difficult. However, viability is not equivalent to death (Liu, 2000). Other findings, moreover, indicate that growth under unconventional and/or oligotrophic conditions may result in successful isolation of new organisms (Aagot *et al.*, 2001; Kashefi *et al.*, 2002). Growth may require narrowly defined experimental conditions. Trace contaminants in laboratory media or water may moreover be toxic and nutritional requirements may not be met by traditional media. VBNC microorganisms may require carefully but poorly defined (i.e., ''complete'') growth medium, such as that used for prokaryotic pathogens or for eukaryotic organisms in cell culture.

IV. Relevant Microbiological Activities, at NASA and Beyond

Activities described in this section are supported by the NASA Astrobiology Program, the U.S. Department of Energy, the National Science Foundation, and private sponsors. The 1976 unmanned Viking 2 mission to Mars seemed not to find extraterrestrial life *in situ* (http://

cmex.arc.nasa.gov/SiteCat/sitecat2/viking.htm). However, the data were interpreted before the extent and diversity of microbial life on Earth were realized, or the variety of biosignatures other than gaseous carbon compounds defined. New, possibly better testing protocols and instrumentation have since been developed and will presumably be used on future missions.

A. Geomicrobiology

The microbiological characterization of unfamiliar or unexplored terrestrial environments has been attracting considerable interest. Sampling of deep-subsurface (i.e., down to 3.3 km) and deep-sea sites has increased, as has recognition of microbial types (and sometimes macroscopic communities) that use unexpected metabolic substrates (Gold, 1992). The combination of environmental characteristics present at any site is rarely exactly duplicated, resulting in identification of subtly different polyextremophiles. Exploration of sites contaminated with physically or chemically hazardous substances such as radioactive or xenobiotic wastes is yielding organisms with novel physiologies, whether for carbon source utilization or for energetic metabolism. These studies strongly support Gale's 1952 Law of Microbial Infallibility: ''No natural organic compound [defined as a product of photosynthesis] is totally resistant to biodegradation.'' Microbiologist Martin Alexander, during the 1960s and 1970s, qualified that assertion by adding the phrase, ''...provided that environmental conditions are favorable,'' (Alexander, 1971, 1977) and discovered that physicochemical bioavailability controls biodegradation (Nam and Alexander, 2001). Recent work, in fact, suggests an additional principle, to include the respiration of all chemical elements. Some environmental isolates have an apparent ability to use manmade elements or materials not available before the mid-twentieth century. This finding, coupled with the known rapid growth rate of microorganisms, suggests that evolution (via genetic drift, horizontal gene transfer, or natural selection) is occurring over human timescales.

B. Physicochemical Studies

Novel microorganisms generally undergo thorough biochemical and physiological characterization if they can be cultured in the laboratory. These studies may require the organism's fractionation; excretion products, such as enzymes or polymers, are often studied as well. The goal of this work is often the identification of marketable products or

activities (Persidis, 1998; Hoyle, 1998). Alternatively, genetic characterization of the isolate is the focus. Physical and chemical studies to determine the size and hydration requirements [including the possibility of life in nonaqueous solvents (Klibanov, 2001)] are sometimes neglected. Fortunately, applied microbiology converges with the search for extraterrestrial life through study of the effects of altered gravity on microbial development and product formation (Fang *et al.*, 1997; Monzer, 1995; Monzer *et al.*, 1994; Moore *et al.*, 1996).

V. Microbiology and Planetary Protection

A. First Principles of Killing

Terrestrial space agencies recognize, but do not necessarily accept, the 1939 concept of panspermia. Indeed, panlithospermia—interplanetary transport of cells associated with solid materials, such as rocks—is being considered instead (Mastrapa *et al.*, 2001; Mileikowsky *et al.*, 2000; Nicholson *et al.*, 2000; Horneck *et al.*, 1996, 2001a,b; Wells *et al.*, 2003). Epidemiological efforts are underway to assure that microbial life is not transferred by or within humans, either to or from a planetary body targeted for exploration (National Research Council, 1998, 2000, 2002a). Humans themselves are contained within closed systems and undergo quarantine after their return to Earth. The relationship of microbiology to human life support has been established (Phillips, 1999; Ball and Evans, 2001; Hurst *et al.*, 2002) and is being updated continually (http://advlifesupport.jsc.nasa.gov/). For inbound flights, sterilization efforts focus instead on decontamination of equipment. Effective sterilization protocols must incorporate knowledge regarding the killing of life forms. Perhaps unfortunately, these efforts are based on life as we know it. Better understanding of the principles surrounding death may be critical to effective decontamination.

