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Soil-Based Gene Discovery: A New Technology to Accelerate and Broaden Biocatalytic Applications

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I. Introduction

Biocatalysis utilizes enzymes or live microbial cultures to catalyze specific reactions or sequences of reactions. Although, in a broad sense, fermentation-based products such as antibiotics and lactic acid are examples of ''biocatalysis'' (Wackett, 2002), they have been excluded from this definition to fit the theme of this chapter. Here, biocatalysis is more narrowly defined with respect to applications of enzyme catalysis in the areas of commodity chemicals, fine chemicals, chiral pharmaceutical intermediates, and agrochemicals (Liese *et al.*, 2000). The examples cited in this chapter pertain to processes wherein substrates are externally supplied and converted to a targeted product in a minimum number of enzymatic steps. Bioremediation of undesirable chemicals can also be included in this definition and there are

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well-documented examples that involve specific enzymes (Wackett *et al.*, 2002; Lange *et al.*, 1998).

Due to potential applications in the pharmaceutical industry for production of chiral drugs, the field of biocatalysis has received considerable attention recently. In 2000, sale of single enantiomer drugs accounted for about $133 billion of the $390 billion in worldwide sales (Erb, 2002; McCoy, 1999). This was a 13% increase over 1999 sales. This number is expected to reach $172 billion by 2005 (McCoy, 1999). This increase is partly due to demand for chiral versions of drugs previously sold as racemic mixtures. Given the high degree of specificity of interaction of small molecules with pharmaceutical target sites, it is not surprising that one stereoisomer may generally be more active or have better properties of absorption, metabolism, and excretion than its opposite enantiomer. In some cases, the inactive isomer may actually be toxic (Erb, 2002; McCoy, 1999). Examples of the strikingly different biological effects of pharmaceutical enantiomers are shown in Table I. Thus, the pure enantiomer of a drug may provide greater efficacy, which, in turn, can accelerate regulatory approval and extend product life cycle (Erb, 2002; McCoy, 1999).

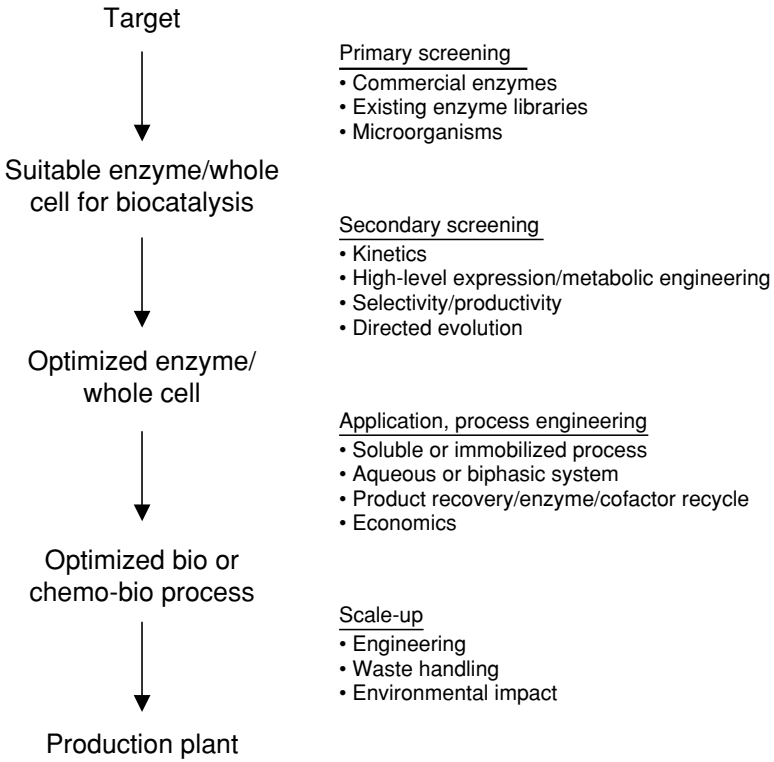
Traditional synthetic methodologies have dominated the field of racemic as well as chiral drug manufacture. However, given the ever-increasing importance of single enantiomers and molecules with multiple chiral centers, there is general realization that enzymes are highly suited for producing optically active small molecules, by virtue of their inherent chemo-, regio-, and enantiospecificity (Erb, 2002; Liese *et al.*, 2000; McCoy, 1999; Patel, 2000). Despite this, commercial application of biocatalysis is not widespread. The applications have

TABLE I

Examples of Select Chiral Molecules and Their Different Effects[a]

Biological effect of right-handed molecule	Drug	Biological effect of left-handed molecule
Orange odor	Limonen	Lemon odor
Causes blindness	Ethambutol	Tuberculosis drug
Extremely toxic	Penicillamine	Antiarthritic
Sedative	Thalidomide	Causes birth defects
Contraceptive	Propranolol	β-Blocker

[a] Used with permission from K. Barry Sharpless and R. Pittman.



SCHEME 1. General scheme for discovery and application of biocatalysis.

been limited to a few hydrolytic reactions such as hydrolytic resolution of racemic esters by lipases, hydrolysis of nitriles by nitrilases, and nitrile hydratases to produce chiral hydroxyacids and amides, respectively (Liese *et al.*, 2000; Ogawa and Simizu, 1999). One reason for this is the difficulty of obtaining enzymes and/or whole cells for specific applications and/or optimal performance. There is a serious lack of large libraries of enzymes from diverse sources for rapid screening against target reactions. In particular, industrially important hydrolytic enzymes such as lipases and nitrilases from diverse microbial sources are not readily available for screening against target reactions.

Various stages of a biocatalytic process are illustrated in Scheme 1. The starting point in the scheme is the discovery of a suitable biocatalyst by screening against a target reaction. Once the biocatalyst is found, the subsequent steps include rigorous characterization in

terms of specificity, productivity, bioprocess development, and manufacturing. If necessary, the biocatalyst can be optimized by directed evolution (Zhao *et al.*, 2002) or by the traditional techniques of mutation and selection. Although the overall scheme has been simplified, it is adequate to illustrate the limitations in developing a biocatalytic process. Diverse families of enzymes for primary screening are not readily available. For example, only about 20 lipases are accessible to screen for hydrolysis or resolution of esters, or for (chiral) transesterification (Bornscheuer and Kazlauskas, 1999). Approximately six enzymes are commercially available to screen for hydrolysis of nitriles to their corresponding amides or (chiral) acids (Biocatalytics, Inc., 2001). Indeed, a large collection of microorganisms is available in public and private collections for screening (Ogawa and Simizu, 1999, 2002; Schmid *et al.*, 2001; Shimizu *et al.*, 1997); however, their numbers are still small relative to the overall microbial diversity that is known to exist in soil and other environments. Even if a microorganism is identified for a specific reaction, there is the added challenge of finding, isolating, and optimizing the gene or genes. Although optimization of microorganisms via mutation and selection has been successful in terms of commercial applications (Liese *et al.*, 2000; Kirk *et al.*, 2002; Ogawa and Simizu, 2002; Schmid *et al.*, 2001), the process is very slow. In today's business environment where speed to market is essential, unless effective biocatalysts are found or optimized and developed rapidly, biocatalysis will not become the method of choice.

Of the three sources listed in Scheme 1 for primary screening against a target reaction, use of microorganisms is most common in spite of serious limitations. These include (1) low activity in the native organism, (2) suppression of genes by the growth media, (3) difficulty of culturing individual microbes in different media, in high-throughput formats, (4) lack of penetration of the target substrates into the cell, (5) loss of the desired product due to further metabolism, and (6) nonavailability of diverse microbes in the soil and other environments for screening due to inability to culture them. It is well documented that only about 1% of soil microbes are culturable (Amman *et al.*, 1995; Robertson *et al.*, 1996; Short, 1997). Nevertheless, screening of microorganisms for biocatalytic applications will continue to be important, especially if the desired reactions are complex. For example, oxygenation or oxidation reactions require multicomponent enzymes coupled to cofactors and intracellular generation of reducing equivalents (Schmid *et al.*, 2001). Another contributing factor is the rapid development of the field of microbial genomics, with approximately 500 bacterial genome sequencing projects now completed or underway in the public domain

(Wackett, 2002). This will significantly impact the discovery of enzymes for biocatalytic applications by providing new pathways and readily available genes to screen. However, for screening simple hydrolytic reactions of commerical interest, there is a dearth of large libraries of enzymes of various classes.

In this chapter, a novel soil-based gene discovery technology is reviewed, which has the potential to accelerate and broaden biocatalytic applications for production of fine chemicals and for bioremediation. Some of the advantages of this technology include (1) access to genes from uncultured microorganisms from a wide range of environments, (2) rapid generation of large families of enzyme libraries for screening, (3) ease of screening using cell lysates or purified enzymes from recombinant hosts or genomic clones, (4) direct access of substrates to enzymes for efficient screening, (5) immediate characterization of selectivity and productivity of specific enzymes in targeted applications, and (6) ready access to multiple genes with desirable phenotypes, for rapid optimization by directed evolution (Zhao *et al.*, 2002).

Three specific examples from the authors' laboratories are reviewed. They demonstrate the ability of soil-based gene discovery to provide libraries of enzymes for industrial applications. The diversity and novelty of genes obtained in all three examples have been validated in terms of their relationships to known sequences. In two cases, where traditional approaches have not yielded an appropriate enzyme, it has been shown that soil-based generation of enzyme libraries and screening can help in the rapid discovery of an appropriate enzyme to enable biocatalytic applications.

II. Diversity in Soil-Based Gene Discovery

Recently, molecular phylogenetic methods have been developed that allow species characterization of entire soil biotopes without individual cultivation (Amman *et al.*, 1995; Robertson *et al.*, 1996; Hugenholtz *et al.*, 1998; Short, 1997, 1999a,b, 2001b,c). Signature analysis of 16 S RNA from soil samples has demonstrated that the multitude of discrete species represented in a single sample can encompass much of the breadth of microbial phylogeny and go far beyond the numbers and the phenotypes of the known cultured organisms (Hugenholtz *et al.*, 1998). The virtue of this analysis is that it demonstrates the availability of an immense trove of genes from organism consortia. For the industrial chemist, it is a potential toolbox of biocatalysts for performing targeted chemical transformations.

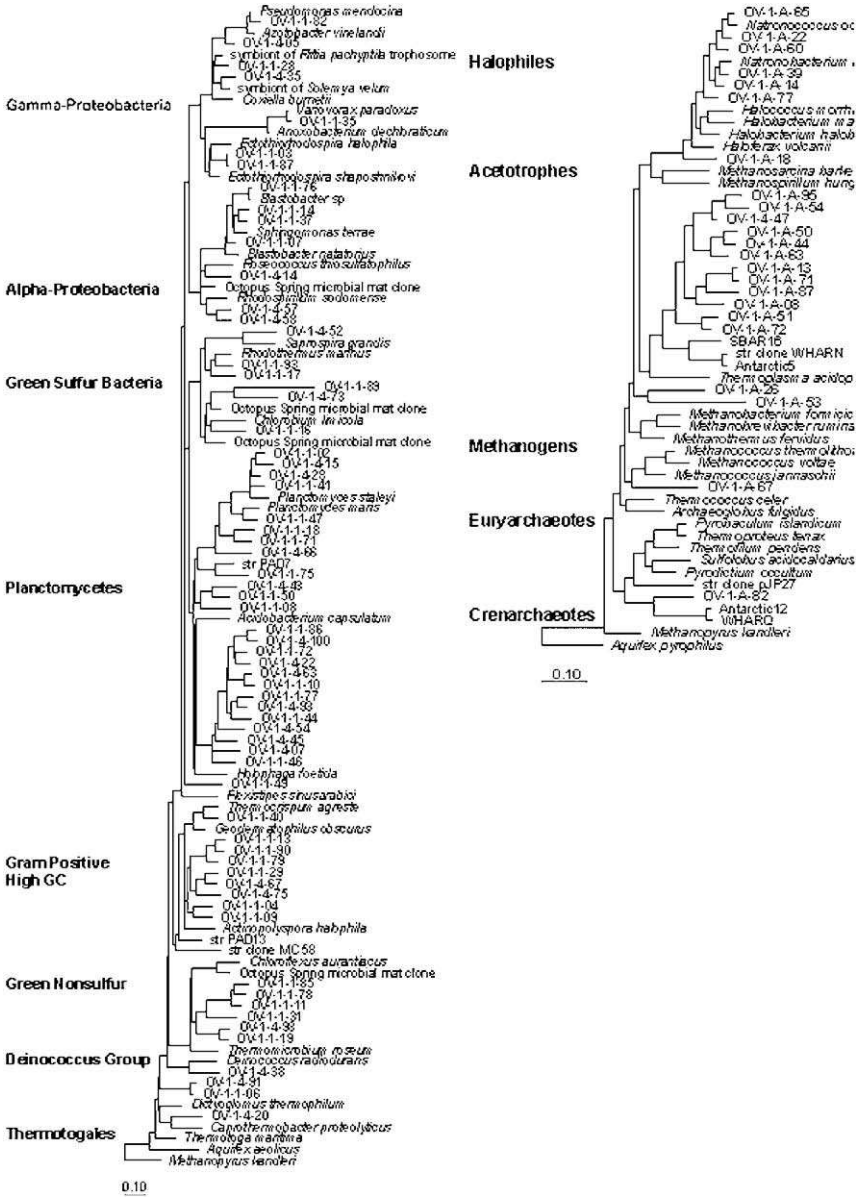


FIG. 1. Partial 16 S RNA sequence analysis of a fraction of DNA isolated from an alkaline desert soil. The figure shows a phylogenetic tree based on 16 S rRNA sequences retrieved from a soil sample obtained from an alkaline desert environment. Dotted lines represent lineages added by the ARB parsimony insertion tool. The right column shows relationships of archaeal clones with *Aquifex* sp. as an out group. The left

Figure 1 shows a partial 16 S RNA sequence analysis of a fraction of DNA isolated from a single 10-g soil sample collected from an arid, alkaline soil environment. All representative sequences, designated **OV-x-x-x**, are referenced to a 16 S phylogeny of cultured soil organisms. The reference sequence hierarchy covers the spectrum of microbial divisions as defined by Hugenholtz *et al.* (1998). Representative sequences from the soil sample are found in all divisions, substantiating the idea of a discrete, complex, interacting consortium supporting the chemical cycle in its discrete microenvironment. Analyzing approximately 5% of the available sequences in the soil sample identified 88 new 16 S signatures with >10% divergence. A conservative extrapolation suggests that this soil biotope supports >1000 unique species of Eubacteria and Archaea. This type of analysis provides a clue to the genetic diversity in the soil consortium. To realize the ultimate utility of gene products from soil for biocatalysis, one must be able to isolate and characterize individual genes from this mixture of complex genomes with respect to desired chemical transformations.

A. Accessing the Genetic and Functional Diversity in Soil

A variety of techniques have been used for the capture of genes from uncultured organisms utilizing both sequence- and activity-based screening methods (Short, 1997, 1999a,b, 2000a,b, 2002a–c). For sequence-based discovery, degenerate polymerase chain reaction (PCR) has been the method of choice. This method relies on the ability to make degenerate oligonucleotides that hybridize with conserved regions within the gene family of interest. Using these primers one can amplify fragments from genomic environmental DNA, fragments corresponding to the sequence family of interest. To capture the full-length genes, further PCR is employed using primers to the initial amplified fragment and random primers that target the unknown flanking sequence. Alternatively, if a small or large insert library is generated from the same environmental DNA, the fragment can be radioactively labeled and larger fragments hopefully containing the full-length gene

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column shows the relationship of bacterial clones with *Methanopyrus lendlari* as an out group. The trees are based on the results of distance matrix analysis including complete or almost complete 16 S rRNA sequences from representative *Archaea* and *Bacteria*. The topology of these trees was evaluated and corrected according to the results of distance-matrix, maximum-parsimony, and maximum-likelihood analysis of various data sets. The bars indicate 10% estimated sequence divergence.

can be pulled out by hybridization. Using these approaches new nitrile hydratases (Precigou *et al.*, 2001), lipases (Belle *et al.*, 2002), and 2,5-diketo-D-gluconic acid reductases (Eschenfeldt *et al.*, 2001) have been isolated. An alternative sequence-based approach for direct access to full-length genes from environmental DNA was reported by Stokes *et al.* (2001). Using a method termed Gene Cassette PCR, a 59-base element family of recombination sites that flank gene cassettes associated with introns was targeted. Contained within these cassettes were novel phosphotransferases and methyl transferases.

Activity-based screening methods have also been successful for soil-based gene discovery (Robertson *et al.*, 1996). For example, Majernik *et al.* (2001) have employed a complementation strategy to find novel Na^+ (Li^+)/H^+ antiporters. In their study, a modified *Escherichia coli* strain that was deficient in the three genes coding for Na^+/H^+ antiporters was used. Environmental DNA libraries were then screened on plates containing lithium chloride. Colonies that grew on these plates were found to contain new antiporter genes. Additionally, Henne *et al.* (1999) were able to screen environmental soil DNA libraries in *E. coli* to discover genes responsible for the utilization of 4-hydroxybutyrate by using this compound as a sole carbon source. Screening large or small insert soil or other environmental DNA libraries for enzyme activity has resulted in the discovery of novel lipases (Henne *et al.*, 2000), chitinases (Cottrell *et al.*, 1999), and amylases (Rondon *et al.*, 2000) as well as an entire biosynthetic gene pathway producing deoxyviolacein (Brady *et al.*, 2001).

One of the most critical steps in constructing a representative environmental DNA (eDNA) library is the preparation of high-quality large-molecular-weight DNA. This is necessary for efficient enzymatic manipulations during subsequent cloning steps. DNA isolated directly from the environmental soil sample or microbial cells may be separated from the soil matrix prior to DNA recovery. The direct lysis technique provides more DNA with a generally higher representation of the microbial community; however, it is smaller in size and more likely to contain enzyme inhibitors than DNA recovered using the cell separation technique. Direct lysis techniques have recently been described that provide DNA of high molecular weight (Zhou *et al.*, 1996; Zengler *et al.*, 2002). To recover DNA of higher purity and higher molecular weight, cell extraction techniques may be used (Holben, 1994; Short, 1999a,b, 2001b,c). This approach uses differential centrifugation to separate the cell fraction from soil matrix debris (Fægri *et al.*, 1977). The cell pellet obtained is lysed by enzymatic and chemical digestions, followed by the isolation and purification of genomic

DNA. To obtain highly purified suspensions of microorganisms even before the extraction of DNA, isopycnic density gradient centrifugation is used with Nycodenz as the density material (Ford and Rickwood, 1982).

The process of DNA extraction captures all eubacterial and archaeal DNA. This DNA is size fractionated and cloned into a common expression vector and expressed in a compatible host system. The size of the DNA library depends upon the number of organisms and individual species represented in the sample. Another critical factor for effective library screening is normalization of the library for representation of individual species' genomes (Short, 1997; Short and Mathur, 1999). Knowing that a microbial community contains many species and that some of those species are represented by significantly more individuals than others, the DNA library may be normalized by GC content, for example, to reflect a uniform representation by each species' DNA. After host transformation each of the members of the resulting clonal population contains an insert of 1–5 kb and the population varies in size from 10^5 to 10^{10} clones.

Soil samples have been collected from a vast diversity of geographic origins and more than 1700 complex DNA libraries have been generated (Short, 1997). The samples used to create these libraries were collected under agreements negotiated between Diversa Corporation and the political entities in control of these natural resources. All collecting was done under the guidelines of the Convention of Biodiversity (Rio Treaty of 1992) that establishes national sovereign rights to natural resources (http://www.biodiv.org). Individual agreements reflect a partnering wherein discovery of genes with industrial utility engenders benefit sharing with the political entity. Collection of individual samples was targeted to biotopes of potential interest, i.e., those in which physical parameters and chemical composition determine aspects of phenotypes of industrial utility and process compatibility. The extrinsic environmental variables of the sample area can be expected to have selected for a population of organisms whose genes have evolved to function optimally under those conditions. For example, the soil sample from the arid, alkaline soil analyzed in Fig. 1 was chosen to yield enzymes with optimal productivity at high pH, temperature, and salinity.