The requirements for life as we know it depend on segregation of cellular life forms from their environment, and distinguish between polar and nonpolar cellular components via the Principle of Uniformity of Nature. Protocols for killing of xenobiotic life forms may appropriately accept that extraterrestrial life could be based on a redox reality different from that on Earth, i.e., that polar compounds could be based on hydrogen and/or an electronegative element other than oxygen. The reader is reminded that it is elemental oxygen, rather than ground-state [triplet] molecular oxygen, that appears to form a chemical basis for life on Earth.

B. Specific Sterilization Practices

1. *Desiccation*

Terrestrial organisms contain significant amounts of liquid water. Water is required for metabolism; the long-term survival of desiccated organisms suggests, however, that simple removal of that solvent does not destroy their information content. Drying is thus a microbistatic, rather than a microbicidal, treatment. Removal of nonaqueous ionic solvents from possible life forms dependent on them may be similarly less than effective.

2. *Irradiation at Low Wavelengths*

Application of energy to hydrous systems, via irradiation, results in cell damage through the formation of active oxygen species—hydroxyl radical, peroxide, superoxide, and possibly singlet oxygen. Death, if defined as loss of fidelity in replication, may be achieved by treatment with ultraviolet light or by exposure to x-rays and gamma rays. But extremophiles capable of surviving exposure to ultraviolet light, and possibly to x-rays or gamma rays, suggest a need for process characterization. Survival of some organisms after long-term exposure to cosmic rays in space may indicate that irradiation has its limits with respect to actual killing.

3. *Physical Disintegration*

Mechanical rupture of cell envelopes can be accomplished via extreme pressure and/or shear, but characterization of xeno-life would be necessary to determine specific energy requirements. The existence of piezophiles suggests a better, quantitative understanding of standard cell disruption processes may be necessary. Titration of force applied within French pressure cells, by microwaving, or during ultrasound (low-energy) treatment may be appropriate to derive information relevant to killing of xeno-life forms.

Chemical rupture of cell envelopes has traditionally been achieved by solubilization of the cell membrane with moderately polar organics such as low molecular weight alcohols. Killing is most effective when the solvents used are hydrous; this latter phenomenon may apply to water-based organisms, such as those on Earth. Yet, the concept of using chemical polarity as a weapon may be a useful basis for treatment of xeno-life forms.

4. *Chemical Inactivation*

Commercial sterilization protocols depend greatly on the use of biocides. These compounds include electronegative elements such as nitrogen (e.g., quaternary ammonium detergents), oxygen (e.g.,

peroxides), or chlorine and other halogens. Specialty biocides include organic compounds that contain reactive functional groups consisting of carbon with single or multiple bonds to nitrogen, oxygen, and/or sulfur. Compounds capable of killing eukaryotic and/or multicellular organisms contain carbon bound to other, less electronegative elements, such as phosphorus or arsenic. This finding suggests that prokaryotes are more resistant to chemical treatment than are higher organisms and emphasizes that the most resistant prokaryotes may be treated effectively with fluorine. Calibration of biocide chemical energy may be useful in assurance of microbical treatment.

Sterilization treatments may use gases such as ethylene oxide; vapors such as hydrogen peroxide, or liquid solutions (usually in water). The role of water here may be as physical carrier rather than as reagent. However, water probably participates in free-radical reactions critical to cell killing. The presence, on the list of commerical biocides, of organometallic compounds containing tin, copper, or other metals suggests that electron-transfer phenomena may be critical to the success of these treatments.

5. *Heat Treatment*

Incineration (''unlimited'' heating, in the absence of water) is usually inappropriate for planetary protection due to resulting destruction of mission-related equipment. Heating of noncombustible materials, such as metals, may be impractical with respect to preservation of mission-related items. But prudence dictates that some such material be sterilized. Where other physical or chemical approaches fail, heat may be the last option.

In industrial practice, sterilization is routinely based on autoclaving, or heat treatment. These protocols generally are based on moist rather than dry heat; it is not clear whether their success is based on elevated pressure or temperature. In the latter case (use of dry heat), autoclaving requires longer periods. The time–temperature relationship for heat treatment can be determined mathematically but must acknowledge the heat-transfer characteristics of the material being autoclaved as well as its physical configuration and the chemistry (pH, density, organic content) of its components. The indicator organisms for successful heat treatment are the spores of thermophilic prokaryotes. However, traditional autoclaving regimes were developed decades ago, before discovery of true extremophiles. New autoclaving protocols that recognize these new microbiological discoveries are needed and possibly must focus on the extremophiles' most resistant forms (spores, cysts, etc.).

6. *Antibiotics?*

In general terms, antibiotics are simply too expensive for use in material decontamination. Moreover, their action depends on the presence of water. But certain antibiotic classes, such as the macrolide antibiotics, possess a chemical structure conducive to electron shuttling. Their critical structural aspect is the presence of conjugated rings, much as those in humic (soil organic) compounds. Electron-shuttling compounds may scavenge bioavailable metallic elements (such as iron, a potential scaffolding element critical to all terrestrial life) and thus interfere with the metabolism of unknown life forms. But cost, relative to other potential electron shuttles, would still make antibiotics impractical for standard practice.

VI. Value of Exomicrobiological Research

A. Discovery

1. *Relationship of Life to Planetary Characteristics*

It is generally accepted that Earth's atmospheric composition is influenced by microbial activity. Accumulated evidence supports the view that Earth's geochemical composition is affected by microorganisms as well. The data are, unfortunately, all contemporary; Earth's characteristics prior to and at the time of the appearance of life are not well defined. Similarly, Earth's future characteristics, and the possible role of life in creating those characteristics, cannot be predicted. Life on this planet evolved at the expense of elements and irradiation present on this planet due to cosmological influences. Coevolution of the planet and the life thereon must be expected to continue, much as pathogens coevolve with their host. Additional data points, obtained from extraterrestrial sources, would allow an understanding of this process.

On a purely biological note, the characteristics of life posited here can only be refined by additional information.

2. *Technological Advances*

Much as Earth's known extremophiles have proven the source of new materials and catalytic methods, greater understanding of possible life dependent on nonaqueous solvents or capable of novel mass transformations or energy conversions would lead to introduction of new, potentially useful industrial products. These new life forms may also illuminate unique approaches to process development, e.g.,

by operation of reactors with unusual solvents or under new gas phases. Unexpected data may actually reveal more than one physical force (other than electromagnetism) available for exploitation by industry.

B. Education

The training of a new generation of scientists and engineers, and the retraining of current ones, could be supported by demonstration of new biological activities. Integration of new structural and functional information into the current gene-centered approach to modern biology would allow a new, finer definition of life's first principles of physics, chemistry, and engineering science. Better still, new knowledge may better integrate biology with the physical sciences and possibly would confer biology with a mathematical rigor. Development of new imaging technologies, and training in their uses, may continue to be important.

VII. Conclusions and Recommendations

NASA's criteria for scientific discovery require that research be interdisciplinary, innovative, international, and integrative. There is already considerable interaction between the American space agency and that of other nations. This interactive model is further supported by collaboration, within the Astrobiology Institute, among astronomers, cosmologists, planetary scientists, physicists, chemists, and microbiologists (National Research Council, 2003). Such collaboration has also been evident throughout the history of microbiology itself, as well as that of space exploration (Table III). Further cooperation could be encouraged and would benefit both NASA and the field of microbiology. Cross fertilization of microbiology with other fields of specialization within biology may also be useful, as demonstrated by listed contributions attributed to nonmicrobial biologists.

In the context of education, collaborative efforts would be served by better integration of biology with chemistry, with physics, with planetary science, and with engineering science. Indeed, the nascent field of geomicrobiology may serve as an example of a successful and productive interaction. But cross-disciplinary education for precollege students through postgraduate students would support a more rapid discovery and exploitation of space through future years.

The systems engineering approach to the search for extraterrestrial life (Table II) may be a useful model for the proposed cross-disciplinary

training effort. A proposed task definition—''How may we design a system that will prepare scientists to work with microorganisms obtained from non-Earth environments?''—could be addressed by an integrative, decentralized team approach. The problem of understanding system complexity (life, whatever the source) will require intellectual contributions from workers in multiple disciplines. Attention to the biological infrastructure for survival and reproduction, rather than solely to its information management aspect, may be useful.