The multigene eDNA libraries are available for high-throughput screening by either sequence identity methods or by activity measurement of expressed genes. The former requires the design of effective sequence probes and captures genes with significant identity to the probe. Iterative screening of the library using newly discovered sequences can

reveal an extended protein sequence phylogeny. Expression screening requires development of a targeted, sensitive assay for gene product activity, amenable to high-throughput, automated technologies. These methods have been used to capture thousands of novel enzymes from complex microbial consortia (Short, 1997; Richardson *et al.*, 2002). These enzymes have been characterized and found to have operating parameters consistent with their source microenvironments.

III. Soil-Based Gene Discovery of Novel Dehalogenases for Remediation and Production of High-Value Chemicals from Haloalkanes

A. Background

Manufacture of propylene oxide and epichlorohydrin as shown in Fig. 2 generates the low-value coproducts 1,2-dichloropropane (DCP) and 1,2,3-trichloropropane (TCP) (Swanson, 1994, 1999; Dravis *et al.*, 2001). TCP could be selectively and hydrolytically dechlorinated via biocatalysis, to dichlorohalohydrin (DCH), and recycled into

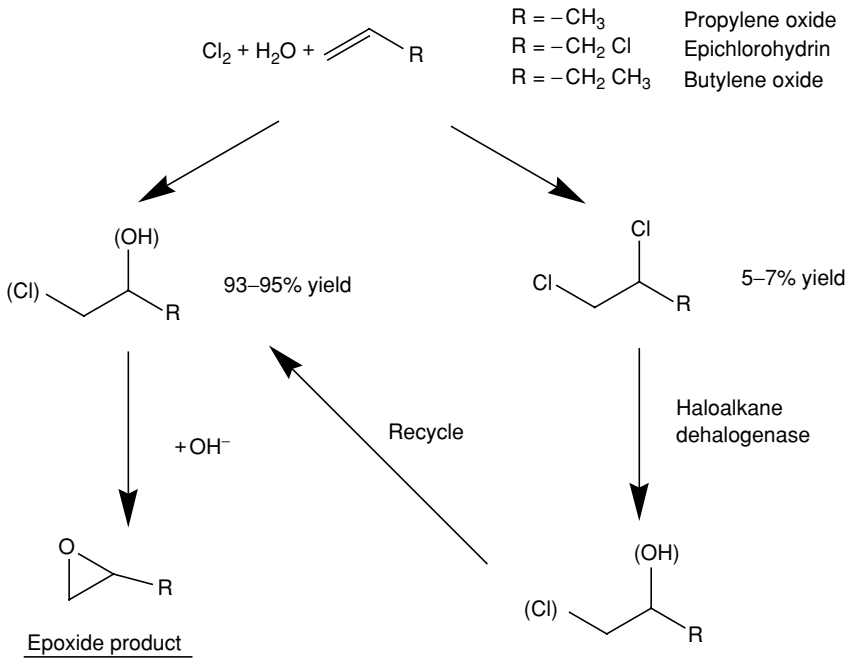


FIG. 2. Manufacture of propylene oxide and epichlorohydrin.

the process. Implementation of biocatalysis can significantly impact the economics of the processes, since the coproducts represent both a 5–7% yield loss and a challenge for remediation. Alternatively, both TCP and DCP could be converted to high-value products such as chiral epichlorohydrin or to polyols via appropriate hydrolytic dehalogenases (EC 3.8.1) (Swanson, 1994, 1999; Dravis *et al.*, 2001).

B. Discovery of Dehalogenases via Traditional Screening of Microorganisms and Selective Enrichment from Soil

Several haloalkane dehalogenases (EC 3.8.1.3) derived from cultured microorganisms are described in the literature (Slater, 1994; Fetzner and Lingens, 1994; Preis *et al.*, 1994; Jansen *et al.*, 1987). However, activity of these enzymes against hindered substrates such as DCP and TCP was found to be poor. Hence, an enrichment program was initiated to select for microorganisms that would utilize TCP and DCP as sole sources of carbon. Diverse soil samples were used as starting material for classic enrichment techniques using mineral salts media in either shake flask liquid culture, minimal agar, or chemostat formats. The volatile chlorocarbons were delivered in the headspace. Despite lengthy enrichments, this procedure did not yield any microorganism that could utilize TCP or DCP as a sole source of carbon (presumably due to the toxicity of downstream metabolites). An alternative enrichment strategy was conducted using monochlorocarbons as the sole source of carbon. Offering 1-chlorobutane, 2-chlorobutane, 1-chloro-2-methylpropane, or 1-chloro-2,2-dimethylpropane as sole sources of carbon successfully provided about 200 microorganisms for further screening against TCP and DCP. Aerobic microbial dehalogenation may occur through hydrolytic or oxidative mechanisms (Fetzner and Lingens, 1994; Preis *et al.*, 1994). Given the fact that oxidative enzymes require added cofactors such as NADH or NADPH, a primary screen was conducted using dialyzed cell-free extracts to select for hydrolytic dehalogenases. This procedure yielded one microorganism, *Rhodococcus rhodochrous* TDTM-003, with the required substrate selectivity on TCP and DCP.

The properties of the *Rhodococcus* haloalkane dehalogenase are summarized in Table II. It is a small monomeric protein (34.5 kDa) tolerant to solvent and stable to oxidation. No metals or coenzymes were needed for dehalogenation of TCP and DCP to the desired products. Sequence and structure analysis (Newman *et al.*, 1999) of this dehalogenase revealed a catalytic triad and other features consist-

TABLE II

PROPERTIES OF WILD-TYPE *RHODOCOCCUS RHODOCHROUS* TDTM-003 HALOALKANE DEHALOGENASE

Haloalkane dehalogenase	Property
Molecular weight	34.5 kDa
Subunit structure	Monomer
Enzyme class	EC 3.8.1, α, β-hydrolase
Catalytic triad	Asp-117, His-283, Glu-141
Bound coenzymes	None
Bound metals	None
K_m (trichloropropane)	1.2 m*M*
K_{cat} (trichloropropane)	0.17 min^{-1}
K_i (2,3-dichloro-1-propanol)	1.3 m*M*
$T^{(0.5)}$ at 55°C	<11 min

ent with a general acid–base mechanism for catalysis. However, poor kinetic properties of the enzyme on the target substrates (Table II) posed a challenge for developing a biocatalytic process. Although hydrolysis of TCP and DCP is thermodynamically favored, productivity of the *Rhodococcus* enzyme was hindered by a low turnover number ($K_{cat} = 0.17$ min^{-1}) and a high K_m (1.3 m*M*) for TCP. Although not inhibited by chloride ion, the product, DCH inhibited the reaction ($K_i = 1.5$ m*M*). These kinetic features seriously limited the utility of this enzyme for biocatalytic remediation and recycling of TCP or DCP.

An effort was made to improve one or more of the kinetic properties of the wild-type *Rhodococcus* dehalogenase by directed evolution. Gene Site Saturated Mutagenesis (GSSM; Gray *et al.*, 2001; Short, 2001a), a technology that systematically alters each of the gene's triplet codons to all of the 63 alternatives, was applied to the wild-type gene to generate a library of variants. Although this procedure resulted in mutants with remarkable thermostability, and some improvement of K_{cat} (Table III), the inhibition caused by DCH was not significantly altered. Clearly, the availability of a large library of hydrolytic dehalogenases was a critical need to find an appropriate enzyme for the desired application (Fig. 2) or to provide a family of closely related genes with appropriate phenotypes for potential optimization by directed evolution, as required.

TABLE III

COMPARISON OF THE PROPERTIES OF WILD-TYPE *RHODOCOCCUS RHODOCHROUS* TDTM-003 HALOALKANE DEHALOGENASE, ITS MUTANT DERIVATIVE, AND HALOALKANE DEHALOGENASES FROM SOIL-BASED GENE DISCOVERY[a]

Enzyme	Source	Amino acid change or % identity	K_{cat} (TCP) (min^{-1})	K_m (TCP) (m*M*)	K_i (DCH) (m*M*)	DCH (enantiomeric excess)
Wild type	*R.r*	—	0.17	1.2	1.5	40
Dhla 8	GSSM of *R.r*	8[b]	0.78	1	1	16
BD2025	Biopanning	55.1	0.41	ND	ND	42
BD2026	Biopanning	62.3	0.14	2	21	57
BD2027	Biopanning	63.8	0.24	5	13	70
BD2084	Biopanning	46	0.19	1.5	>100	55

[a] Data on Dhla 8, a highly thermostable mutant of wild type, was obtained from Gray *et al.* (2001). TCP, trichloropropane; DCH, dichlorohalohydrin; *R.r, Rhodococcus rhodochrous*; ND, not determined.

[b] This mutant derived had eight amino acid changes from the parent.

C. SOIL-BASED DISCOVERY OF DEHALOGENASES

The application of conventional sequence-based screening methodology, such as plate-based hybridization, becomes impractical with DNA libraries containing 10^6 to 10^9 clones. In the present work, a proprietary technology termed biopanning (Short and Mathur, 1999; Short, 2000a,b) was used. This technique allows for enrichment of specific gene sequences. An overall scheme for the biopanning technology is shown in Fig. 3 (see color insert). In brief, the method relies on access to a known gene or set of genes and uses the gene(s) or a fragment with internally conserved motifs as a probe. These degenerate primers can then be used to prescreen eDNA from soil and other environmental libraries. Positive libraries yield fragments of genes that can then be used as probes to pull out full-length sequences with significant identity to the original probe by Biopanning. The degenerate PCR approach was used to discover novel haloalkane dehalogenases from DNA libraries constructed directly from soil (Short and Mathur, 1999; Short, 2000a,b). The set of sequences for the initial probe development used two known haloalkane dehalogenase genes (Newman *et al.*, 1999; Nagata *et al.*, 1997). A third gene from *Mycobacterium tuberculosis* (Poelarends *et al.*, 1999) postulated to be a dehalogenase by BLAST

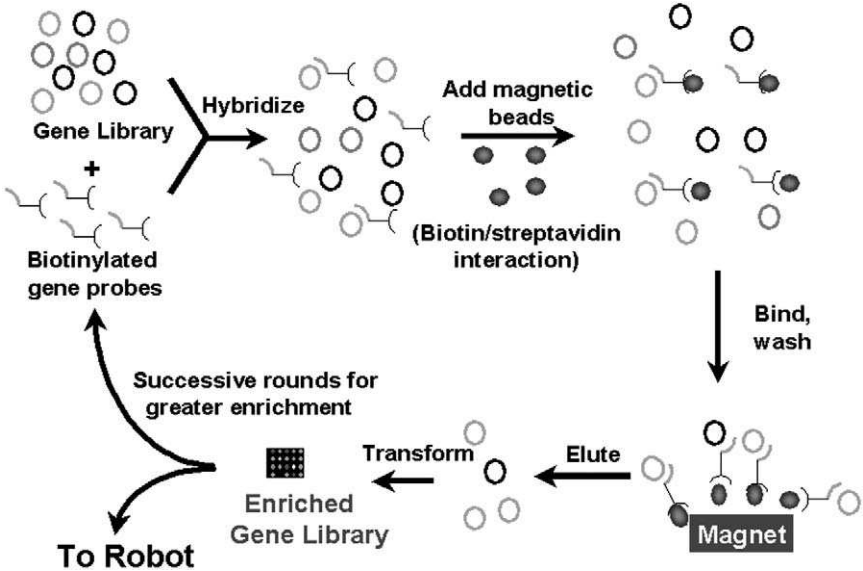


FIG. 3. An overall scheme for discovery of dehalogenases from soil using Biopanning. Complex, multiclonal DNA libraries, with 1 to 5-kb fragments incorporated into phage constructs, are screened for sequence orthologs using gene-specific probes. The biotinylated probes, which hybridize under conditions of defined stringency, are recaptured using streptavidin-labeled magnetic beads. Streptavidin–biotin pairs are washed and the captured DNA fragments are eluted from the magnetic beads. The process can be used in an iterative mode to successively enrich libraries for specific gene sequence orthologs.

analysis following a survey of the nonredundant database at the National Center for Biotechnology Information (NCBI), was also included. When these three genes were aligned, two regions of high identity were found. In total approximately 300 eDNA preparations were screened.

A total of 461 DNA samples and gene libraries were prescreened by PCR with degenerate primers (Damborsky *et al.*, 1997) designed from an alignment of the haloalkane dehalogenase genes of *R. rhodochrous, M. tuberculosis*, and a *Pseudomonas* sp. A series of experiments was conducted to determine that the degenerate primers could amplify their cognate sequences whereas the primers did not amplify any sequences in *E. coli*. Seventy unique clones greater than 400 bp in length were found to have significant identity to the dehalogenase probes, of which 19 were identified as full-length haloalkane dehalogenase genes. The sequences of the 19 genes were aligned and subjected to phylogenetic analysis with the known dehalogenase genes (Cowan *et al.*, 1998). The resulting dendrogram is shown in Fig. 4 (see color insert). The

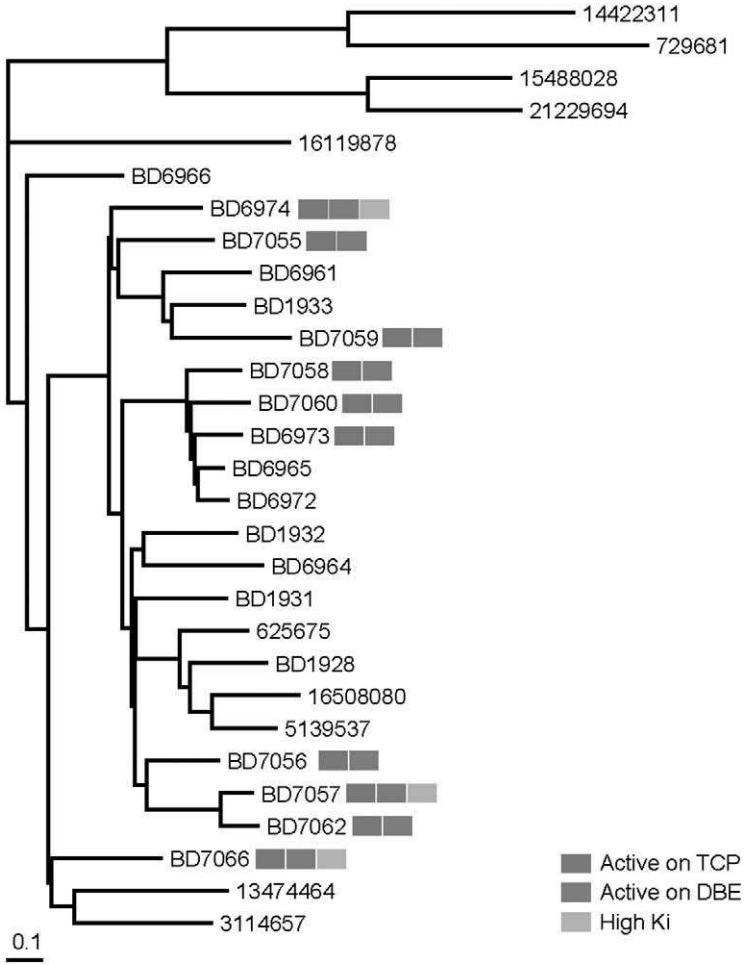


FIG. 4. Phylogenetic analysis of haloalkane dehalogenase genes discovered from Biopanning. The phylogenetic tree compares biopanned genes (BD numbers) with dehalogenases from the Genbank public database. The tree was generated using the program Phylip (version 4.0) using the nearest-neighbor joining method. The branch length represents average amino acid substitutions per 100 residues. The sequences group into subclades distinct from any of the previously deposited sequences. The enzymes were tested for activity on TCP and DBE (dibromoethane) and specificity for these substrates was found in the indicated areas of the phylogeny. Genbank (GI numbers): *Rhodococcus rhodochrous* (3114657), *Pseudomonas paucimoblis* (625675), *Mesorhizhobium loti* (13474464), *Mycobacterium bovis* (5139537), *Agrobacterium tumefaciens* (16119878), *Photobacterium profundum* (15488028), *Mycobacterium avium* (14422311), *Xanthomonas campestris* (21229694), *Xanthobacter autotrophicus* (729681), and *Mycobacterium smegmatis* (16508080).

sequences fall into a number of subbranches and clearly describe new niches in dehalogenase sequence space. The tree as shown is rooted to known dehalogenase phylogeny, and the new genes significantly extend the areas of sequence space heretofore defined only by dehalogenases from *Mycobacterium* and *Rhodococcus*. Note that each branch length corresponds to degree of relatedness. Also isolation of dehalogenases from cultured microbial sources has previously resulted in only two new enzymes.

The sequences represented in the Fig. 4 dendrogram are interesting for their individual phenotypes of specificity and product inhibition. The full-length genes have been subcloned and characterized for their activity on TCP as well as on a model substrate, 1,2-dibromoethane (DBE) and product inhibition by DCH. Most full-length genes have activity on both substrates, although there are notable exceptions of tighter specificities as noted in Fig. 4. Moreover, three of the 19 genes exhibited reduced product inhibition following catalysis of the industrially targeted substrates. Clearly, this set of genes has added significantly to the search for unique process parameters in terms of dehalogenation of haloalkanes.

D. Properties of Dehalogenases Discovered by Soil-Based Screening

A large number of the biopanned genes are expressed as insoluble, misfolded proteins in an *E. coli* host expression system. The enzymes that were expressed in the soluble fraction were chosen for purification and characterization. All genes were His tagged at the C-terminus and purified on Ni^{2+}-NTA chromatography. The properties of four of the biopanned genes with respect to activity verses TCP are compared to that of wild type and one of its mutant derivatives, Dhla8 (Gray *et al.*, 2001), in Table III. The new genes had between 46 and 64% identity to the wild type. Two of the four genes had significantly higher activity on TCP than the wild type, although K_m for the substrate was in the same range. Interestingly, three of the four biopanned genes were significantly desensitized to inhibition by the product DCH. In particular, BD2084 was completely insensitive to inhibition by DCH. This was one of the significant problems with respect to the application of the original gene and dhla8 (Gray *et al.*, 2001) for the conversion of TCP to DCH. Another commercially interesting property of the new gene, BD2027, is the 70% enantioselectivity in conversion of TCP to DCH. Although the data are not shown in Table III, all four biopanned genes also catalyzed the dehalogenation of TCP analogs, dichloropropane, 1,3-dichloropropane, and monochlorohalohydrin. Other biopanned

genes (Fig. 4) have not been fully characterized. Nevertheless, in one iteration of direct haloalkane dehalogenase discovery from soil, 19 new genes were discovered. In addition to full-length genes, several partial genes were also discovered, which could serve as new probes in subsequent iterations of Biopanning. The full-length genes producing active enzymes can be used for future applications, which previously were not available by the traditional screening methodology. In addition, a family of related haloalkane dehalogenase sequences (full length and partial) with multiple phenotypes is now available for improvement by directed evolution, along multiple parameters (Gray *et al.*, 2001; Zhao *et al.*, 2002).

IV. Soil-Based Gene Discovery of Novel Nitrilases for Synthesis of Chiral Amino Acids and Hydroxyacids

A. Background

Nitrilases (EC 3.5.5.1) catalyze the hydrolysis of natural and xenobiotic nitriles, under mild conditions, and often with very high enantioselectivity, to their corresponding carboxylic acids (Kirk *et al.*, 2002; Ogawa and Simizu, 2002; Schmid *et al.*, 2001). Hence, this class of enzymes is of great interest to chemical industries with respect to manufacture of fine chemicals. Highly specific nitrilases have been employed in the manufacture of fine chemicals such as acrylic acid (Ogawa and Simizu, 2002). However, nitrilases are not readily available for screening against cyanohydrins and aminonitriles for the generation of interesting chiral hydroxyacids and amino acids. Soils are clearly a potential source of microbial communities harboring nitrile-hydrolyzing activity. Organocyanide compounds are widespread in nature as intermediates in the metabolism of cyanide by plants, animals, and microbes (Wieser and Nagasawa, 2000). Hydrolytic degradation of nitriles has been observed in eukaryotes and, more widely, in microorganisms (Wieser and Nagasawa, 2000). Fewer than 15 nitrilase sequences have been reported in the literature (Cowan *et al.*, 1998) and these are all very similar to each other in DNA sequence and substrate chemo- and enantiospecificity. Using the all-inclusive screening of eDNA, the potential exists for the discovery of a toolbox of nitrilase enzymes categorized by substrate specificity and by their abilities to set the desired chiral centers in synthetically valuable carboxylic acids.

B. Soil-Based Selection Screening of DNA Libraries for Nitrilases

An *E. coli* screening host strain was optimized for selections on a nitrile substrate (DeSantis *et al.*, 2002). This host was infected with various eDNA libraries such that complete coverage of the library was achieved. Transformed clones were inoculated into a medium containing 10 m*M* concentration of an appropriate nitrile substrate as the sole nitrogen source. General aliphatic nitrile substrates such as adiponitrile were employed to capture general nitrile hydrolyzing activity. α-Methylbenzyl cyanide as a mandelonitrile surrogate or 4-chloro-3-hydroxybutaronitrile as a mimic of 1,4-dicyanobutyrate were also employed as industrially relevant substrates for capture of highly specific nitrilases. Each of the latter compounds is implicated in stereosynthesis of a desired pharmaceutical intermediate (DeSantis *et al.*, 2002). Positive nitrilase cultures were identified by growth, due to the ability to hydrolyze the nitrile substrate into acid and ammonia products for nitrogen utilization. The DNA from positive secondary cultures exhibiting regrowth was isolated and sequenced to confirm discovery of a nitrilase gene.

C. Characterization of Soil-Derived Nitrilases

The nitrilase sequence dataset consisted of 170 unique sequences derived from selection screening of more than 1700 soil and environmental DNA libraries. Nine bacterial nitrilase sequences available from GenBank are also included in the phylogenetic analysis shown in Fig. 5 (see color insert). For rooting the tree, we used the published sequence of *Arabidopsis thaliana* nitrilase (Hillebrand *et al.*, 1998). The sequences were aligned in BioEdit using Clustal W followed by manual refinement. In this dataset the nitrilases are between 304 and 385 amino acids long, most of them being in the 320–340 range. The variation in length is due primarily to extensions at their C-terminal ends. The sequences all exhibit the conserved catalytic triad Glu-Lys-Cys and appear to have structural elements common to the reported α–β–β–α-fold of this subfamily (DeSantis *et al.*, 2002).

For the phylogenetic analysis, the small regions in the global alignment that could not be reliably aligned were masked out. A maximum likelihood analysis was performed in ProML (Phylip 3.6; Felsenstein, 1989), using the JTT substitution model (Jones *et al.*, 1992) with equal rates, global rearrangements, and three random sequence addition replicates. The large dataset made the use of more complex evolutionary models computationally prohibitive. Bayesian phylogenetic analysis

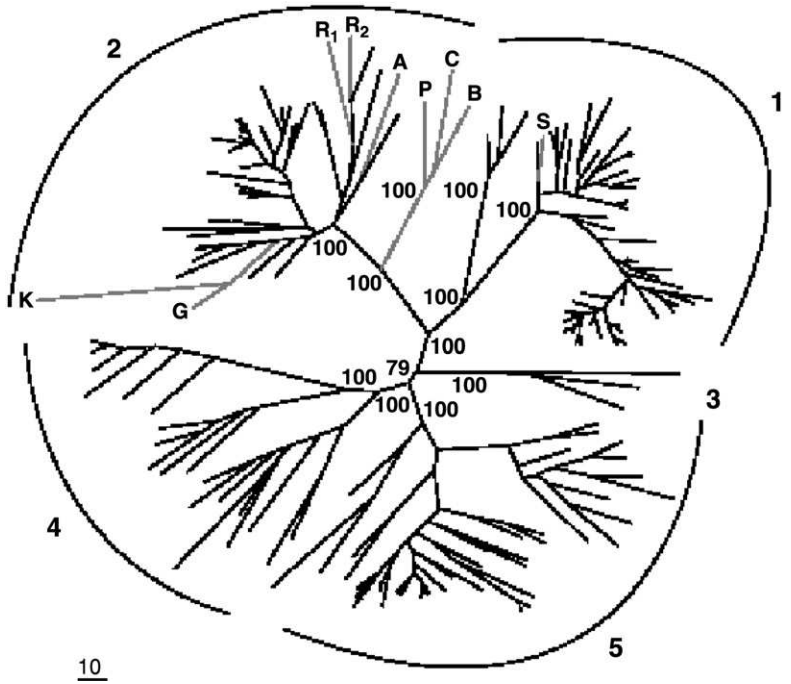


FIG. 5. Phylogenetic analysis of nitrilase genes discovered from eDNA libraries. The tree encompassing 179 novel nitrilase sequences was generated by maximum likelihood analysis. The numbers at branch points are an indication of the statistical confidence for a particular clade and were derived by Baysian inference (Huelsenbeck and Ronquist, 2001). The sequences group into five general clades with all public database sequences shown in red. Public database sequences are indicated by letter codes followed by accession numbers. Genbank GI numbers: A, *Alcaligenes faecalis*, P20960; B, *Bacillus* sp., BAA90460; C, *Comamonas testosteroni*, JC4212G; G, *Gordonia* sp., AAE06465; K, *Klebsiella pneumoniae*, P10045P; P, *Pseudomonas stutzeri*, BAA11653S; S, *Synechocystis* sp., BAA10717R1; R_1, *Rhodococcus rhodochrous*, Q02068R2; R_2, *Rhodococcus rhodochrous*, Q03217.

was also performed on the dataset, using MrBayes (Huelsenbeck and Ronquist, 2001). Four Monte Carlo Markov chains were run for 700,000 generations after stabilization of the likelihood values, generating 7000 trees. A majority rule consensus tree was generated and the percentage of the time a particular clade occurred, i.e., its posterior probability, was recorded at the nodes. Values higher that 80–85% are considered a strong support and are equivalent to high confidence values obtained by bootstrap analysis. The topologies of the maximum likelihood tree and that of the Bayesian consensus tree are virtually

identical. The same topology was also obtained by a neighbor joining analysis. Rooting of the tree with the *Arabidopsis* sequence did not affect the internal topology of the individual clades.

Inspection of the tree reveals the presence of several distinct and highly supported sequence clades. Interestingly, all but one of the public bacterial database sequences cluster within what we call clade 2. The other sequence, from the cyanobacterium *Synecocystis* sp., belongs to clade 1B. The degree of sequence conservation varies between and within the individual clades. Clusters of sequences occur within clades, in which sequences share over 75% identity. Between clades, the average percentage identity is 40–60%.

The nitrilase library generated from soil was further characterized with regard to substrate and enantiospecificity (DeSantis *et al.*, 2002). Three diagnostic and industrially relevant nitrile classes and their derivatives were chosen as target substrates, viz., mandelonitrile, 3-hydroxyglutaronitrile, and arylcyanohydrins. Of the 179 enzymes shown in the phylogenetic tree, 138 were assayed for turnover, rate, and selectivity on these substrate groups.

(*S*)- and (*R*)-Mandelic acids, the products of enantioselective mandelonitrile hydrolysis, are important in the production of pharmaceutical and agricultural intermediates (Coppola and Schuster, 1997). None of the previously described nitrilase enzymes provides optimal selectivity for economical synthesis of the desired enantiomers. Activity screening of the newly discovered nitrilase library, however, resulted in identification of 27 enzymes that provide mandelic acid with >90% enantiomeric excess (ee) (DeSantis *et al.*, 2002). One enzyme in particular exhibited high turnover, high yields, and >98% ee for (*R*)-mandelic acid. This enzyme also showed high specificity for various *ortho*-, *meta*-, and *para*-substituted mandelonitriles (DeSantis *et al.*, 2002).

Hydrolysis of cyanohydrin substrates to yield chiral aryllactic acids is another important industrial target. Aryllactic acids are valuable starting materials for fine chemical synthesis (Coppola and Schuster, 1997; DeSantis *et al.*, 2002). The library of nitrilases was again screened and a number of enzymes with attractive operating parameters were identified. In particular, one nitrilase was highly selective, i.e., >96% ee at 84% yield for the conversion of phenylacetaldehyde cyanohydrin to *S*-3-phenyllactic acid (DeSantis *et al.*, 2002). The superior productivity and enantioselectivity of this enzyme is compared to the existing commercial nitrilases in Table IV. Finally, the new nitrilase library was screened for the ability to desymmetrize the prochiral substrate 3-hydroxyglutarylnitrile. The library provided four enzymes that

TABLE IV

COMPARISON OF THE SPECIFIC ACTIVITY AND ENANTIOSELECTIVITY OF COMMERCIALLY AVAILABLE NITRILASES TO THAT OF A GENE DISCOVERED DIRECTLY FROM SOIL, WITH RESPECT TO CONVERSION OF PHENYLACETALDEHYDE CYANOHYDRIN TO (*S*)-3-PHENYLLACTIC ACID[a]

Enzyme source	Specific activity (nmol/min/mg protein)	Enantiomeric excess (%)
Nitrilase 001	48	69.5
Nitrilase 002	88	93.6
Nitrilase 003	31	83.8
Nitrilase 004	10	ND
Nitrilase 005	0	ND
Nitrilase 006	<3	ND
Diversa BD5070 (from eDNA nitrilase library)	2380	96

[a] Nitrilases 001–006 were obtained from Biocatalytics, Inc. (2001). Assays were carried out in cell-free lysates using a colorometric assay for liberation of ammonia (Fawcett and Scott, 1959). Enantiomeric excess of 3-phenyllactic acid was determined by LC on Chiraldex R (Astec).

hydrolyzed the substrate to give the (*R*)-derivative at >90% ee and 22 enzymes that converted the substrate to the (*S*)-enantiomer in high yield with ee >70% (DeSantis *et al.*, 2002).

These results demonstrate the utility of screening an enzyme library with a large degree of sequence space. The nitrilases discovered from uncultured organisms collected in various biotopes provide new substrate specificities at high productivity for some very important industrial syntheses applications. Clearly, the exploration of protein sequence space and development of screenable enzyme libraries are important for implementation of mild, selective enzyme catalysis.

V. Soil-Based Discovery of Novel Esterases and Lipases

Esterases and lipases are subclasses of the hydrolase superfamily of enzymes. Lipases have been historically differentiated from esterases by their ability to hydrolyze glycerol esters of long-chain fatty acids, as well as being enzymatically activated at the lipid–water interface. The bacterial lipases/esterases encompass a large superfamily of enzymes that has been classified into eight major families (Reetz, 2002). This classification was based primarily on sequence identity and the biological properties of the enzymes. Additional researchers have also set up lipase/esterase databases that can be accessed through the WWW. These databases classify lipolytic enzymes from a variety of

species including eukaryotic organisms. Of special note is the ESTHER database, which is dedicated to members of the a/b hydrolase superfamily (http:www.ensam.inra.fr/cholinesterase) and the Lipase Engineering Database (http://www.led.uni-stuttgart.de/), where lipases are classified into 16 superfamilies.

Esterases and lipases isolated from cultured bacteria and fungi have been extensively used in industrial applications. This work has been well documented and reviewed (Bornscheuer and Kazlauskas, 1999) and will not be covered further in this study. However, it is clear from the literature that the range and scope of current biotechnological applications for lipases/esterases are expanding.

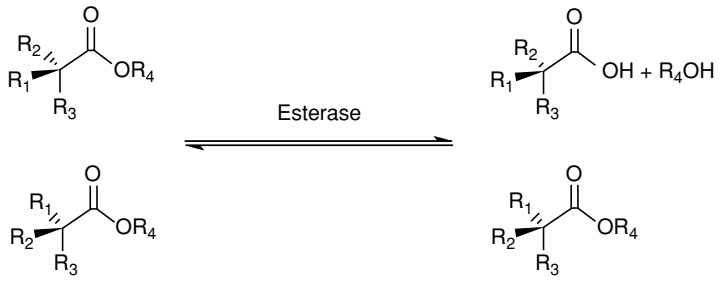
Two approaches have been taken to discover esterolytic enzymes from eDNA via high-throughput expression screening. In the first approach, a generic esterase substrate was used to screen a large number of eDNA libraries (Robertson, 1999; Short, 1999a,b, 2001b,c). This substrate is a short-chain fatty acid coupled to a chromophore that allows hydrolytic enzymes to be identified with high sensitivity. In this manner, over 350 unique and novel esterase enzymes were discovered. Bioinformatic analysis of this pool of esterase genes revealed that many of the encoded enzymes clustered into the known families previously reported (Reetz, 2002). However, despite the simplicity of the screening substrate a significant proportion of the esterases discovered had remote or no identity to previously described esterases/lipases and have been classified into novel enzyme families.

With such a vast number of enzymes discovered, it was of interest to characterize them to determine whether their individual activities or specificities are unique. In short, characterization showed that the enzymes exhibited a broad range of substrate specificities and, most important for industrial catalysis, they showed a range of enantiospecificities for chosen substrates. Table V illustrates enantiospecificities for a simple target reaction, namely resolution of a racemic mixture of a tertiary ester. Here, a diagnostic substrate was tested for hydrolysis and specificity using a subset of >50 enzymes from the esterase/lipase collection. These data indicate that some of the esterases recognized this substrate and that they resolved the racemic mixture with differing degrees of specificity. In fact, at least one enzyme was found with near absolute specificity for a single enantiomer. These data again confirm the utility of the exhaustive search through enzyme sequence space to solve important catalytic problems.

Screening of the 350 esterases discovered on the generic substrate revealed that less than 10% of the enzymes have lipolytic activity.

TABLE V

ACTIVITY AND SELECTIVITY OF FIVE BIOPANNED ESTERASES VERSUS TERTIARY ESTER[a]



Esterase	Enantiomeric excess (%)	% Conversion
BD 45	0	100
BD 138	60	40
BD 202	30	30
BD 236	40	25
BD 265	>99.8	50

[a] The reaction shown above was chosen to demonstrate the novelty of newly discovered esterases. Commercially available esterases do not have adequate selectivity on the tertiary ester. Screening was performed on extracts following subcloning and expression of the indicated enzymes. Hydrolysis of the substrate was followed by high-performance liquid chromatography.

Therefore an alternative approach to direct discovery of lipases from eDNA libraries was developed. In this method a lipid substrate is combined with a colorimetric agent to monitor the hydrolysis of the glycerol fatty acid esters. A screening program, currently in progress, has led to the discovery of enzymes that can be designated as lipases both in terms of activity and sequence homologies. Furthermore, the power of this alternative approach is demonstrated by the fact that several novel lipases have been discovered in libraries previously screened using the generic esterase screen.

These studies have demonstrated the power of using expression screening to discover lipases and esterases that differ significantly from known enzymes in both sequence and activities. Extensive characterization of these esterases and lipases is currently in progress. A wide variety of applications are being considered as targets for use of

these enzymes. These targets include biocatalytic syntheses, desymmetrizations, and kinetic resolutions. In addition, the lipolytic enzymes are being tested for utility in oleochemicals applications, such as synthesis of structured lipids and selective hydrolysis of fatty acid esters.

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The Potential of Site-Specific Recombinases as Novel Reporters in Whole-Cell Biosensors of Pollution

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I. Introduction

The presence of xenobiotic molecules in natural environments has led to the adaptation of some bacterial species to utilize such molecules as carbon and/or energy sources. Through the development of complex webs of microbial enzymes, capable of transformation of xenobiotics to metabolic intermediates, bacteria are able to derive energy even from the most recalcitrant or toxic pollutants. The regulation of genes encoding enzymes involved in pollutant degradation is typically geared toward whether the pollutant molecule itself is present in the environment of the bacterium. Such regulation is dependent on control at the transcriptional level. Genes are organized into operons so that typically, a single promoter controls manufacture of all enzymes necessary for transformation of a xenobiotic to more easily utilizable intermediates. Regulation usually relies on specific

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transcriptional activators with a promoter located immediately upstream of the degradative operon. Xenobiotic molecules of related structure bind to a recognition domain on the transcriptional activator (also known as the regulator protein) enhancing its DNA binding properties. Interaction of the transcriptional activator with several other proteins at the promoter leads to the efficient initiation of transcription.

Conventional methods of determining the presence and degradation of pollutants present in natural environments have depended largely on chemical analysis techniques (Weiman and Bojesen, 1998). Application of these methods to complex environments such as soil is technically complicated and very expensive (Martinez *et al.*, 1996; Wise and Kuske, 2000). With increasing legislation demanding that industries become accountable for the chemical pollution that they produce, there has been an ever-increasing demand for more cost-effective and precise alternatives. This is because chemical methods tend to overestimate the bioavailable portion of pollutants. In complex environments, it is known that the distribution of pollutants among solid, liquid, and gaseous phases can depend both on the pollutant itself and the type of polluted matrix in which it resides. These factors determine whether, and to what extent, compounds are accessible to bacterial degradation (Heitzer *et al.*, 1992, 1994). It has been shown that metals and xenobiotics usually exist in an insoluble form in the natural environment (Sticher *et al.*, 1997; Chaundrie *et al.*, 1999) and polyaromatic hydrocarbon degradation is controlled by the dissolved fraction of these compounds (Stucki and Alexander, 1987).

Development of whole-cell biosensors has been restricted by our limited understanding of the underlying regulation of the genetic systems that control pollutant degradation (Wise and Kuske, 2000). However, new developments in this field have determined that many pollutant-responsive systems appear conserved in many aspects of regulation. This has led to more interest in the development of biosensors capable of detecting pollutant molecules (Beaton *et al.*, 1999). A major advantage is that whole-cell biosensors can determine the bioavailable fraction of pollutant molecules, that is, the actual concentration that the microbial community encounters within a particular environment (Hestbjerg and Sorensen, 2000). Conventional, largely chemical methods, often overestimate pollutant bioavailability by measuring molecules to which microbes are not exposed. For example, in terrestrial ecosystems, access of microbes to xenobiotic molecules may be limited because of the following:

1. Many xenobiotics have low aqueous concentrations and as a consequence their bioavailability is low (Jaspers *et al.*, 2001).
2. Some chemicals have a tendency to adsorb to surfaces, dissolve into organic matrices, or form nonaqueous phases, reducing accessibility to soil microbial communities (Ramaswami and Luthy, 1997).
3. The solid phase of soil environments reduces the mobility of microorganisms, separating microbe and pollutant. For example, hydrophobic molecules often diffuse into inaccessible soil micropores (Harms, 1996).

Microbial biosensors are therefore greatly valued for their potential ability to detect the presence of environmental pollution, determine if the toxic molecules are accessible to biological communities, and assess the potential for their degradation.

Here we briefly review the major, most widely used whole-cell biosensors as well as the mechanisms by which the presence of a target pollutant is reported. The potential of DNA recombinase systems as reporters of pollutants is then described.

II. Well-Characterized Biosensors

A. Microbial Xenobiotic Biosensors

Biosensors used to detect xenobiotics may be classified as either **nonspecific**, by which toxicity exerts a detectable and usually deleterious effect on communities or individual species, or **specific**, which depend on induction/repression of genes within individual species. In the latter case, fusion of the promoter of a degradative operon with a reporter gene is exploited to record changes in transcriptional activity reflected by differences in the levels or activity of a ''reporter'' molecule.