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References

Aagot, N., Nybroe, O., Nielsen, P., and Johnsen, K. (2001). An altered *Pseudomonas* diversity is recovered from soil by using nutrient-poor *Pseudomonas*-selective soil extract media. *Appl. Environ. Microbiol.* **67,** 5233–5239.

Alexander, M. (1971). ''Microbial Ecology.'' John Wiley and Sons, Inc., New York, NY.

Alexander, M. (1977). ''Introduction to Soil Microbiology,'' 2nd Ed. Krieger Publishing Co., New York, NY.

Angert, E. R., Clements, K. D., and Pace, N. R. (1993). The largest bacterium. *Nature* **362,** 239–241.

Ball, J. R., and Evans, C. H., Jr., Eds. (2001). ''Safe Passage: Astronaut Care for Exploration Missions.'' National Academies Press, Washington, DC.

Barnes-Svarney, P., Ed. (1995). ''New York Public Library Science Desk Reference.'' Stonesong Press, New York, NY.

Bennett, J., Shostak, S., and Jakosky, B. (2003). ''Life in the Universe.'' Addison-Wesley, San Francisco, CA.

Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W., and Stetter, K. O. (1997). *Pyrolobus fumarii* gen. and sp.nov. represents a group of archaea, extending the upper temperature limit for life to 113 °C. *Extremophiles* **1,** 14–21.

Boddy, L., and Wimpenny, J. W. T. (1992). Ecological concepts in food microbiology. *J. Appl. Bacteriol. Symp. Supp.* **73,** 23S–38S.

Brock, T. D. (1999). ''Milestones in Microbiology: 1546–1940.'' American Society for Microbiology Press, Washington, DC.

Brown, T. A. (1995). ''Gene Cloning.'' Chapman and Hall, London, U.K.

Bunch, B., and Hellemans, A. (1993). ''The Timetables of Technology.'' Touchstone Press, New York, NY.

Cady, S. L. (2001). Paleobiology of the Archaean. *Adv. Appl. Microbiol.* **50,** 3–36.

Carlile, M. J. (1980). From prokaryote to eukaryote: Gains and losses. *Symp. Soc. Gen. Microbiol.* **30,** 1–40.

Chang, S. (1988). Planetary environments and the conditions of life. *Philos. Trans. R. Soc. Lond. A* **325,** 601–610.

Chyba, C. F. The search for life: A core mission for NASA in the 21st century. *In* ''Space Policy in the Twenty-First Century'' (W. H. Lambright, ed.). Johns Hopkins Univ. Press, Baltimore (in press).

Clark, S. (2000). ''Life On Other Worlds and How To Find It.'' Praxis Publishing, Ltd., Chichester, UK.

Clements, K. D., and Bullivant, S. (1991). An unusual symbiont from the gut of surgeonfishes may be the largest known prokaryote. *J. Bacteriol.* **173,** 5359–5362.

Colwell, F. S., Onstott, T. C., Dilwiche, M. E., Chandler, D., Fredrickson, J. K., Yao, Q.-J., McKinley, J. P., Boone, D. R., Griffiths, R., Phelps, T. J., Ringelberg, D., White, D. C., LaFreniere, L., Balkwill, D., Lehman, R. M., Konisky, J., and Long, P. E. (1997). Microorganisms from deep, high temperature, sandstones: Constraints on microbial colonization. *FEMS Microbiol. Rev.* **20,** 425–435.

Crawford, R. L., Paszczynski, A., Land, Q., Cheng, I. F., Barnes, B., Anderson, T. J., Wells, R., Wai, C., Corti, G., Allenbach, L., Erwin, D. P., Park, J., Assefi, T., and Mojarradi, M. (2001). *Icarus* **154,** 531–539.

Deamer, D. W. (1997). The first living systems: A bioenergetic perspective. *Microbiol. Mol. Bio. Rev.* **61,** 239–261.

Deamer, D., Dworkin, J. P., Sandford, S. A., Bernstein, M. P., and Allamandola, L. J. (2002). The first cell membranes. *Science* (submitted).