B. Nonspecific Microbial Biosensors

Toxicity of pollutants to microorganisms can be measured at different hierarchical levels that range from entire microbial communities to individual taxa and down to individual species. Ecotoxicological analysis often examines entire populations using techniques such as measurement of CO_2 (respiratory activity), analysis of total biomass, or determination of numbers of live organisms recovered from the test environment. Such methods give a general overview of what is

occurring, but due to the multifactorial nature of complex environments such as soil, interpretation of the results is often difficult and inconclusive. For example, the use of algae as biosensors for the toxicity of polychlorinated phenols (PCP) is now a standard test for contamination of waste water. However, at high levels of inorganic nutrients such as phosphorous and nitrate, the inhibitory effects of PCPs are masked by these elevated micronutrients and algae can flourish in contaminated waters (Hund, 1997).

Methods based on the recovery of microorganisms from environmental samples are also questionable due to the uncertainty associated with the ability to culture organisms (Colwell *et al.*, 1985). Studies attempting to recover the entire microbial population from environmental samples are doomed to failure because only a small proportion of organisms is culturable. For example, only approximately 1% of cells from a lake water bacterial population are culturable by available methods (Morgan *et al.*, 1989).

At the species level, a novel new technology has been discovered to measure nonspecific toxicology using the luciferase assay (Horsburgh *et al.*, 2002; Paton *et al.*, 1995, 1997; Turner *et al.*, 2001). Initial research used the naturally luminescent marine bacterium *Vibrio fischeri*. The organism was marketed as Microtox and has been used as a rapid screen test (Steinberg *et al.*, 1995; Doherty 2001). One of the limitations of the Microtox test was that it used a saltwater bacterium that was not ecologically representative of those present in soil or lake water. Because there has not been any recorded isolation of a luminescent organism in such environments, the operon encoding luminescence has been transferred into a range of bacterial species.

In general, luminescent bacterial biosensors offer a rapid real-time analysis of the toxicity of xenobiotic pollutants. However, sensitivity is very low compared with algal biosensors. For example, changes in photosynthetic activity of algae in reponse to herbicides showed sensitivity in the low microgram per liter range (Strachan *et al.*, 2001). In contrast the most sensitive bacterial biosensor detection range was 10–400 mg/liter. Despite such drawbacks, luminescence-based biosensors have been and are continuing to be used to determine the toxicity of a range of substances such as tetracyclines (d'Haese *et al.*, 1997; Hansen and Sorensen, 2000b), metals (Paton *et al.*, 1995, 1997; Palmer *et al.*, 1998), and xenobiotics (Bundy *et al.*, 1997; d'Haese *et al.*, 1997; Reid *et al.*, 1998; Sousa *et al.*, 1998; Beaton *et al.*, 1999).

C. Specific Biosensors

Studies of specific gene expression in bacteria have been greatly facilitated by the use of reporter genes (Slauch and Silhavy, 1991) of which there are numerous examples.

1. *Chromogenic Reporter Genes*

Chromogenic reporter genes encode enzymes that produce a colored product when supplied with the appropriate substrate and the most widely used examples are *xylE*, which encodes catechol 2,3-dioxygenase (C230), *lacZ*, which encodes β-galactosidase, and *gusA*, which encodes *Escherichia coli* glucuronidase (reviewed in Jefferson, 1989).

The *xylE* gene was originally found on the TOL plasmid of *Pseudomonas putida* pWW0 (Nakai *et al.*, 1993) as part of the *meta* operon. It has additionally been found on the lower NAH, *bph*, and *dmp* operons in *Pseudomonas* spp. and has subsequently been cloned and sequenced (Noh *et al.*, 2000). xylE is an important component in the degradation pathways of toluene and xylenes and catalyzes the dioxygenolytic cleavage of the aromatic ring (Franklin *et al.*, 1981). It encodes the enzyme catechol 2,3-dioxygenase (C230), which catalyzes the conversion of the colorless intermediate catechol into hydroxymuconic semialdehyde, which is yellow in color. This yellow compound can be measured spectrophotometrically to provide a measure of gene expression in *xylE*-containing recombinant cells when catechol is added (Schweizer, 1993; Zukowski *et al.*, 1983).

Promoter-less derivatives of *xylE* have been successfully expressed in different microorganisms, including *P. putida*, *Streptomyces lividans*, *Mycobacterium smegmatis*, and *Neisseria gonorrhoeae* (Ingram *et al.*, 1989; Hahn *et al.*, 1991; Curcic *et al.*, 1994; Danaher *et al.*, 1994). However, levels of *xylE* activity can vary with both the type of promoter used and the host organism itself. It has been shown that there is increased expression in *Pseudomonas* and *Actinobacter* compared with enteric bacteria and that the degree of induction can vary considerably between different constructs (Prosser, 1994).

xylE is an attractive choice of reporter gene due to the relatively simple, rapid, and reliable detection of its gene product. However, although the gene itself is uncommon in environments not previously exposed to aromatic hydrocarbon contamination, its applicability for use in contaminated environments would be limited by the presence of indigenous *xylE* genes producing the C230 product, which would interfere with any reporter functions. Additionally, it has been demonstrated that C230 is sensitive to hydrogen peroxide (H_2O_2) when used

as a reporter in gene fusion constructs. Caution must be used in interpreting data derived from these constructs under aerobic conditions, during oxidative stress, or when catalase-deficient strains are used (Hassett *et al.*, 2000).

The *lacZ* gene encodes β-galactosidase (Pardee *et al.*, 1959) and is widely used as a reporter in whole-cell biosensors. Its enzyme product β-galactosidase normally cleaves the disaccharide lactose into glucose and galactose. However, it can also cleave commercially developed substrates such as X-gal to produce a blue pigment that is detectable spectrophotometrically. It is a relatively easy, reliable, and quick reporter assay to apply to whole-cell biosensors but as described for *xylE*, its main disadvantage is its widespread presence in bacteria. This is particularly a problem in soils rendering *lacZ* problematical for work *in situ*. Additionally, promoter fusions to *lacZ* are highly dependent on the characteristics of fused upstream regions, thus making comparative analysis between promoters complicated (Linn and St. Pierre, 1990).

2. *Fluorescent Reporter Genes*

The fluorescent reporter gene group consists of the fluorescent proteins *gfp* (green fluorescent protein), *yfp* (yellow fluorescent protein), *bfp* (blue fluorescent protein), and *rfp* (red fluorescent protein), but the most widely used and characterized is *gfp* derived from the jellyfish *Aequorea victoria* (reviewed in Tsien, 1998). Luminescence is common in a variety of marine invertebrates; many Cnidarians and probably all Ctenophores emit light when they are mechanically disturbed. The phylum Cnidaria, including *A. victoria*, emit green light due to the GFP class of proteins. Such jellyfish are generally translucent but are frequently decorated by brightly luminescent structures such as the green fluorescent lining along the margins of the jellyfish bell (Misteli and Spector, 1997). The GFP protein of *A. victoria* is highly fluorescent and is activated *in vivo* via a Ca^{2+}-activated photoprotein (Prasher, 1995) and two closely associated proteins making up the photoprotein, aequorin and the Ca^{2+}-binding apoprotein (apoaequorin), facilitate fluorescence activation in a calcium-dependent manner. Coelenterazine (an organic substrate) and molecular oxygen are also involved (Inouye and Tsuji, 1994a,b). The activated aequorin bound with Ca^{2+} triggers an intramolecular reaction in which coelenterazine is oxidized to coelenteramide yielding as products a blue fluorescent protein (coelenteramide bound to apoaequorin), CO_2, and light (Johnson and Shimonura, 1978). The activated blue fluorescent protein (aequorin)

complex transfers energy indirectly to GFP to trigger the release of green light.

The absorbance/excitation peak of the wild-type GFP is at 395 nm with a minor peak at 475 nm and the emission is at 508 nm (Tsien, 1998). The primary structure of GFP and aequorin has been deduced from the nucleotide sequence (Inouye *et al.*, 1985; Prasher *et al.*, 1992). Both proteins are made up of a single polypeptide chain and GFP and aequorin were found to be 27-kDa and 21.4-kDa protein monomers, respectively (Prendergast and Mann, 1978; Shimonura, 1979). It has been suggested that the primary structure of GFP undergoes modification to form a chromophore during expression, probably by modification of certain amino acid residues within the polypeptide (Prasher *et al.*, 1992). The chromophore of mature purified GFP is extremely stable to a wide variety of conditions and persists not only through exposure to heat (up to 65°C) and extreme pH (pH 2 to pH 11), but also through treatment with formaldehyde (Ward and Bokman, 1982) and chemical denaturants [such as 1% sodium dodecyl sulfate (SDS)]. The actual formation of the chromophore is species independent (Kain *et al.*, 1995) and is thought to result from spontaneous cyclization and oxidation of the sequence -Ser^{65} (or Thr^{65})-Tyr^{66}-Gly^{67} (Cody *et al.*, 1993; Cubitt *et al.*, 1995). This process is oxygen dependent and occurs gradually after translation has occurred (Inouye and Tsuji, 1994a). The crystal structure of GFP has been solved and shown to be a remarkable barrel-like arrangement (Ormo *et al.*, 1996; Wachter *et al.*, 1997; Yang *et al.*, 1996) of 11 β-sheets surrounding a single central α-helix of approximately 9 to 13 residues that contains the fluorescent chromophore center.

GFP is rapidly becoming an important reporter molecule for environmental applications (Kain *et al.*, 1995) and is seen to be advantageous over other more established reporter techniques in a number of ways. On the whole it has been found that GFP provides a powerful, nondestructive method for *in situ* monitoring of gene expression in a wide range of species and even in paraformaldehyde-fixed dead cells (Eberl *et al.*, 1997). Its expression in *E. coli* was found to be identical to that in *A. victoriae* (Inouye and Tsuji, 1994b), indicating that the gene itself contains all the information necessary for the posttranslational synthesis of the chromophore with no jellyfish-specific enzymes being needed. Because of this, GFP has been transferred to and expressed in an extremely wide range of organisms (Chalfie *et al.*, 1994), including mammals (Pines, 1995; Ludin *et al.*, 1996), fish (Moss *et al.*, 1996), *Saccharomyces cerevisiae* (Niendenthal *et al.*, 1996), *Drosophila melanogaster* (Brand, 1995), plants (Haseloff and Amos, 1995), *Dictyostelium*

(Hodgkinson, 1995), as well as a wide range of bacteria such as *E. coli* (Inouye and Tsuji, 1994b; Andersen *et al.*, 1998) and *Pseudomonas* spp. (Bloemberg *et al.*, 1997; Suarez *et al.*, 1997; Andersen *et al.*, 1998; Errampalli *et al.*, 1998, 1999; Joyner and Lindow, 2000). Recently it has been used as a biosensor for the detection of the induction of the SOS response as an indicator of genome damage (Ptitsyn *et al.*, 1999; Kostrzynska *et al.*, 2002).

The wild-type GFP gene has been mutated to allow different variants of fluorescent proteins to become available for biological applications. For example, Crameri *et al.* (1996) made GFP with an improved codon usage screening for the brightest transformants and eventually securing a GFP with a 45-fold increase in fluorescence. This is particularly useful when analysis at the single-cell level is required. Additionally, variants in the excitation spectra of GFP have been isolated to produce ''red shifted'' excitation (excitation spectrum shifted from 395 to 488 nm) by mutagenesis of one residue adjacent to the chromophore Thr-203 or His (Delgrave *et al.*, 1995) and blue fluorescent protein (Zhao *et al.*, 2000), which both allow the dual detection of various fluorescent strains within the same sample. Finally, the wild-type GFP has been destabilized to allow more sensitive responses to induction and transient gene expression (Zhao *et al.*, 1999).

3. *Bioluminescent Reporter Genes*

Bioluminescence refers to the process of visible light emission in living organisms mediated by enzyme catalysis, and the most frequently reported bioluminescent systems for use in whole-cell biosensor applications are from the naturally luminescent marine bacteria *Vibrio fischeri* or *Vibrio harveyi* and the North American firefly *Photinus pyralis*. Both prokaryotic (*lux*) and eukaryotic (*luc*) luminescence systems have been extensively reviewed (Zeigler and Baldwin, 1981; Campbell, 1989; Stewart and Williams, 1992; Meighen, 1988, 1991, 1993; Lindow, 1995; Steinberg *et al.*, 1994) and are known to be highly suitable as reporters of gene expression (Olsson *et al.*, 1988).

A large number of studies in microbial ecology have used bacterial luciferases as reporter genes to confer bioluminescence. The light-emitting reaction involves an intracellular, luciferase-catalyzed, oxidation of the reduced form of flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde by molecular oxygen. The *in vitro* reaction is

$$FMNH_2 + O_2 + R - CO - H \xrightarrow{\text{Luciferase}} FMN + R - COOH + H_2O + \text{blue-green light}$$

The *lux* operon consists of a number of genes that have been cloned, sequenced, and analyzed (Belas *et al.*, 1982; Engebrecht *et al.*, 1983; Boylan *et al.*, 1985; Cohn *et al.*, 1983, 1985; Delong *et al.*, 1987; Foran and Brown, 1988). *luxA* and *luxB* code for the α- and β-subunits of the bacterial luciferase enzyme, respectively. In the heterodimeric enzyme complex, it has been shown that the active site is located primarily in the 40-kDa α-subunit (Gunsalus-Miguel *et al.*, 1972) that is essential in the reaction. However, although the β-subunit is not required for the catalytic processes of luciferase, it is required in a regulatory sense to maintain the active conformation of the α-catalytic subunit (Cline and Hastings, 1972; Meighen and Bartlet, 1980; Cohn *et al.*, 1985; 1980). *luxC*, *luxD*, and *luxE* collectively encode for the fatty acid reductase required for conversion of fatty acids to the long-chain aldehyde required for the luminescence reaction. In addition, there are regulatory genes present in the operon. *luxI* and *luxR* are involved in the autoregulation of the wild-type *lux* operon (extensively reviewed in Blouin *et al.*, 1996; Meighen, 1988, 1991).

The *lux* genes can be cloned to function as a reporter system containing a complete *luxCDABE* cassette or the *luxAB* genes only. The advantage of this second approach is the elimination of the complex enzymatic subprocesses involved in the recycling of the natural lucifiern substrate. Commonly, only the *luxAB* gene fragment is fused to the promoter. However, when *luxC*, *luxD*, and *luxE* are omitted, the long-chain aldehyde substrate is not produced and must be added exogenously. This is a point to consider when designing whole-cell biosensors containing *lux* genes as reporters.

Bacterial *lux* genes have been widely used in whole-cell biosensor technology and there are many examples of environmental pollution detection utilizing the reporter *lux*, such as the detection of mercury (Selfinova *et al.*, 1993; Hansen and Sorensen, 2000a; Rasmussen *et al.*, 2000), organic carbon (Yeomans *et al.*, 1999), salicylate (Heitzer *et al.*, 1994), polychlorinated biphenyls (Layton *et al.*, 1998), nickel (Guzzo and De Bow, 1994), BTEX (Applegate *et al.*, 1998), and general toxicity (Weitz *et al.*, 2001; Bechor *et al.*, 2002). Recombinant *E. coli* containing *lux* has also been used to monitor radiation levels (Min *et al.*, 2000).

Similarly to *lux*, the eukaryotic-derived *luc* gene–based whole-cell biosensors have been successfully used in a wide range of applications, for example, in the detection of toluene (BTEX, Willardson *et al.*, 1998), antimonite and arsenite (Taurianen *et al.*, 1997), cadmium (Taurianen *et al.*, 1998), and arsenite and mercury (Taurianen *et al.*, 1999), and have been successfully used in a wide variety of host cells (Millar *et al.*, 1992; Wood, 1995).

D. Limitations of Reporter Genes for *in Situ* Applications

β-Galactosidase and luciferase assays have allowed detailed analysis of gene expression for *in vitro* monocultures of a variety of bacterial species (Andersen *et al.*, 1998). However, as more investigators have worked with these reporter genes, specific problems have been encountered for certain applications. For example, the population size of an environmental microorganism will often be very low compared with that used in the laboratory and therefore the efficiency of the reporter gene is an important issue. Both β-galactosidase and luciferase require large cell numbers for detection. This is also the case for ice nucleation reporter gene *inaZ* (Miller *et al.*, 2001).

In the case of *luxAB*, placing the entire *lux* operon onto the bacterial chromosome can compensate for the requirement of substrate addition but becomes an energy burden on cells (De Weger *et al.*, 1991). The *lux* bioluminescence assay requires metabolic energy in the form of oxidized cofactor $FMNH_2$ for the luciferase reaction. Bacteria present in their natural oligotrophic habitat are commonly extremely energy limited, thus reducing the efficiency of this biomarker *in situ*. However, attempts have been made to overcome this by linking *lux* genes to stress promoters (Ben-Israel *et al.*, 1998). Additionally, the *lux* bioluminescence reaction requires oxygen and therefore is limited to use with aerobes.

For *in vivo* applications of microbial communities the reporter gene of choice has recently switched to *gfp*. Not only does GFP not require any chromogenic substrates to be detected, but it is also very stable, requiring no time limit for bacteria to be recovered from a complex environment and analyzed. *gfp* has no background problems, under normal circumstances is not a burden to energy resources (Andersen *et al.*, 1998), and does not require cellular disruption for analysis. However, there are several drawbacks to using the *gfp*. First, as is the case with the *lux* bioluminescence reaction, GFP manufacture has a requirement for molecular oxygen. Second, analysis requires very expensive equipment such as epifluorescence microscopy or flow cytometry (Lowder *et al.*, 2000). Finally, interpretation of GFP intensities as a function of promoter induction experienced by individual cells remains a major difficulty with this reporter gene (Jaspers *et al.*, 2001).

An interesting application of promoter-driven reporter gene cassettes for monitoring the presence of bactericidal agents has been described by Goulsbra *et al.* (2001). FIS is expressed from the *fis* gene in a growth-dependent manner so that the *fis* promoter is active only in growing cells. This property was exploited to construct *fis* promoter

(P) cassettes in *E. coli* using three different reporter genes to produce P_{fis}–*lux*; P_{fis}–*gfp*, and P_{fis} *lacZ*. The reporter genes would be expressed only in growing cells and this was used to develop a monitoring protocol for the efficacy of different bactericidal agents whereby low to no reporter gene expression was indicative of killing or growth inhibition. The *lux* construct proved to be of no use because expression occurred only over a narrow range of cell densities. Likewise the *lacZ* construct proved to be a poor sensor as it lacked sensitivitiy (high cell numbers required) and was difficult to monitor in real time. The *gfp* construct proved to be an excellent reporter of growth inhibition (killing) by disinfectants as it could be monitored continuously, cells were highly fluorescent when growing, and the fluorescence of a cell was stable for long periods, which meant cells samples could be collected, stored, and analyzed later (Goulsbra *et al.*, 2001).

E. Characteristics Required in a Reporter Gene

Because of the wide range of different types of reporter genes available for use in whole-cell biosensors or technology, considerations regarding their individual characteristics, advantages, and disadvantages must be taken into account. Desirable properties required in a reporter gene include the following.

- **Stability.** The genetic stability of the reporter is paramount as is the stability of the output product. Stability requirements will vary depending on each individual application. For example, a rapid, short-lived assay would require a gene product with less stability, whereas long-term applications would require a more stable, long-lasting gene product.
- **Sensitivity.** A high sensitivity of detection is usually essential, particularly if detection of gene expression is monitored in a single or a few cells.
- **Specificity.** The reporter must be specific for the response of the organism to the molecule that is to be detected and must not be influenced by the presence of potentially competing substances.
- **A large dynamic range.** The ability to detect reporter output over several orders of magnitude is a desirable characteristic to allow for a wide concentration of the target pollutant or environmental condition.