Des Marais, D. J., Harwit, M., Jucks, K., Kasting, J. F., Lunine, J. I., Lin, D., Seager, S., Schneider, J., Traub, W., and Woolf, N. (2002). Remote sensing of planetary properties and biosignatures on extrasolar terrestrial planets. *Astrobiology* (in press).

Drossel, B. (1999). Simple model for the formation of a complex organism. *Phys. Rev. Lett.* **82,** 5144–5147.

Dworkin, J. P., Deamer, D. W., Sandford, S. A., and Allamandola, L. J. (2001) Self-assembling amphiphilic molecules: Synthesis in simulated interstellar/precometary ices. *Proc. Nat. Acad. Sci. USA* **98,** 815–819.

Edelman, G. W., and Gally, J. A. (2001). Degeneracy and complexity in biological systems. *Proc. Nat. Acad. Sci. USA* **98,** 13763–13768.

Ewald, P. W. (1996). Guarding against the most dangerous emerging pathogens: Insights from evolutionary biology. *Emerg. Infect. Dis.* **2,** 245–257.

Fang, A., Pierson, D. L., Mishra, S. K., Koenig, D. W., and Demain, A. L. (1997). Gramicidin S production by *Bacillus brevis* in simulated microgravity. *Curr. Microbiol.* **34,** 199–204.

Fox, S. W. (1973). Origin of the cell: Experiments and premises. *Naturwissenschaften* **60,** 359–368.

Fredrickson, J. K., and Onstott, T. C. (1997). Microbes deep inside the earth. *Sci. Am.* **275,** 68–75.

Garcia-Pichel, F. Manuscript in preparation; http://www.spacedaily.com/news/life-01zh.html.

Gladman, B. J., Burns, J. A., Duncan, M., Lee, P., and Levison, H. F. (1996). The exchange of impact ejecta between terrestrial planets. *Science* **271,** 1387–1392.

Gold, T. (1992). The deep hot biosphere. *Proc. Nat. Acad. Sci.* **89,** 6045–6049.

Gorbushina, A. A., Krumbein, W. E., and Volkmann, M. (2002). Rock surfaces as life indicators: New ways to demonstrate life and traces of former life. *Astrobiology* **2,** 203–213.

Haldane, J. B. S. (1947). ''What is Life?'' Boni and Gaer, New York, NY.

Harris, M. J., Wickramasinghe, N. C., Lloyd, D., Narlikar, J. V., Rajaratnam, P., Turner, M. P., Al-Mufti, S., Wallis, M. K., Ramadurai, S., and Hoyle, F. (2001). The detection of living cells in stratospheric samples. *Proc. Soc. Int. Soc. Opt. Eng. [SPIE] Conf.* **4495,** (in press).

Hellemans, A., and Bunch, B. (1991). ''The Timetables of Science.'' Touchstone Press, New York, NY.

Hoover, K., Bhardwaj, M., Nancy Ostiguy, N., and Thompson, O. (2002). Destruction of bacterial spores by phenomenally high efficiency non-contact ultrasonic transducers. *Mat. Res. Innovat.* **6,** 291–295.

Horneck, G., Rettberg, P., Rabbow, E., Strauch, W., Seckmeyer, G., Facius, R., Reitz, G., Strauch, K., and Schott, J.-U. (1996). Biological dosimetry of solar radiation for different simulated ozone column thicknesses. *J. Photochem. Photobiol. B. Biol.* **32,** 189–196.

Horneck, G., Rettberg, P., Reitz, G., Wehner, J., Eschweiler, U., Strauch, K., Panitz, C., Starked, V., and Baumstark-Khan, C. (2001a). Protection of bacterial spores in space, a contribution to the discussion on Panspermia. *Orig. Life Evol. Biosphere* **31,** 527–547.

Horneck, K, Stöffler, D., Eschweiler, U., and Hornemann, U. (2001b). Bacterial spores survive simulated meteorite impact. *Icarus* **149,** 185–193.

Hoyle, R. (1998). In hot pursuit of extremophiles. *Nature Biotechnol.* **16,** 312.

Huber, H., Hohn, M. J., Rachel, R., Fuchs, T., Wimmer, V. C., and Stetter, K. O. (2002). A new phylum of Archaea represented by a nonosized hyperthermophilic symbiont. *Nature* **417,** 63–67.

Hurst, C. J., Roman, M. C., Garland, J. L., Obenhuber, D. C., and Brittain, A. M. (2002). Microbiological aspects of space exploration. *Am. Soc. Microbiol. News* **63,** 611–615.

Ideker, T., Galitski, T., and Hood, L. (2001). A new approach to decoding life: Systems biology. *Ann. Rev. Genomics Hum. Genet.* **2,** 343–372.

Kashefi, K., Holmes, D. E., Reysenbach, A.-L., and Lovley, D. R. (2002). Use of Fe(III) as an electron acceptor to recover previously uncultured hyperthermophiles: Isolation and characterization of *Geothermobacterium ferrireducens gen. nov., sp. Nov.* **68,** 1735–1742.

Klibanov, A. M. (2001). Improving enzymes by using them in organic solvents. *Nature* **409,** 241–246.

Kobayashi, H., Takaki, Y., Kobata, K., Takami, H., and Inoue, A. (1998). Characterization of α-maltotetraohydrolase produced by *Pseudomonas* sp. MS300 isolated from the deepest site of the Mariana Trench. *Extremophiles* **2,** 401–407.

Koch, A. L., and Silver, S. (2003). The first cell. *FEMS Microbiol. Rev.* (in press).

Leadbetter, E. R., and Poindexter, J. S. (1985). ''Bacteria in Nature, Vol. 1: Bacterial Activities in Perspective.'' Kluwer Academic/Plenum Publishers, New York, NY.

Liu, S. V. (2000). Viable but nonculturable (VBNC) microorganisms: A misnomer or a whistle-blower? *Logical Biol.* **1,** 17–20.

Madigan, M. T., Martinko, J. M., and Parker, J. (1997). ''Brock Biology of Microorganisms,'' 8th Ed. Prentice-Hall, Upper Saddle River, NJ.

Margulis, L. (1993). ''Symbiosis in Cell Evolution,'' 2nd Ed. Freeman Publishers, New York, NY.

Margulis, L. (1998). ''Symbiotic Planet: A New Look at Evolution.'' Basic Books, New York, NY.

Martin, W., and Russell, M. (2002). On the origins of cells: A hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells. *Philosoph. Trans. Royal Soc. B,* published online, doi: 10.1098/rstb.2002.1183.

Mastrapa, R. M. E., Glanzberg, H., Head, J. N., Melosh, H. J., and Nicholson, W. L. (2001). Survival of bacteria exposed to extreme acceleration: Implications for panspermia. *Earth Planet. Sci. Lett.* **189,** 1–8.

Mileikowsky, C., Cucinotta, F., Wilson, J. W., Gladman, B., Horneck, G., Lindegren, L., Melosh, J., Rickman, H., Valtonen, M., and Zheng, J. Q. (2000). Natural transfer of viable microbes in space, part 1: From Mars to Earth and Earth to Mars. *Icarus* **145,** 391–427.

Monzer, J. (1995). Cellular graviperception in the basidiomycete *Flammulina velutipes*—Can the nuclei serve as fungal statoliths? *Eur. J. Cell Biol.* **71,** 216–220.

Monzer, J., Haindl, E., Kern, V. D., and Dressel, K. (1994). Gravitropism of the basidiomycete *Flammulina velutipes*: Morphological and physiological aspects of the graviresponse. *Exp. Mycology* **18,** 7–19.

Moore, D., Hock, B., Greening, J. P., Kern, V. D., Novak-Frazer, L., and Monzer, J. (1996). Gravimorphogenesis in agarics. *Mycol. Res.* **100,** 257–273.

Morowitz, H. J., Heinz, B., and Deamer, D. W. (1988). The chemical logic of a minimum protocell. *Origins Life Evol. Biosphere* **18,** 281–287.

Nam, K., and Alexander, M. (2001). Relationship between biodegradation rate and percentage of a compound that becomes sequestered in soil. *Soil Biol. Biochem.* **33,** 787–792.

National Research Council (1998). ''Evaluating the Biological Potential in Samples Returned from Planetary Satellites and Small Solar System Bodies: Framework for Decision Making.'' National Academies Press, Washington, DC.