- **Appropriateness.** Although there are characteristics desirable to most possible applications of reporter genes, specific consideration must be given to the specific application or role of the reporter gene. A reporter system for a defined application or environment may not necessarily be the most suitable for another.
- **Physiological state of cells.** Energy requirements for expression and detection of the reporter signal may be critical. For example, *luxAB* and *luc* need to be present in metabolically active cells and thus will have no phenotype in the absence of cellular, and this can limit their applicability in anaerobic environments. Ideally the reporter output should be long-lived and be detectable whether the cell is ''alive'' or dead, without requirement for the microorganism to be recovered and cultured in order to detect or induce reporter gene output.
- **Signal intensity.** Expression needs to be at a detectable level. Occasionally a weak signal can be increased by using multicopy plasmids.
- **Uniqueness.** The reporter gene should be present within the indigenous population in the natural habitats that may be the target site for biosensor applications. For example, *xylE* is native to the soil bacterium *P. putida* and background *xylE* could interfere with detection and quantification of *xylE* signals in recombinant whole-cell biosensor bacterial strains.
- **Substrate availability.** In some cases, reporter gene products require an exogenous substrate to produce a signal, e.g., *xylE*, *lacZ*, and *luxAB*.
- **Ease of detection.** Laborious protocols to detect reporter gene output are time consuming and usually expensive; rapid inexpensive equipment for detection of reporter gene expression is therefore important.

III. DNA Recombination as a Potentially Novel Reporter System

Here we briefly review site-specific recombination and describe how it could be developed to produce a reporter for *in situ* gene expression. The advantages/disadvantages of recombinases for this purpose are discussed.

The ability to respond rapidly to environmental changes is of paramount importance for microbial survival. Cells must detect changes and react rapidly and appropriately by the expression/repression of appropriate genes. Regulation of gene expression typically involves the transient interaction of a specific binding protein with a site on the

DNA adjacent to the regulated gene (Silverman *et al.*, 1979). The paradigm of the lactose operon as a model for this type of regulation holds true for regulation of a variety of operons from amino acid biosynthesis to DNA repair (Plasterk and van de Putte, 1984a,b; Plasterk *et al.*, 1985). In these systems, DNA is not directly involved in regulation and remains unchanged throughout. There are, however, other phenomena that exist to regulate gene expression. One such variation involves site-specific recombination of DNA.

A. Site-Specific Recombination

Bacteria are increasingly being studied and viewed as multicellular populations whereby survival of the population as opposed to the individual cell is paramount (Dybvig, 1993). The continuous generation of mixed phenotypes ensures that no matter how sudden the environment changes, a percentage of the population is already equipped with the capacity to express appropriate gene or genes for survival. Such subpopulations can be generated by DNA rearrangements.

Homologous or RecA-dependent recombination promotes exchange between sequences that are generally closely related in sequence, but that may under certain circumstances have as little shared homology as 20–100 bp. Generalized recombination mediated by RecA can be utilized to create significant variation in particular alleles. Perhaps the best example occurs in pilus variation in *Neisseria gonorrhoeae* where over a million combinations of sequences can theoretically be generated in the pilin subunit (Dybvig, 1993; Saunders, 1999). In this case, variation and ON↔OFF switching is mediated by *recA*-dependent recombination between an expressed locus (*pilE*) and one of a number of truncated silent pilin sequences (*pilS*). More commonly, qualitative and/or quantitative switches in gene expression are achieved by RecA-independent mechanisms. Frequently, such DNA rearrangements involve site-specific recombination as opposed to the generalized form of recombination promoted by RecA.

B. DNA Inversion

Site-specific recombination leading to DNA inversion is widespread in both Gram-positive and Gram-negative bacteria. Inversion has been shown to occur not only on chromosomes, but has also been found on plasmids and bacteriophages with inverted DNA sizes ranging from as

little as 314 bp to 35 kb. Frequencies of inversion are typically in the order of 10^{-3} to 10^{-4} (Dybvig, 1993).

DNA inversion acts in the simplest case as an ON/OFF switch, typically placing or removing a promoter from upstream of the coding region of a gene. Therefore, the gene is expressed only if the invertible element is in the correct orientation. For example, the Hin invertase of *Salmonella typhimurium* inverts an ON/OFF switch for flagellar phase variation (Silverman *et al.*, 1979). In more complicated systems represented by Shufflons (Komano *et al.*, 1995), combinations of inversions and shuffling of segments internal to a coding region may affect variation at specific sites in a genome.

C. Families of Recombinase

There are two recombinase enzyme families catalyzing single-stranded DNA inversions that can be distinguished by amino acid sequence comparison. The first class is the **DNA Invertase** (DIN) family, a subset of the transposon Tn*3* resolvase family of site-specific recombinases (Sherrat, 1989). The DIN family recombinases are believed to promote inversion by a mechanism involving double-stranded DNA breaks where inverted repeats in the DNA are aligned in parallel and a subsequent cleavage, rotation, and religation of the two DNA strands cause a sequence inversion (Stark *et al.*, 1992).

The second family of recombinase is the **DNA Integrases**, so called because they show homology with phage λ integrase and they catalyze inversions, deletions, and intramolecular reactions. They promote pairwise single-stranded exchanges by the formation of Holliday junctions. (Craig, 1988; Stark *et al.*, 1992). An example of such a system is the variation of type 1 fimbriation in *E. coli* in which related proteins FimB and FimE regulate a molecular switch for inverting the promoter for the *fim* operon (Glasgow *et al.*, 1989).

D. DIN

The DIN family consists of four known systems in prokaryotes; the Hin system of *Salmonella typhimurium*, the Cin system of phage P1, the Pin system of *E. coli*, and the Gin system of phage Mu and all are known to be functionally interchangeable (Craig, 1988). The bacterial DNA invertases are a particularly well-characterized family of closely related site-specific recombination systems (Dybvig, 1993), but each system encodes its own specialized recombinase that functions at

unique DNA sites assisted by additional cellular proteins. All invertases are known to be able to complement each another (Plasterk and van de Putte, 1984a) and each system is discussed in detail below.

1. *The Pin System*

A cryptic episome-like element has been found to be present in the *E. coli* K-12 chromosome and this has been named e14 (Greener and Hill, 1980). It is present as a 14.4-kb DNA circle that is induced to excise from the *E. coli* chromosome upon activation of the SOS pathway by stimuli such as ultraviolet (UV) light or thermal shifting (Brody *et al.*, 1985). el4 is considered to possibly be a defective prophage and it has been found that *E. coli* that harbor this element can complement mutants lacking the *gin* recombinase gene to allow inversion of the phage Mu G-Segment. Therefore, it is referred to as a recombinase, Pin, that appears to control the inversion of an internal 1.8-kb portion of the element known as the P-Segment. This inversion appears to control alternate expression of two proteins that are encoded partially within this inverting P-Segment. The Pin recombinase system and its relationship to other recombination systems have been reviewed in more detail in Plasterk and van de Putte (1984a) and Glasgow *et al.* (1989).

2. *The Hin System*

The Hin system is found in *S. typhimurium* and the inversion of the H-Segment mediates the reciprocal expression of two sets of tail fiber genes by switching the orientation of their promoter (Silverman *et al.*, 1979). The Hin system consists of a 995-bp DNA fragment (Zeig and Simon, 1980) adjacent to the H2 structural gene and the orientation of this inversion region controls expression of the *fliC/fljB* genes for H2 flagellin and the FljB/FljA repressor of an alternate flagellin gene. In one orientation, there is no expression of the H2 flagellin gene whereas in the other, H2 is expressed. The invertible segment is flanked by the two recombination sites *hix*L and *hix*R (Zeig and Simon, 1980; Johnson and Simon, 1985; Haykinson *et al.*, 1996) that are bound by a dimeric Hin recombinase protein to allow cleavage and exchange of the two DNA strands with the aid of a host *E. coli*-encoded factor of 98 amino acids, FIS that binds to a recombinational enhancer (Johnson *et al.*, 1988; Hiechman and Johnson, 1990). This results in inversion of the region at a frequency ranging from 10^{-3} to 10^{-5} per bacterium per generation (Glasgow *et al.*, 1989; Henderson *et al.*, 1999).

3. *The Cin System*

Inversion of a 3-kb DNA segment flanked by 0.6-kb inverted repeats (Chow and Bukhari, 1976) on the bacteriophage P1 genome is mediated by the P1-encoded site-specific Cin recombinase. Cin binds to the *cix*L and *cix*R inverted repeat recombination sequences to allow inversion of the C-Segment allowing infectivity of the phage P1 on *E. coli* K-12 to be altered depending upon C-Segment orientation (Iida *et al.*, 1982). This system is also sometimes referred to as the Gin system of phage P1 as the genes encoded by the invertible C-Segment, 19 and 19′ (alternate tail fibers) and *tfs* (tail fiber specificity) share extensive homology with corresponding regions of phage Mu.

E. The Gin System

Along with Hin, the most extensively studied DIN system is the Gin system. The Gin regulator of phage Mu is highly homologous to the above regulators. It shows 60–70% amino acid identity with Hin (van de Putte and Goosen, 1992), 70% nucleotide sequence identity with *pin* (Plasterk *et al.*, 1983a), and the G-Segment at the 5′ end is identical to the P1 invertible C-Segment (Chow and Bukhari, 1976). The alternate orientation of an invertible G-Segment allows alternate tail fibers to be expressed, hence altering the phage host cell specificity. The Gin invertase system allows the alternate expression of tail fiber genes *S* and *U* by switching genetic information on and off (Howe, 1980; van de Putte *et al.*, 1980; Symonds, 1982; Plasterk *et al.*, 1984) but this switching is of relatively low efficiency *in vitro*, about 10^{-6} per lytic cycle in Mu (Plasterk *et al.*, 1983b). When Mu particles are grown by induction of a lysogen, about half the particles will contain DNA with one orientation referred to as G(+) and the rest of the particles will be in the reverse orientation G(−). However, when Mu phage is grown by infection, almost all particles will be G(+), leading to the conclusion that the G(−) orientation does not have ability to adsorb properly to the bacterial cells (Bukhari and Ambrosio, 1978; Kamp *et al.*, 1978). It has been shown that the host range control of adsorption properties is caused by a difference between G(+) and G(−) tail fibers in their binding to lipopolysaccharide (LPS) receptors on host bacteria (van de Putte *et al.*, 1980; Kamp *et al.*, 1984; Kamp and Sandulache, 1983). Therefore, it is clear that the different host ranges of the two phage types is caused by recognition of different receptors by the tail fiber structures that allow interaction with the surface receptors of different bacteria. G(+) and G(−) phage particles themselves do not seem

to differ grossly in structure by EM analysis alone. However, antisera analysis allowed visible differences between the two serotypes to be observed (Kamp *et al.*, 1984), providing further evidence for control at the tail fiber level. The alternate host ranges of the G(+) and G(−) phage particles have been indicated as follows: G(+) is infectious for *E. coli* K-12 (Bukhari and Ambrosio, 1978; Kamp *et al.*, 1978), whereas G(−) phage particles can infect other enteric bacteria such as *Citrobacter freundii*, *Enterobacter cloacae*, and *Serratia marcescens* (van de Putte *et al.*, 1980).

1. *Structure of the Gin Invertase System*

The alternate tail fibers determining phage host range specificity are encoded by two genes (*S* and *U* or *S′* and *U′*) that are located within the 3-kb invertible DNA sequence itself (Howe *et al.*, 1979; Plasterk *et al.*, 1983b). The Gin system structurally consists of a 3-kb invertible DNA region known as the G-Segment. Located on the right of the other known genes in the Mu genetic map (Daniell *et al.*, 1973), this region is flanked by two 34-bp inverted repeats (*gix* sites) that are the sites of recombination (Plasterk *et al.*, 1983a). The inversion is catalyzed by the expression of the DNA invertase Gin in the β region of the Mu genome and the binding of this dimerized Gin to the two *gix* sites.

There are four genes encoded by the G-Segment proposed to be involved in tail fiber specificity, *S*, *U*, *S′*, and *U′*, of which *S* and *U* are expressed in the G(+) orientation and *S′* and *U′* in the inverted orientation, G(−). Giphart-Gassler *et al.* (1982) presented a model of tail fiber gene organization and reported that the *S* and *S′* genes are partially located outside the G-Segment and share a common NH_2-terminal region located in the α region, known as *Sc* (constant). The two different COOH-terminal parts of the gene, *Sv* and *Sv′* (variable), are spliced to the constant region by inversion of the G-segment.

The *gix* recombination sites flank the G-Segment and are organized as inverted repeats of 34 bp (Plasterk *et al.*, 1983a; Mertens *et al.*, 1988). Each *gix* site consists of two 12-bp inversely orientated binding sites for the recombinase Gin (half-sites) separated by an asymmetric 2-bp core region called the crossover region (Klippel *et al.*, 1988). The sequence polarity of this region confers directionality on the *gix* sites. A study by Klippel *et al.* (1988) determined that the central dinucleotide within the inverted repeat is the actual position of single-stranded Gin cleavage.

The recombinase Gin was originally identified as a prerequisite for G-Segment inversion by Allet and Bukhari (1975). It is a very hydrophobic 21.7-kDa Mu-encoded invertase and is stimulated by

interaction with *E. coli* host protein FIS. In Mu, expression of Gin function appears to be constitutive (Koch and Kahmann, 1986) with the native *gin* promoter being located within the right side *gix* site (IR-R) of the G-Segment (Plasterk *et al.*, 1983a). *Gin* is expressed at very low levels of efficiency of approximately 10^{-6} switching per generation (Plasterk *et al.*, 1983b) and this is attributed to the low efficiency of both transcription and translation initiation of the gene (Plasterk *et al.*, 1983b). Plasterk *et al.* (1983a) determined the nucleotide sequence of *gin* and identified a number of features. First, the initiation triplet codon is unusual in that it is GTG rather than ATG and it has been suggested that this contributes to the low translation efficiency of the gene as discussed above. The consensus Shine–Dalgarno sequence for aiding 16 S ribosome binding during the initiation of transcription was also indicated to be 8–13 bp downstream of the initiation codon. Second, the termination codons found at the end of *gin* are immediately followed by a methylation site (GATC). The methylation site is within the promoter of the next Mu gene, *mom*, and this overlap could indicate some coordinate expression between the two. Additionally, in the same study, Plasterk *et al.* (1983a) investigated the level of identity between Pin and Gin recombinases and found that there was 70% homology between the two and that they could productively complement each other. It was previously found that Gin can complement Hin and Cin (Chow and Bukhari, 1976; Iida *et al.*, 1982; Kutsukake and Iino, 1980).

The Gin protein product exists as a dimer in solution and this dimerization is probably stabilized by hydrophobic cross-linking interactions between the subunits by disulfide bond formation between the cysteine residues present in the protein (Spaeny-Dekking *et al.*, 1995). Gin has been studied extensively primarily by producing overexpressing Gin mutant strains to overcome the low transcription rate of the wild-type (Kahmann *et al.*, 1984; Mertens *et al.*, 1984). Gin catalyzes efficient site-specific recombination between the inverted repeat sequences *in vivo* and *in vitro* in the presence of host factor FIS and the recombinational enhancer by introducing specific single-stranded breaks into the duplex DNA fragments containing the IR.

The G-Segment is flanked by α and β regions to its left and right, respectively, and their roles in inversion of the G-Segment have been studied (Kahmann *et al.*, 1984). It has been found that the α sequence can be reduced in size from 2000 to 50 bp without measurable effects on inversion. However, when the 200-bp β segment is reduced in size, inversion is completely abolished. This indicates α and β sequences are unequal partners in the inversion reaction. Additionally, it was

found in the same study that β can substitute for α but α cannot replace β. This points to the existence of a site in β that is essential for inversion. This was later found to be the site of *gin*, as discussed above.

2. *Inversion of the G-Segment*

It has been shown that the following conditions are necessary for G-Segment inversion: two inversely orientated recombination sites flanking the G-Segment, the Gin recombinase and its enhancer site (*sis*), a negatively supercoiled substrate, and a host-encoded protein, FIS (Crisona *et al.*, 1994).

3. *Activity of Gin*

As discussed previously, the Gin recombinase exists as a dimer in solution held together by hydrophobic interactions between the subunits (Spaeny-Dekking *et al.*, 1995) and when it binds to the 34-bp IR sites, it catalyzes the inversion of the G-Segment by causing bending of the DNA substrate with the cooperation of FIS (Mertens *et al.*, 1988). After binding, a complex is formed between the two Gin dimers bound to the IR and a third DNA sequence, *sis*, to which the host factor is bound. In this active synaptic complex, each monomer of Gin introduces a single-strand nick. Klippel *et al.* (1988) established that the central dinucleotide within the inverted repeat sequences was the exact position of Gin cleavage. After the introduction of single-strand nicks, the Gin recombinase remains covalently linked to the 5′-phosphate of the DNA at the position of cleavage via a phosphoserine (Klippel *et al.*, 1988). Gin contains serine at nine different positions (Plasterk *et al.*, 1983a), but Klippel *et al.* (1988) established that the serine at position nine situated within the highly conserved N-terminal portion was at the center of catalytic activity in Gin. The introduction of single-strand nicks by this catalytic Gin results in a double-strand break within the IR at staggered positions allowing subsequent DNA inversion and relegation of the DNA to occur.

4. *The* sis *Enhancer*

The Gin invertase alone is not sufficient to catalyze inversion of the G-Segment. An important cofactor is the *sis* (sequence for inversion stimulation) enhancer and its binding with FIS host protein (reviewed in Johnson and Simon, 1987). The presence of the *cis*-acting enhancer and its associated protein FIS strongly stimulates inversion (Koch and Kahmann, 1986); it can increase inversion rates over 100-fold (Johnson,

1991) and it has been shown that as long as the enhancer is present, it functions independently of both orientation and distance from the *gix* recombination sites (Kahmann *et al.*, 1985). The enhancer is located within the *gin* recombinase gene itself at its 5′ end (Plasterk *et al.*, 1983a), it is 60 bp in length, and contains two 15-bp FIS binding sites (Plasterk *et al.*, 1984) at a fixed distance from one another of exactly 48 bp (Kanaar *et al.*, 1989a). This means there is a spacing of approximately 4.5 turns of the DNA helix between the FIS binding sites, which therefore places the bound FIS molecules at each binding site on nearly opposite sides of the DNA helix. This reinforces the importance of maintaining the exact spacing between the two FIS molecules bound to the DNA (Johnson *et al.*, 1987; Hubner *et al.*, 1989) The enhancer sequence itself has been shown to be symmetric and has a 2-fold axis of rotation (Kanaar *et al.*, 1989b). It is this symmetry that allows the enhancer to function in an orientation-independent manner and independently of the mode of interaction with the recombination sites (Kanaar *et al.*, 1989a). Kanaar *et al.* (1989a) additionally identified the exact center of symmetry within the FIS–DNA complex. It has been suggested that the enhancer is required at early stages of recombination, perhaps to allow formation of the synaptic complex itself (Kanaar *et al.*, 1990).

5. *FIS (Factor for Inversion Stimulation)*

FIS was first identified as a host *E. coli* factor required for *in vitro* activity of phage Mu Gin invertase and *S. typhimurium* Hin invertase systems (Koch and Kahmann, 1986). This host factor was found to act at the *sis* enhancer site and provide stimulatory effects on the Gin recombinase (Kahmann *et al.*, 1985). In fact, stimulation was increased by 20-fold by the presence of FIS *in vitro* (Kahmann *et al.*, 1985; Koch and Kahmann, 1986). Kahmann *et al.* (1985) showed using *E. coli* K-12 cell extracts that a host factor was indeed required for efficient inversion and also that this FIS stimulation increased inversion only in plasmid substrates that contained a *sis* site, suggesting a direct interaction between the two. FIS is a basic, heat-stable, 11.2-kDa protein of 98 amino acids (Johnson *et al.*, 1988; Koch *et al.*, 1988) and is abundant in exponentially growing cells (Thompson *et al.*, 1987). The number of FIS molecules per cell has been shown to rapidly and dramatically change when growth conditions are varied. A greater than 500-fold increase is seen in FIS being induced when the bacterial cells switch from stationary to exponential growth phase (Ball *et al.*, 1992).

FIS exists as a homodimer in solution (Koch and Kahmann, 1986), each of which is 98 amino acids (Johnson *et al.*, 1988; Safo *et al.*, 1997) and each subunit is known to consist of four connected α-helices (Kostrewa *et al.*, 1991). The FIS protein can be divided into two domains, the *C*-terminal domain containing a defined helix-turn-helix motif (Kostrewa *et al.*, 1991) that allows DNA binding and an N-terminal domain that allows stimulation of G-Segment inversion (Koch *et al.*, 1991). It is known that the N-terminal amino acids extend from the FIS subunit and it was found that this extended activation region consists of two β-hairpin arms acting as a regulatory motif (Safo *et al.*, 1997). The most critical activation residues were shown to be located near the tips of the β-hairpin arms by saturation mutagenesis experiments and it was shown that contacts between these β-hairpin tips with the Gin recombinase activate the Gin protein in assisted formation of the invertasome complex when the two arms are linked together (Safo *et al.*, 1997). FIS interacts with adjacent major grooves on the enhancer DNA and interacts noncooperatively with two sites on the enhancer (Plasterk *et al.*, 1984). Also, this binding of FIS serves to induce the DNA bending that is important for invertasome assembly and subsequent single-stand recombination of DNA within it. FIS bound to DNA migrates as a highly bent complex in gel electrophoresis (Johnson *et al.*, 1987; Hubner *et al.*, 1989), which supports this role of the FIS protein binding to the DNA and inducing a large conformational change.

6. *DNA Supercoiling*

Negative supercoiling plays an important role in nearly all organisms, particularly in bacteria, and a number of reactions depend on supercoiling. Negative supercoiling promotes processes that require unwinding of the double helix (for example, transcription and replication), toroidal winding of DNA around proteins, and an intramolecular juxtaposition of two DNA segments (for example, recombination). Negative supercoiling has been shown to be obligatory in the DNA invertase family of site-specific recombinases as they require a supercoiled substrate containing two recombination sites. Benjamin *et al.* (1996) proved that a relaxed synaptic complex does not in fact recombine and that the mechanisms of recombinases may be constructed to depend on supercoiling as a regulatory signal and selectivity determinant, rather than an obligatory contributor to the chemistry or thermodynamics.

F. The *FIM* System

Escherichia coli uses many types of adhesin molecules to attach to eukaryotic cells. Type I fimbriae have been suggested to play an important role in communicability and in urinary tract infections (Bloch and Orndorff, 1990; Gally *et al.*, 1993). However, these molecules are also excellent immunogens, and the ability to switch off expression may be critical to the survival of bacteria within a host. Control of expression of type I fimbriae is attributable to the inversion of a 314-bp invertible region, the *fim* switch (*fimS*) (Abraham *et al.*, 1985). *fimS* contains a promoter for *fimA*, the gene encoding the major structural subunit of type I fimbriae (Olsen and Klemm, 1994). Inversion of *fimS* in one orientation places the promoter adjacent to *fimA* (the ON orientation), allowing transcription (see Fig. 1). In the other (OFF orientation) type I fimbriae are not produced. Two tyrosine recombinases of the lambda integrase family are responsible for inversion of *fimS*, FimB mediates both ON-to-OFF and OFF-to-ON switching at approximately the same frequency, whereas FimE mediates predominantly ON-to-OFF switching (Blomfield *et al.*, 1991b; McClain *et al.*, 1993; Gally *et al.*, 1996; Kulasekara and Blomfield, 1999). Type 1 pili also contain FimH, a minor mannose-binding lectin that promotes binding of bacteria to mucosal surfaces and allows $FimH^+$ bacteria to survive in macrophages following phagocytosis.

Chromosomally encoded FimB promotes recombination with little orientational bias at a rate of 10^{-3} to 10^{-4} per cell per generation, whereas FimE has been shown to have a much faster switching frequency of at least 1×10^{-2} per cell per generation (Blomfield *et al.*, 1991a; Kulasekara and Blomfield, 1999). In an *E. coli* K-12 background (*fimB*$^+$, *fimE*$^+$), ON-to-OFF switching occurs at a very high frequency, up to 0.75 per cell per generation (Dove and Dorman, 1996), due to

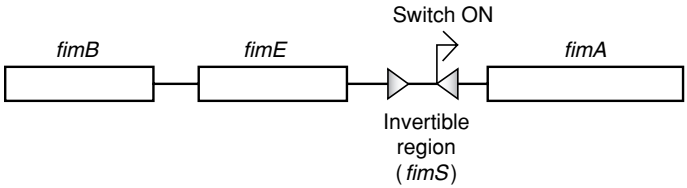


FIG. 1. The *fim* switch and upstream regulatory genes. *fimB* and *fimE* are shown in their native orientations adjacent to *fimS*. The arrow illustrates the orientation of the promoter (located with the invertible region) of *fimA*, the gene that encodes the major structural protein of type I fimbriae.

the overriding effect of FimE-mediated switching. Thus the afimbriate state is predominant under all conditions (Spears *et al.*, 1986; McClain *et al.*, 1993).

1. *The Function of the* fim *System*

As with many other bacterial virulence factors, type I fimbriae are not constitutively expressed. Instead, intimate association with the host is required before the pathogenicity genes are expressed. The environmental triggers for such expression include physical parameters such as pH, temperature, or osmolarity as well as chemical signals such as the presence or absence of an amino acid. Such control of expression prevents wasteful production of virulence genes that may significantly influence the survival of the pathogen outside the host cell.

Survival inside of the host depends on the interaction between proteins expressed on the bacterial cell surface and the host immune system. Type I fimbriae are expressed externally and are therefore immunogenic. Expression of genes in every cell of the bacterial population in response to an environmental cue (stereotypic control), which works very well to prevent wasteful expression of virulence genes outside of the host, would be disastrous for control of fimbrial expression. The immune response of the host to the antigenic fimbriae could result in the elimination of the entire bacterial population. To avoid detection the antigen may be varied within all cells of the bacteria population (antigenic variation), or different cells of the same population may vary the expression of the virulence factor at random (phase variation). Phase variation is employed in the expression of type I fimbriae allowing a small number of bacteria that are not expressing the antigenic fimbriae to survive upon the elicitation of an immune response by the host. Variants expressing different fimbriae can then be generated at a later time.

2. *Protein Cofactors That Regulate Expression of* fim

In addition to FimB and FimE, at least three global regulators influence inversion of the *fimS*. Mutations in *ihfA* or *ihfB* encoding the site-specific DNA binding protein integration host factor (IHF) result in a marked decrease in both FimB- and FimE-promoted switching (Blomfield *et al.*, 1997). The leucine-responsive regulatory protein (Lrp) is also a requirement for normal FimB- and FimE-mediated switching. The *fim* system is unique since it is the only known example in which Lrp acts as a positive regulator with leucine, as well as other aliphatic amino acids (alanine, isoleucine, and valine) amplifying this effect (Blomfield *et al.*, 1993; Gally *et al.*, 1993). Temperature has also

TABLE I

SUMMARY OF *FIM* SWITCH REGULATION

Regulatory factor	Description	Effect on *fim* transcription
FimB	Recombinase	Promotes ON-to-OFF and OFF-to-ON inversion
FimE	Recombinase	Promotes ON-to-OFF inversion
IHF	Bends DNA	Helps promote inversion, enhances *fimA* promoter
Lrp	Bends DNA	Helps promote inversion
H-NS	Binds curved DNA	Inhibits inversion, inhibits *fimA*, *fimB*, *fimE* promoters
DNA topoisomerase I	Relaxes negatively supercoiled DNA	Facilitates ON-to-OFF and OFF-to-ON inversion
DNA gyrase	Relaxes positively supercoiled DNA, negatively supercoils DNA	Retards OFF-to-ON inversion

been demonstrated to affect *fimS* inversion. The nucleoid-associated protein H-NS has been implicated in the thermal regulation of a number of operons (Donato *et al.*, 1997).

Despite the in-depth characterization of environmental factors controlling *fim* expression, it is still unclear exactly how these diverse regulatory inputs come together at the molecular level to optimize *fim* gene expression. H-NS is a *trans*-acting repressor of the *fim* switch that binds DNA nonspecifically to exert its effect. Unlike many other proteins that interact with DNA, H-NS does not specifically bind unique DNA sequences, but instead forms associations with curved DNA formations. In fact, all promoters regulated by H-NS have such a conformation including *hns*, its own promoter (allowing autoregulation). H-NS transcription is activated during DNA synthesis and is repressed in the stationary phase. This probably represents the need to maintain an approximately equal DNA:H-NS ratio. Factors important for regulation of Fim recombinases are summarized in Table I.

3. *DNA Supercoiling and* fim *Inversion*

In light of the fact that DNA bending proteins H-NS, IHF, and Lrp are involved in the regulation of the *fim* switch, a role for other mechanisms controlling DNA topology have also been explored. Local DNA supercoiling occurs during the moving of complexes such as DNA and RNA polymerases due to the unwinding of the DNA duplex.

Normally within the cell, counteracting enzymes DNA gyrase (introduces negative supercoils) and topoisomerase I (removes such negative coils) avert supercoiling. However, the physiological status of the cell is significant in determining supercoiling. Variations in supercoiling have been shown to occur in response to temperature, oxygen availability, osmotic stresses, and other parameters (Dorman, 1995). These are likely to cause inactivation of topoisomerase and/or DNA gyrase resulting in local supercoiling. It has been demonstrated that such supercoiling can have a profound effect on promoter function as well as site-specific recombination.

The introduction of mutations into the *topA* gene, encoding topoisomerase I, prevents the inversion of the *fimA* promoter (Higgins *et al.*, 1988). Cells containing this mutation have higher levels of negatively supercoiled DNA than wild-type cells. However, it is local supercoiling that is significant in the *fim* switch. The global level of supercoiling does not correlate with the inverting of the DNA segment, instead, levels of topoisomerase I influence inversion suggesting topoisomerase I is required to relax negatively supercoiled DNA in or near the site of DNA inversion in the *fim* switch. The inhibition of DNA gyrase with novobiocin has been found to have a specific effect on the *fim* switch. Phase-ON cells are unaffected by the antibiotic. However phase-OFF cells are strongly affected and invert the *fimA* promoter in the ON orientation.

4. *Use of the* fim *System as a Transcriptional Biosensor*

An important consideration of using the *fim* system as a biosensor is internal regulation of this system and whether these parameters can be controlled. As mentioned in the previous section, *fim* is regulated by differing environmental conditions, including temperature and media. It is essential that the signal given from the biosensor is not significantly changed due to superimposed regulation of the reporter system under differing physiological and environmental changes. Unlike other reporter genes (such as *lacZ*) where a substrate is added in excess and the kinetics of the enzyme-catalyzed reaction are directly proportional to the enzyme concentration, the substrate in this case (i.e., *fimS*) will clearly not be in excess. The invertible element substrate is at single copy on the chromosome and accordingly, during the recombination reaction, the recombinase is likely to be in excess and the invertible element, the rate-limiting factor. It may be envisaged therefore that this system cannot quantify transcription levels due to the fact that increases in transcription (from a promoter fused to a suitable recombinase-reporter gene) will not be detectable because there will be

insufficient substrate for this excess enzyme to convert. When analyzing the whole-cell biosensor at a single-cell level transcriptional quantification would be difficult. However, this potential problem may be resolved by analyzing a population of bacteria. Here, increasing recombinase levels should elevate the rate of inversion in the bacterial population; indeed inversion rates have been shown experimentally to be faster for the multicopy versus the monocopy recombinase. The use of recombinase genes as reporters of transcription in general may be problematic due to the reporting range of invertible elements. At a population level, the reporting range of a recombinase is determined by its rate of inversion. A rapidly switching recombinase gives a small range and a slow switcher a much larger range. However, there will always be a point at which further increases in enzyme levels will no longer be detectable because the recombinase has inverted all of the substrate invertible elements in the population. Therefore the rate at which this critical threshold is reached is the most important consideration in determining the potential of using recombinases as transcriptional reporters. Environmental and physiological factors have been shown to regulate the rate of *fimS* inversion, as has the quantity of recombinase (Gally *et al.*, 1993; Blomfield *et al.*, 1993, 1997). To test the properties of a recombinase suitable for biosensor applications, experimental data published on *fim* recombinases were compared. *fim* is an extremely well-characterized system and data include many experimental conditions, which should allow an accurate assessment of whether these recombinases will be useful for biosensor development.

Inversion rates of *fim* recombinases are now determined by a standard method (Eisenstein, 1981). The method utilizes an *E. coli* strain (such as AAEC370A) that has a *lacZ* fusion to *fimA* (Fig. 2). Because the *fimA* promoter lies within the invertible region, a single cell can either have *fimS* with the *fimA* promoter (P_A) reading into *fimA*, termed the ON orientation, or with *fimA* promoterless, termed the OFF orientation. The *lacZ* fusion to the *fimA* gene open reading frame thus

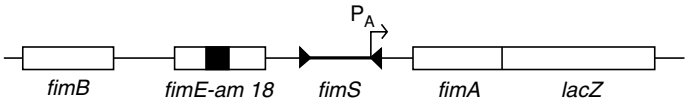


FIG. 2. Schematic illustration of the relevant parts of the *fim* operon of *E. coli* strain AAEC370A. AAEC370A contains a functional copy of *fimB* but the *fimE* gene contains an amber mutation. The *fimA* gene is fused with the *lacZ* reporter gene allowing the switch orientation to be determined when cells are grown on the appropriate indicator plates (see text). P_A is the *fimA* promoter. *fimS* starts in the ON orientation.

allows the orientation of the *fim* invertible element to be determined. Experiments to determine the switching frequency of *fim* recombinases involve growing a culture of a *lacZ*-fusion strain (e.g., AAEC370A) to the exponential phase. The culture is diluted appropriately and plated out onto media containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and colonies are allowed to develop. Colonies expressing *lacZ* will convert the X-gal substrate to a blue product indicating *fimS* is in the ON orientation, whereas white colonies indicate *fimS* is in the OFF orientation. Because a single colony is derived from a single bacterial cell, and assuming that *fimS* does not undergo rapid switching during the incubation of the indicator plates, the proportion of ON and OFF colonies can be assumed to correspond to the proportion of ON and OFF bacteria within the bacterial population of the original culture. The switching frequency is expressed per cell per generation and is calculated from the number of generations a culture has undergone and the proportion of ON to OFF ''bacteria'' by using Eq. (1) (Gally *et al.*, 1993):

$$\text{Probability of switching per cell per generation} = 1 - \sqrt[n]{1 - x} \quad (1)$$

where $n =$ number of generations,

$$x = \frac{(\text{number of ON colonies})}{(\text{total number of colonies})}$$

One problem of applying this method is that under the standard conditions used (37°C in defined rich medium) it cannot measure recombinases with very high switching frequencies. In this instance, switching would occur during incubation of the indicator plates affecting the orientation of *fimS* and therefore reporter gene *lacZ* transcription. To overcome this assessment of rapid-switching recombinase *fimE*, it is necessary to incubate the indicator plates at 42°C and plate cells onto minimal medium to reduce this frequency below 1×10^{-2} (Gally *et al.*, 1993).

To assess the usefulness of *fim* recombinases for applications as a whole-cell biosensor, a comparison of recombinase switching frequencies by mathematical modeling was made (Fig. 3). This allowed comparison of how rapidly the recombinases could switch *fimS* to the 100% inversion equilibrium over time. Figure 3 shows that when expressed from the chromosome FimE has a very rapid switching frequency and is capable of inverting *fimS* completely to the OFF orientation within only a few bacterial generations. Chromosomally

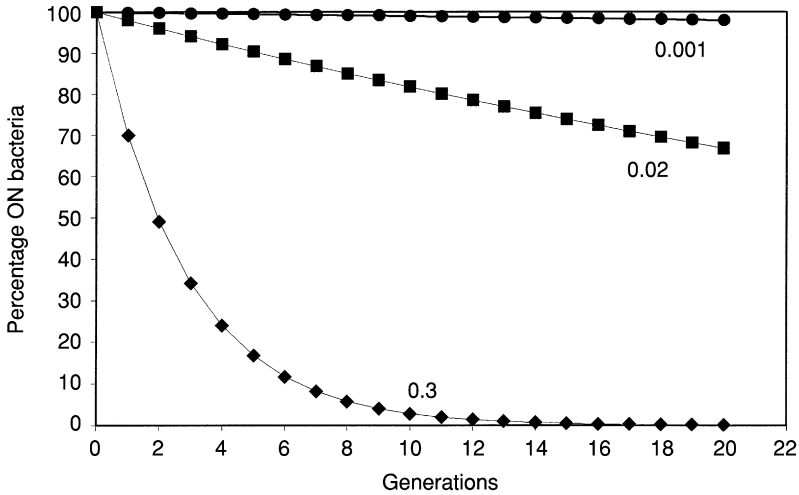


FIG. 3. Mathematically modeled switching rates of *fim* recombinases. (●) *fimB*, chromosomally expressed (*fimB*$^+$, *fimE*$^-$), (■) *fimB* expressed from a multicopy plasmid (pSLD203), (◆) *fimE*, chromosomally expressed (*fimB*$^+$, *fimE*$^+$). Switching frequencies data shown on the graph for *fimS* are for the ON to OFF orientation and were determined by using the indicator plate method. Data were calculated from published switching frequencies (Gally *et al.*, 1993; Dove and Dorman, 1996).

expressed FimB, in contrast, has a very slow switching frequency. Additionally, it is clear that the amount of recombinase affects the switching frequency. When *fimB* is expressed from a multicopy plasmid the recombinase has more than a 20-fold increased switching rate compared to when the gene is expressed from the chromosome.

Chromosomally expressed *fimB* is unlikely to be useful as a transcriptional reporter recombinase since a detectable polymerase chain reaction (PCR) signal would require about 50 bacterial generations. On the other hand, the extremely rapid switching frequency of FimE would be ideal for application in a recombinase-based biosensor whose promoter is extremely tightly regulated. However, basal expression may result in the *fim* switch being converted to its threshold level without promoter induction, which is completely to the OFF orientation for *fimE*. The data for *fimE*-mediated switching were obtained from chromosomal expression for this gene from its native promoter. Switching rates for plasmid-expressed *fimE* would be expected to be even higher, as is seen for the *fimB* multicopy data. Because molecular biological manipulations allow cloning into plasmids with far greater

ease than chromosomal insertion, initially the biosensor design focused on the use of multicopy *fimB*. The value for the switching rate of plasmid-encoded *fimB* has been estimated to be more than 0.02 per cell per generation (Dove and Dorman, 1996). This value is the maximum switching frequency determinable by using the indicator plate method described above when incubation of indicator plates was carried out under standard growth conditions (37°C). Modification of the incubation conditions, as was carried out for FimE switching frequencies, was not done for multicopy *fimB*. As a result, questions about the true switching rate of multicopy *fimB* still remain, as does the question of whether recombinase production is proportional to the switching rate in a dose-dependent fashion.

IV. Strategy for Recombinase-Based Biosensors

A. Advantages of Recombinase-Based Reporter Genes

Recombinase-based whole-cell biosensors potentially have a number of advantages that overcome some of the limitations of other reporter genes. For instance, detection of gene expression will not require culture and recovery of the organism from the test environment. Efficient methods for extraction of total DNA from environmental samples (including complex ones such as peat and soil) are now tried and tested. They produce DNA of sufficient purity and quality for amplification of target gene sequences using the PCR. It is therefore feasible to detect DNA fragments that contain the whole invertible region by PCR. Such an approach has many potential advantages:

- Substrates need not be added to detect reporter gene expression.
- Once recombination has occurred it will provide a permanent record of gene expression allowing large sample numbers to be tested without the requirement for immediate processing.
- The reaction should take place irrespective of the physiological status of cells. In environments such as soils, a metabolically shut-down state is the most common status for most nondifferentiationg cells.
- Many studies have shown that cells introduced into nutrient-limited environments typically become nonculturable after residence for prolonged periods (Morgan *et al.*, 1989). As a result, large cell numbers are required for detection of expression for most reporter genes and often cells must be recovered from the test environment (e.g., *lacZ*) and hence cannot be used for this type of analysis. These problems should be overcome with a DNA-based

reporter gene system. Recombinases also have a number of inherent disadvantages. Chief among these is the problem of controlling switching rates in such a way as to reflect pollutant concentration. Once induced the recombinase has the potential to continue its activity either until all the invertible region has switched from one orientation to another, e.g., all ON to OFF, or if switching is reversible as in Gin, until a 50:50 ratio of ON:OFF has been attained.