National Research Council (1999). ''Size Limits of Very Small Microorganisms: Proceedings of a Workshop.'' National Academies Press, Washington, DC.

National Research Council (2000). ''Preventing the Forward Contamination of Europa.'' National Academies Press, Washington, DC.

National Research Council (2002a). ''The Quarantine and Certification of Martian Samples.'' National Academies Press, Washington, DC.

National Research Council (2002b). ''Signs of Life: A Report Based on the April 2000 Workshop on Life Detection Techniques.'' National Academies Press, Washington, DC.

National Research Council (2003). ''Life in the Universe: An Assessment of U.S. and International Programs in Astrobiology.'' National Academies Press, Washington, DC.

Nicholson, W., Munaka, N., Horneck, G., Melosh, H. J., and Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microb. Mol. Biol. Rev.* **64,** 548–572.

Orgel, L. (1973). ''The Origins of Life: Molecules and Natural Selection.'' John Wiley and Sons, New York, NY.

Pace, N. R. (1991). Origin of life—Facing up to the physical setting. *Cell* **65,** 531–533.

Persidis, A. (1998). Extremophiles. *Nature Biotechnol.* **16,** 593.

Petras, E., and Bisa, K. (1968). Microbiological studies on the radiation environment of the ionosphere and stratosphere. *Life Sci. Space Res.* **6,** 115–122.

Phillips, J. N., Jr. (1999). Closed ecological systems for space travel and extraterrestrial habitation. *J. Indus. Microbiol. Biotechnol.* **22,** 216–224; originally published in *Dev. Indus. Microbiol.*, 1961.

Rothschild, L. J. (2002). Life in extreme environments. *Ad Astra* **14,** 32–40.

Schleper, C., Pühler, G., Holz, I., Gambacorta, A., Janekovic, D., Santarius, U., Klenk, H.-P., and Zillig, W. (1995). *Picrophilus* Gen. Nov. Fam. Nov.: A novel aerobic, heterotrophic, thermoacidophilic genus and family comprising archaea capable of growth around ph zero. *J. Bacteriol.* **177,** 7050–7059.

Sharma, A., Scott, J. H., Cody, G. D., Fogel, M. L., Hazen, R. M., Hemley, R. J., and Huntress, W. T. (2002). Microbial activity at gigapascal pressures. *Science* **295,** 1514–1516.

Sherratt, D. (2001). Bacteria rule!—The start of a new era in bacterial microbiology? *Eur. Molec. Biol. Org. Rep.* **21,** 175–176.

Shultz, H. N., Brinkhoff, T., Ferdelman, T. G., Hernández Mariné, M., Teske, A., and Jørgensen, B. B. (1999). Dense populations of a giant sulfur bacterium in Namibian shelf sediments. *Science* **284,** 493–495.

Skidmore, M. L., Foght, J. M., and Sharp, M. J. (2000). Microbial life beneath a high Arctic glacier. *Appl. Environ. Microbiol.* **66,** 3214–3220.

Stevenson, D. J. (2000). Planetary science: A space odyssey. *Science* **287,** 997–1005.

Tindall, B. J., Ross, H. N. M., and Grant, W. D. (1984). *Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaebacteria. *Syst. Appl. Microbiol.* **5,** 41–57.

Uwins, P., Webb, R., and Taylor, A. (1998). Novel nano-organisms from Australian sandstones. *Am. Mineralogist* **83,** 1541–1550.

Vreeland, R. H., Rosenzweig, W. D., and Powers, D. W. (2000). Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature* **407,** 897–900.

Ward, P. D., and Brownlee, D. (2000). ''Rare Earth.'' Springer-Verlag, New York, NY.

Wells, L. E., Armstrong, J. C., and Gonzalez, G. (2003). Reseeding of early earth by impacts of returning ejecta during the late heavy bombardment. *Icarus* **162,** 38–46.

Woese, C. R., Kandler, O., and Wheelis, M. L. (1990). Towards a natural system of organisms: Proposal for the domains *Archaea*, *Bacteria*, and *Eucarya*. *Proc. Natl. Acad. Sci. USA* **87,** 4576–4579.

Wolstencroft, R. D., and Raven, J. A. (2002). Photosynthesis: Likelihood of occurrence and possibility of detection on Earth-like planets. *Icarus* **157,** 535–548.

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