To develop a PCR-based recombinase biosensor that can respond to differing levels of promoter-driven transcription the following are required:

- The recombination event must be clearly detectable by PCR giving a stable, irreversible signal.
- The invertible switch region must contain a restriction site that cuts asymmetrically within this invertible element to allow the determination of its orientation.
- The PCR signal given should also preferably increase in a dose-dependent fashion with increased promoter induction.
- The candidate recombinase must not have an intrinsically rapid switching frequency, such that the recombinase switches the invertible element to its maximum extent/signal without significant increases in transcriptional activity from the fused promoter.
- Ideally there should be no significant basal or ''leaky'' expression from the promoter or transcriptional readthrough from a vector backbone.
- Levels of recombinase may be sufficient to recombine the invertible element to its maximum signal. Clearly this is undesirable since further increases in transcriptional activity will not be detectable.
- Ideally, the organism from which the invertible system is derived must not be naturally resident in the test environment. However, this may be compensated for by genetically engineering hybrid invertible switch regions that include DNA sequences not found in nature. These sequences may then be used as the PCR inversion assay primer template.

B. Construction and Testing of Model Systems

The components required for a whole-cell biosensor would be a pollutant-sensitive promoter (to provide specificity), the specific regulator (to induce expression from this pollutant-specific promoter), and expression of either an integrase or invertase DNA recombinase linked

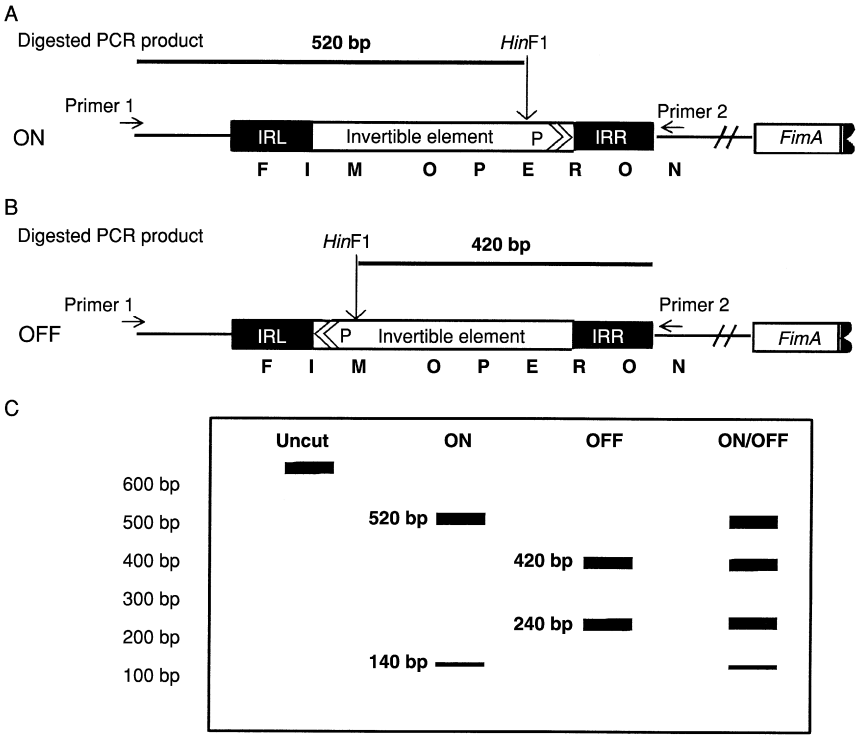


FIG. 4. PCR inversion assay of the *fim* switch. (A) *fim* switch in the ON orientation; (B) *fim* switch in the OFF orientation; (C) illustration of agarose gel electrophoresis carried out on *Hin*F1 digested *fim* switch PCR products. IRL, left invertible repeat; IRR, right.

to an invertible fragment of DNA. Detection of recombination should be possible by PCR amplification of the invertible region followed by restriction digestion of an asymmetrically located restriction site. A model system based on the *fim* system is described in Fig. 4 in which the invertible region has such an asymmetric restriction site, *Hin*F1. After inversion from ON to OFF the expected fragment patterns obtained in each an orientation after PCR amplification are shown in Fig. 4C.

To test the feasibility of this model we constructed a range of constructs based on the arabinose-inducible promoter P_{bad} (Guzman *et al.*, 1995). Figure 5 shows a general overview of how the constructs were made using either a range of recombinases or *gfp* as reporters. In series 1, arabinose was used as a model pollutant (Fig. 5A) and tested for its

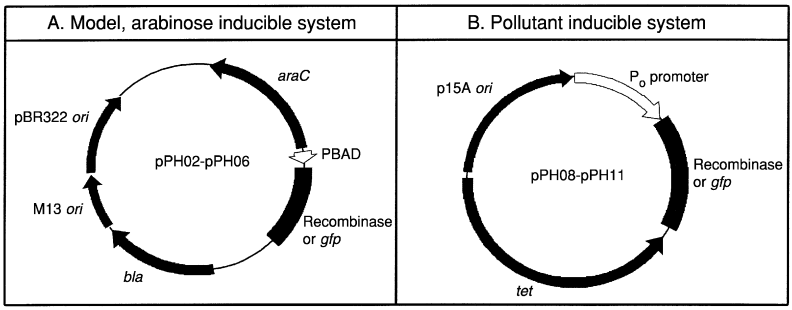
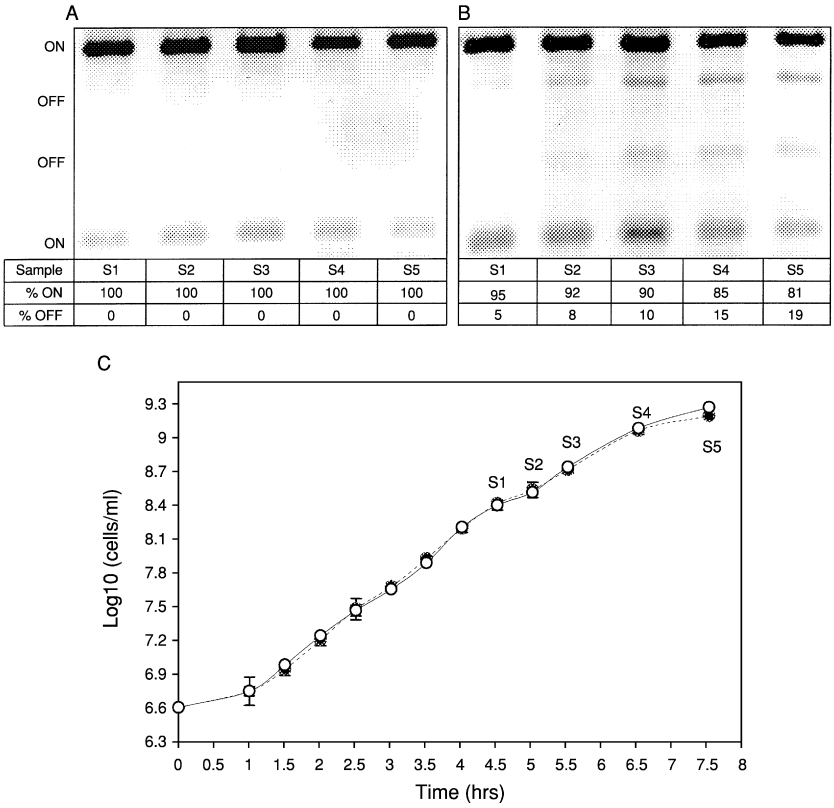


FIG. 5. Overview of genetic constructs. (A) Vector series that includes recombinases (*fimB*, *fimE* two *fimE* mutants) and *gfp* cloned into pBAD18. (B) Vector series that includes recombinases (*fimE*, two *fimE* mutants) and *gfp* cloned adjacent to a pollutant-sensitive promoter (P_o) in pAW9.

ability to induce expression of the recombinase detected by the PCR assay described in Fig. 4. A second series of plasmid vectors used the promoter P_o that is activated by the presence of dimethylphenol. Degradation of (methyl)phenols in *Pseudomonas* sp. strain CF600 occurs when the *dmp* operon is expressed. This operon is located on a catabolic megaplasmid (pVII50) that encodes a number of enzymes required for aromatic catabolism (Shingler *et al.*, 1992). Pseudomonads harboring pVII50 can grow on phenol, monomethylated phenols, and 3,4-dimethylphenol as a sole source of carbon and energy (Shingler *et al.*, 1989) since such substrates are transformed to Krebs cycle intermediates pyruvate and acetyl coenzyme A via the *meta*-cleavage pathway (Powloski and Shingler, 1994). The promoter of the *dmp* operon, P_o, is tightly controlled by regulatory protein DmpR, which for the vectors in Fig. 5B was located on the chromosome.

Figure 6 shows an experiment designed to test arabinose as the model pollutant to induce a *fimE* recombinase as a reporter system expressed by the arabinose-inducible promoter P_{bad}. This experiment involved growing recombinant *E. coli* with or without arabinose, sampling at known intervals during growth (Fig. 6C), and testing for DNA inversion from 100% ON at time 0 to OFF as the recombinase became inducible (Fig. 6A and B). Two features emerge; the recombinase was inducible only in the presence of arabinose and was most inducible toward the end of growth. This experiment showed the feasibility of using a recombinase as a reporter of the presence of a defined molecule. However, it also highlighted unexpected problems of relatively low levels of expression (only 19% of cells have inverted to the OFF



Sample	S1	S2	S3	S4	S5
% ON	100	100	100	100	100
% OFF	0	0	0	0	0

S1	S2	S3	S4	S5
95	92	90	85	81
5	8	10	15	19

FIG. 6. FimE (P74) switching during different phases of growth in rich medium. *E. coli* AAEC374A pPHO6 cells starting with *fimS* ON were inoculated at a concentration of 4×10^6 cells/ml into LB medium (A) without arabinose and (B) with arabinose. (C) Growth curve of cultures. Dashed line, filled circles, without arabinose; black line, open circles, with arabinose. Data labels (S1–S5) correspond to the points at which subsamples were taken for the PCR inversion assay. Overproduction of FimB was induced by adding 0.02% arabinose at $t = 0$.

by the end of growth) and there appeared to be a cell density effect that governed expression. In further work, we have shown that the recombinase inversion system partially detects phenol in a DmpR/P_o construct, but that the P_o promoter is leaky so that expression of the recombinase occurs in the absence of the inducer, dimethylphenol (Fig. 7). This also occurs if *gfp* replaces the *fimE* recombinase, suggesting that it is a feature of the promoter rather than the recombinase.

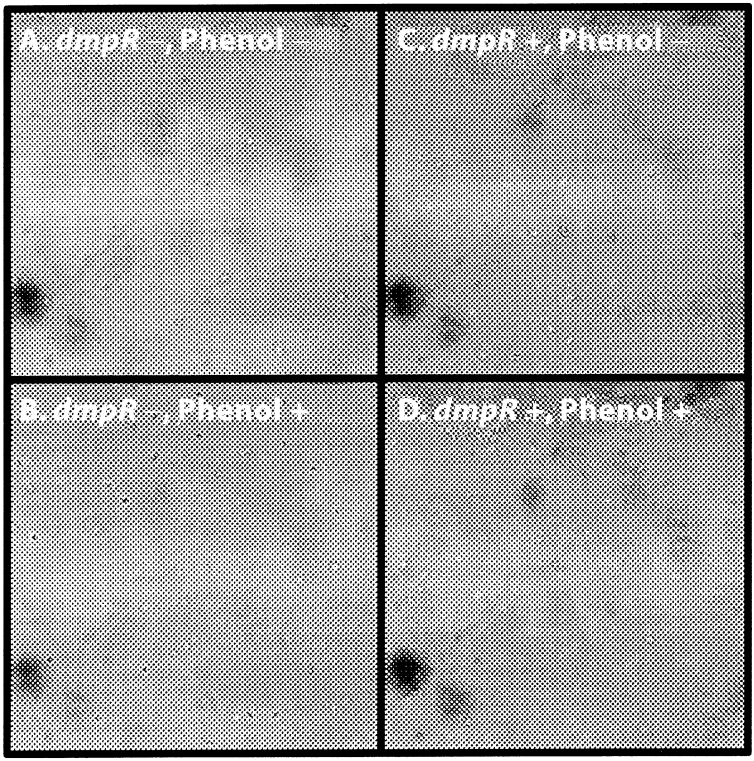


FIG. 7. *dmpR*-mediated transcription from the P_o monitored with the *gfp* gene. *E. coli* AAEC374A (*dmpR* negative—A and B) or PHECO2 (*dmpR* positive—C and D) transformed with pPH10 (pAW9-P_o-*gfp*) were grown in LB medium overnight. Phenol (5 m*M*) was added as indicated. Photographs are representations of triplicate data.

V. Summary

DNA recombinases show some promise as reporters of pollutants providing that appropriate promoters are used and that the apparent dependence of expression on cell density can be solved. Further work is in progress using different recombinases and other promoters to optimize recombinase expression as well as to test these genetic constructs in contaminated environmental samples such as soil and water. It may be that a graded response reflecting pollutant concentration may not be possible. However, they show great promise for providing definitive detection systems for the presence of a pollutant and may be applicable to address the problem of bioavailability of pollutants in complex environments such as soil.

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Microbial Phosphate Removal and Polyphosphate Production from Wastewaters

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I. Introduction

Phosphorus (P) is an essential macronutrient for all organisms and plays some part in almost all life processes. Yet despite this well-accepted central metabolic role, inorganic phosphate, in excess, represents a potentially serious environmental and ecological problem. In particular, the enrichment of water bodies with phosphate makes an important contribution to the process of eutrophication, which has developed into a serious water management problem throughout the world. Population growth and the intensification of farming have led to the collapse of the natural P cycle and its replacement with a system whereby phosphate is extracted from rock, passed through crops, animals, and humans, to end up either in landfill or in the aquatic environment; some 140 million tons of rock phosphate are cycled annually in this way (Yeoman *et al.*, 1988). The excessive growth of algae or higher plants that occurs as a consequence of eutrophication leads to many water quality problems, including oxygen depletion, increased water purification costs, a decline in the amenity and conservation value of waters, loss of livestock, and the possible sublethal effects of algal toxins on humans using eutrophic water supplies for drinking.

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To put eutrophication into perspective, P concentrations in natural, noneutrophic, freshwaters are usually below 25 μg/liter. Concentrations above 50 μg P/liter are generally the result of anthropogenic inputs. A survey of rivers across Europe found that of the 1000 monitoring stations analyzed, 90% had levels of total phosphate in excess of 50 μg P/liter (Centre Européen d'Études des Polyphosphates, 1999), whereas a survey by the former U.K. National Rivers Authority found that 23% of lakes in Britain are ''severely'' affected by eutrophication (ENDS Report, 1997). Increased licensing charges and more severe legislation now put strict limits on the levels of phosphate that can be discharged from such point sources as municipal and industrial wastewater treatment plants. Within Europe this has been reflected in the imposition of the Urban Wastewater Treatment Directive 91/271, which specifies compliance limits for P removal from wastewaters and in particular for those treatment works discharging into areas designated as ''eutrophication sensitive'' (Council of the European Community, 1991). To meet these increasingly stringent targets, which generally require P removal efficiencies from sewage of over 80%, two main processes are employed, either separately or in combination: (1) chemical precipitation and (2) ''Enhanced Biological Phosphate Removal.''

Chemical precipitation is the traditional, and still the most common, method of P removal from wastewater streams; it involves the use of ferric, ferrous, aluminum, or calcium salts (reviewed in Yeoman *et al.*, 1988; Brett *et al.*, 1997). However, the technique—although reliable and capable of meeting the phosphate discharge limits—is expensive in terms of the cost of precipitants, the possible need for an additional tertiary filtration step because of the potential for heavy metal contamination of receiving waters, and the necessity to dispose of the large excess volumes of sludge generated [sludge volumes may be increased by up to 20% through chemical P precipitation (Cooper *et al.*, 1994, 1995)]. The remainder of this review will concentrate on the physiological and biochemical basis of enhanced biological P removal by microorganisms.

II. The Enhanced Biological Phosphorus Removal Process

Our awareness of the phenomenon of Enhanced Biological Phosphorus Removal dates back to 1955 when Greenberg *et al.* (1955) proposed that activated sludge had the ability, under certain circumstances, to accumulate phosphate in excess of that required for balanced microbial growth. While studying the feasibility of growing rice plants on the surface of an activated sludge plant, Srinath *et al.*

(1959) found that the plants suffered from the characteristic symptoms of phosphate deficiency exemplified by excessive vegetative growth and diminished grain formation. This depletion of soluble P was traced to its excessive accumulation by the biomass. Levin and Shapiro (1965) demonstrated that such excess phosphate removal was biologically mediated; addition of 2,4-dinitrophenol to the biomass inhibited phosphate uptake. To wastewater treatment engineers the phenomenon of ''luxury'' phosphate uptake suggested a potential biotechnological approach to phosphate removal from waste streams; its exploitation ultimately led to the development of the Enhanced Biological Phosphorus Removal (EBPR) process.

In general EBPR is characterized by the exposure of activated sludge to periods with and without aeration—the ''aerobic'' and ''anaerobic'' phases. This is achieved by configuring the activated sludge system such that an anaerobic zone is added upstream of the traditional aerobic phase; influent wastewater is introduced into the anaerobic zone (reviewed in Yeoman *et al.*, 1988; Kortstee *et al.*, 1994; van Loosdrecht *et al.*, 1997; Brett *et al.*, 1997; Mino *et al.*, 1998). In the initial anaerobic phase of the treatment process it is believed that phosphate-accumulating microorganisms (PAOs) take up the short-chain fatty acid molecules such as acetate that accumulate as fermentation products in the absence of oxygen or nitrate, and convert these to a carbon storage polymer. These intracellular carbon reserves consist, for the most part, of poly-β-hydroxybutyrate (PHB), although poly-β-hydroxyvalerate (PHV) has also been observed. The energy required for the uptake and synthesis of PHB comes at the expense of a second biopolymer that is accumulated by PAO cells, namely polyphosphate (polyP). PolyP consists of a linear chain of phosphate residues linked together by high-energy phosphoanhydride bonds and ranges in length from 3 to greater than 1000 orthophosphate residues (Kulaev, 1979) (see Section III.A). The hydrolysis of intracellular polyP reserves under anaerobic conditions to provide an energy source for the uptake of short-chain fatty acids and accumulation of PHB by PAOs is accompanied by the release of phosphate into the extracellular medium.

In the subsequent, aerobic, stage of EBPR those microorganisms containing stored PHB/PHV replenish their internal polyP reserves, and in doing so take up not only the phosphate released during the anaerobic phase of the process but also nearly all the available phosphate from the surrounding environment. As much as 30% of the PHB/PHV formed during the anaerobic phase is consumed during the aerobic uptake of phosphate and its conversion to polyP. The exact physiological reason why EBPR microorganisms should replenish polyP

reserves at the expense of PHB/PHV, instead of utilizing these biopolymers solely for growth and biomass production, is at present unknown (Kortstee *et al.*, 2000).

Under favorable conditions EBPR plants can remove 80–90% of influent phosphate, achieving residual P levels in effluent of less than 1 mg/liter and producing sludges with a P content of up to 15%; this compares with the 20–40% P removal typical of conventional wastewater treatment (Streichan *et al.*, 1990). Various full-scale process configurations exist for EBPR, including the University of Capetown Process, Phostrip Process, Modified Bardenpho Process, Three Stage Phoredox Process, A/O Process, Rotanox Process, and the Modified University of Capetown Process (reviewed by Yeoman *et al.*, 1988; Brett *et al.*, 1997).

The selective enrichment of microorganisms capable of polyP accumulation through the cycling of activated sludge between anaerobic and aerobic zones is therefore the basis of conventional EBPR technology; phosphate removal occurs only during the aerobic phase. Recent investigations have, however, demonstrated that phosphate uptake and polyP formation may not necessarily require an aerobic zone and that under anoxic conditions nitrate can provide an alternative electron acceptor (Kuba *et al.*, 1993; Jorgensen and Pauli, 1995; Barker and Dold, 1996; Barak and van Rijn, 2000; Egli and Zehnder, 2002). Nitrification-Denitrification Biological Enhanced Phosphate Removal (NDBEPR) may in fact have a number of advantages over traditional EBPR, including considerable energy savings, reduced biomass production, and a maximization of the amount of COD available for both nitrogen and P removal (Kuba *et al.*, 1993). Various laboratory scale studies have demonstrated that NDBEPR sequence batch reactors can achieve results comparable to those of the conventional EBPR process, albeit at lower biomass levels and with different P release and uptake rates (Kerrnjespersen and Henze, 1993). Microbial community analysis has revealed that those microorganisms capable of conventional EBPR, using oxygen as the terminal electron acceptor (see Section IV), may also be those responsible for the utilization of nitrate in an NDBEPR system (Dabert *et al.*, 2001).

III. Polyphosphate Metabolism in Microorganisms

A. Polyphosphate: An Introduction

Whatever the configuration chosen for a biological P removal facility, the process is necessarily dependent on the ability of sludge microorganisms to take up phosphate and to store it intracellularly in

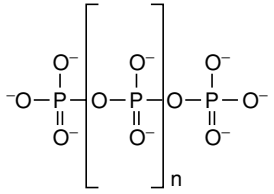

FIG. 1. The structure of inorganic polyphosphate.

the form of polyP. The polymer was first identified by Wiame (1947) as the major component of the intracellular ''volutin'' granules that stain metachromatically with basic dyes, and has been described in the microbiological literature since the early 1900s (Wiame, 1947, 1948). Today it is recognized as one of the most widely distributed natural biopolymers, having been detected in many bacteria, fungi (including yeasts), plants, and animals (Dawes and Senior, 1973; Kulaev and Vagabov, 1983; Kulaev *et al.*, 1999). PolyP consists of a linear chain of phosphate residues linked together by high-energy phosphoanhydride bonds and ranges in length from 3 to greater than 1000 orthophosphate residues (Kulaev, 1979) (Fig. 1): intracellular polyP inclusions appear as dark granules of very variable size and number when examined by electron microscopy (Fig. 2). Under optimal conditions polyP may amount to 10–20% of the cellular dry weight and as such greatly exceeds the P requirements of the cell, suggesting that it may perform metabolic roles other than simply that of a phosphate reserve material (Pick *et al.*, 1990). Many reviews detail both the physical and chemical properties of polyP as well as its predicted role in prebiotic evolution (Harold, 1966; Dawes and Senior, 1973; Kortstee *et al.*, 1994; Kulaev *et al.*, 1999; Kornberg and Fraley, 2000).

B. Enzymes of Polyphosphate Synthesis in Microorganisms

1. *Polyphosphate Kinase in Prokaryotes*

Bacterial polyP synthesis is primarily catalyzed by the enzyme polyphosphate kinase (polyphosphate:ADP phosphotransferase; PPK; EC 2.7.4.1) (Kornberg, 1995). PPK has been extensively characterized in a number of prokaryotes, including *Vibrio cholera, Pseudomonas aeruginosa, Propionibacterium shermanii, Acinetobacter* sp., *Neisseria meningitidis, Arthrobacter atrocyaneus, Corynebacterium xerosis, Salmonella minnesota, Burkholderia cepacia, Sulfolobus acidocaldarius*,

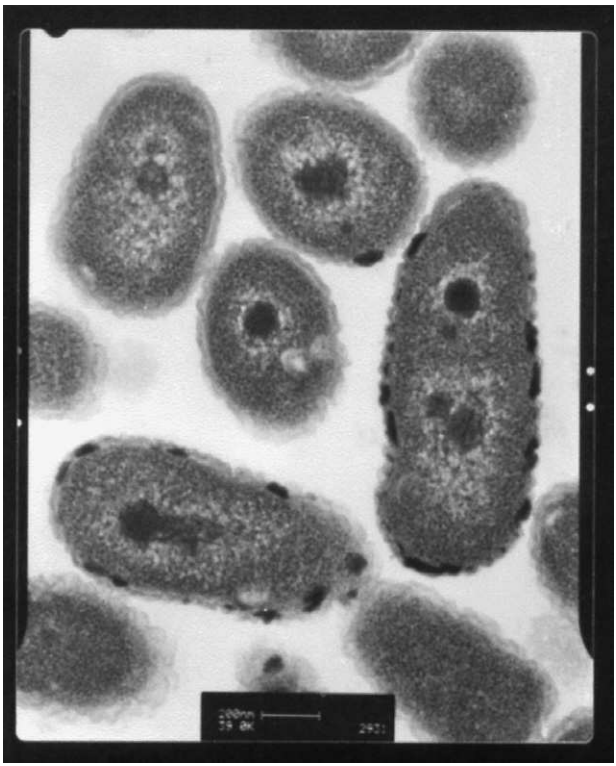

FIG. 2. Transmission electron micrographs of *Burkholderia cepacia* AM19. Large dark inclusions represent intracellular polyphosphate. Bar = 200 nm.

and, most extensively, in *Escherichia coli* (Kulaev *et al.*, 1999; Kornberg and Fraley, 2000).

E. coli PPK is a membrane-bound homotetramer with a subunit molecular mass of 80 kDa. The enzyme catalyzes the progressive synthesis of the polyP chain through the reversible transfer of the gamma phosphate from adenosine triphosphate (ATP) to polyP [Eq. (1)] (Akiyama *et al.*, 1992; Kato *et al.*, 1993; Tinsley and Gotschlich, 1995; Geißdörfer *et al.*, 1998). The polymerization reaction in both *N. meningtidis* and *E. coli* proceeds via an N-linked phosphoenzyme that serves as an intermediate of the phosphotransfer reaction. The amino acid residues His-441 and His-460 are particularly important in the mechanism of PPK activity: site-directed mutagenesis of these residues rendered mutant proteins incapable of polyP formation (Kumble *et al.*, 1996).

$$\text{PolyP}_n + \text{ATP} \longleftrightarrow \text{PolyP}_{n+1} + \text{ADP} \quad (1)$$

E. coli PPK also catalyzes the reverse reaction—the formation of ATP from adenosine diphosphate (ADP) and polyP; however the PPK of *Acinetobacter* sp. strain ADP1 will catalyze only the forward reaction (Ahn and Kornberg, 1990; Trelstad *et al.*, 1999). Activation by divalent cations and in particular Mg^{2+} is a characteristic common to all prokaryotic PPKs (Murata *et al.*, 1988; Tinsley and Gotschlich, 1995; Trelstad *et al.*, 1999). The K_m values for purified PPKs from *E. coli* (Ahn and Kornberg, 1990), *N. meningtidis* (Tinsley *et al.*, 1993), and *Acinetobacter* sp. strain ADP1 (Trelstad *et al.*, 1999) are 2.0, 1.5, and 1.0 m*M* ATP, respectively.

Of particular interest from the studies on prokaryotic PPKs is the apparent stimulation of kinase activity in the presence of phosphate and/or polyP. The PPK activities of *N. meningtidis*, *P. shermanii*, and *B. cepacia* AM19 were stimulated 6-fold, 10-fold, and 30-fold, respectively, by the addition of phosphate to the reaction buffer (Robinson *et al.*, 1987; Tinsley *et al.*, 1993; Mullan *et al.*, 2002b). In *B. cepacia* AM19, in the absence of free phosphate, addition of polyP (chain length 75; 5 m*M*) stimulated PPK activity 6-fold (Mullan *et al.*, 2002b); similar results have been observed for *P. shermanii* (Robinson *et al.*, 1987). These results lend support to the hypothesis that phosphate or polyP may serve as primer for PPK activity, although this was not true of the *E. coli* enzyme (Kornberg *et al.*, 1999).

In addition to both polyP and ATP synthesis *E. coli* PPK exhibits a range of other activities, including that of a general nucleoside-diphosphate kinase (Kornberg *et al.*, 1999), in the catalysis of the synthesis of linear guanosine 5′-tetraphosphate (ppppG), and in the autophosphorylation of the PPK enzyme itself (Tzeng and Kornberg, 2000). However, recent evidence from *P. aeruginosa* PAO1 suggests that the proposed reverse reaction of ATP formation from polyP catalyzed by PPK and known as polyphosphate:ADP phosphotransferase may, at least in this organism, be catalyzed independently of PPK (Section III.C.5) (Ishige and Noguchi, 2000, 2001).

2. *Polyphosphate Kinase in Eukaryotes*

Although the discovery of inorganic polyP in *Saccharomyces cerevisiae* dates back over 100 years the enzymatic basis of polyP synthesis in eukaryotes remains unresolved (Kulaev *et al.*, 1999). To date only one description of putative PPK activity from yeast cell extract exists (Fetler and Stahl, 1973). Recent investigations have, however, shown that the activity described was actually that of diadenosine-5′,5‴-P^1,P^4-tetraphosphate α,β-phosphorylase working in concert with exopolyphosphatase (Section III.C.1) rather than PPK (Booth and Guidotti,

1995); failure to assay the alleged PPK by formation of polyP resulted in the misrepresentation of the enzyme activity in the original report (Fetler and Stahl, 1973; Booth and Guidotti, 1995). [Similarly PPK activity has not been identified in the domain Archaea; an alleged *S. acidocaldarius* glycogen-bound PPK has recently been reclassified as a thermostable glycogen synthase (Cardona *et al.*, 2001).]

The lack of a definitive activity in eukaryotic cell extracts analogous to prokaryotic PPK is puzzling, especially given the likely role of lower eukaryotes in phosphate removal from wastewaters (Melasniemi and Hernesmaa, 2000). This failure may, however, be due in part to a shortage of suitable methods for the quantification of PPK and polyP. Current methods of enzymatic analysis of polyP synthesis are technically difficult and inexact and the development of improved methodologies that directly measure changes in polyP concentration should facilitate an increased understanding of those enzymes involved in polyP turnover during the EBPR process. Such a system, based on the well-established metachromatic reaction of toluidine blue with polyP, has recently been shown to be effective in the characterization of PPK activity in crude extracts of an environmental *B. cepacia* isolate (Mullan *et al.*, 2002b).

3. *Regulation of Polyphosphate Kinase*

Polyphosphate kinase is encoded by the *ppk* gene, which, like the enzyme itself, has undergone extensive study particularly with regard to its regulation. The *ppk* genes of many organisms including *Klebsiella aerogenes*, *E. coli*, *N. meningitidis*, *P. aeruginosa*, and various *Acinetobacter* spp. have been cloned, sequenced, and characterized (Ahn and Kornberg, 1990; Akiyama *et al.*, 1992, 1993; Kato *et al.*, 1993; Tinsley and Gotschlich, 1995; Trelstad *et al.*, 1999; Zago *et al.*, 1999; Gavigan *et al.*, 1999). There is, however, disagreement as to the regulation of *ppk* within these organisms and in particular to the involvement of the *pho* or phosphate regulon (Gavigan *et al.*, 1999).

The *pho* regulon describes those phosphate starvation-inducible genetic elements involved in all aspects of phosphate assimilation (Wanner, 1990, 1993). The gene cluster includes the *pst SCAB-phoU* operon for the high-affinity phosphate-specific transporter (Pst) system, the alkaline phosphatase gene, the glycerol-3-phosphate transport (Ugp) system, and the genes for phosphonate utilization (Metcalf *et al.*, 1990; Metcalf and Wanner, 1991). Typically a *pho*-controlled gene is induced more than 100-fold by phosphate limitation and is under the control of a two-component sensor–regulator complex that consists of the proteins PhoB and PhoR (Wanner, 1990, 1993). Regulation is achieved via

the PhoB DNA-binding protein, which acts as a transcriptional activator. PhoB is itself activated through the action of the second protein, PhoR, which phosphorylates the PhoB protein during times of phosphate stress (and vice versa under conditions of phosphate excess) (Wanner, 1990, 1993). Overseeing the action of the PhoR protein as a protein kinase is the Pst system and an accessory protein known as PhoU. The Pst system acts as a cell surface receptor complex and is involved in the detection of extracellular phosphate levels. This Pst system, in tandem with the PhoU protein, regulates the phosphorylating/dephosphorylating activities of the PhoR protein (Wanner, 1990, 1993).

Disagreement exists, however, as to whether PPK and polyphosphatase (PPX), the major polyP-degrading enzyme in bacterial cells (Section III.C.1), are under the control of the *pho* regulon. In *E. coli* both *ppk* and *ppx* are located on the same operon; interruption of *ppk* results in the loss of *ppx* expression (Ahn and Kornberg, 1990; Akiyama *et al.*, 1992, 1993). The promoter region contains two putative PhoB boxes; despite this, however, expression of the *ppk* operon is not solely responsive to P_i but rather also requires amino acid starvation. A separate study using an *E. coli ppk::lacZ* gene fusion found that levels of expression doubled upon phosphate starvation. Similar results were, however, observed using both *phoB* and *phoU* mutant host strains making it unlikely that the *pho* regulon is involved in the regulation of *E. coli* PPK (Lee *et al.*, 1999). It has recently been shown that *E. coli* or *Synechocystis* cells lacking a functional *phoU* accumulated up to 6-fold more polyP than their corresponding wild types (Morohoshi *et al.*, 2002).

Transcription of the *ppk* gene is nevertheless induced by phosphate starvation in both *Acinetobacter* sp. ADP1 (Trelstad *et al.*, 1999) and *Acinetobacter baumannii* 252—by a factor of 11 in the latter organism (Gavigan *et al.*, 1999). No *pho* box-type sequences are present either in *Acinetobacter* sp. ADP1 or *A. baumannii* 252 nor have any *phoB* or *phoR* genes been cloned from these organisms, although it is likely that they do contain analogous genes (Gavigan *et al.*, 1999). Unlike the situation in *E. coli* the *ppk* genes of *Acinetobacter* sp. ADP1 and *A. baumannii* 252 are not followed by an exopolyphosphatase-encoding *ppk* gene. To date *ppx* has not been identified in any *Acinetobacter* species (Gavigan *et al.*, 1999).

PolyP accumulation by PPK may also be regulated at the enzymatic level. The *ppk* and *ppx* genes of *P. aeruginosa* 8830 are transcribed divergently and their expression is not coordinated (Zago *et al.*, 1999). Under such circumstances polyP accumulation may be regulated at the enzymatic level through inhibition of PPX. In response to amino acid starvation *E. coli* increases its intracellular polyP levels 1000-fold,

whereas the cell-free activities of both PPK and PPX remain unaltered (Kuroda *et al.*, 1997); polyP accumulation is achieved by selective inhibition of PPX *in vivo* by either guanosine tetraphosphate or guanosine pentaphosphate generated in response to the amino acid starvation (Kuroda *et al.*, 1997; Ault-Riche *et al.*, 1998; Rao *et al.*, 1998). Additionally the response of *E. coli* to other conditions such as osmotic stress (Ault-Riche *et al.*, 1998) is under the control of additional stress-induced proteins such as the sigma factor RpoS. RpoS may act, in concert with other regulatory signals, to either inhibit PPX or stimulate PPK (Ault-Riche *et al.*, 1998). The allosteric activation of PPK by phosphate as described above for *N. meningtidis*, *P. shermanii*, and *B. cepacia* AM19 may provide a further control of PPK activity whereby the enzyme is activated in response to high intracellular phosphate concentrations allowing excess phosphate to be stored predominantly as polyP.

It is clear, therefore, that a detailed understanding of the regulation of PPK (and PPX) at the levels of both their synthesis and activity is still lacking, even in well-studied laboratory isolates, whereas even more work is required to elucidate the exact mechanisms of the control of polyP biosynthesis in those environmental microorganisms that may be important in wastewater treatment. Without a thorough knowledge of these control mechanisms, however, the molecular basis of this central aspect of the EBPR process will not be fully understood.

4. *Other Polyphosphate-Synthesizing Enzymes*

It is clear that enzymes other than PPK do exist for the intracellular production of polyP. Evidence for such pathways mainly lie in the ability of *ppk*$^-$ mutants to maintain a residual level of polyP formation. Pathways for polyP synthesis that do not involve PPK have been proposed for the *ppk*$^-$ mutants of *E. coli* (Crooke *et al.*, 1994; Castuma *et al.*, 1995), *N. meningitidis* (Tinsley and Gotschlich, 1995), *P. aeruginosa* 8830 (Zago *et al.*, 1999), *Acinetobacter* sp. strain ADP1 (Trelstad *et al.*, 1999), and also in *Saccharomyces carlsbergensis* and *S. cerevisiae* (Kulaev and Kulakovskaya, 2000).

These possible alternative routes include the involvement of the enzyme 1,3-diphosphoglycerate-polyphosphate-phosphotransferase (EC 2.7.4.17), which has been shown to form polyP at the expense of 1,3-diphosphoglycerate (Kulaev, 1979), or the reversal of those enzymes involved in polyP catabolism, i.e., exopolyphosphatase (Section III.C.1) and endopolyphosphatase (Section III.C.2). As yet no experimental evidence exists to support the latter suggestion, although membrane-bound polyphosphatases exist widely in eukaryotes; the

binding of enzymes to membranes may change the direction of the catalyzed reaction (Kulaev and Vagabov, 1983; Kulaev and Kulakovskaya, 2000). In addition the production of surface high-molecular-weight polyP in yeasts is known to be synthesized by a pathway closely resembling that involved in the biosynthesis of cell-wall mannoproteins (Kulaev and Kulakovskaya, 2000). Synthesis occurs in the membrane fraction of the endoplasmic reticulum and involves the enzyme dolichylpyrophosphate-polyphosphate-phosphotransferase (EC 2.7.4.20) (Kulaev and Kulakovskaya, 2000). Finally, the recent failure to detect a *ppk* gene in *S. cerevisiae* using genomic expression analysis has led to the suggestion that intracellular yeast polyP synthesis may involve vacuolar membrane-bound enzymes working in concert with the proton motive force to provide energy for the direct elongation of the polyP (Ogawa *et al.*, 2000). Further research will, however, be required to elucidate these other putative pathways of polyP biosynthesis.

C. Enzymes of Polyphosphate Utilization in Microorganisms

In addition to PPK (polyP:ADP phosphotransferase—Sections III.B.1 and III.C.5) a variety of hydrolases and phosphotransferases are known to utilize polyP as a substrate.

1. *Exopolyphosphatase (EC 3.6.1.11)*

This enzyme catalyzes the processive hydrolytic cleavage of P_i from the end of the polyP chain; the reaction may continue until only pyrophosphate (PP_i) remains (Kornberg *et al.*, 1999):

$$\text{PolyP}_n + H_2O \longrightarrow \text{PolyP}_{n-1} + P_i \qquad (2)$$

A diversity of exopolyphosphatases has been identified from bacterial and eukaryotic sources. The *E. coli* enzyme (PPX) is a peripheral membrane protein that requires K^+ for maximal activity and has a high affinity for polyP ($K_m = 9$ n*M*). It is encoded in the same operon as PPK; the two enzymes are believed to act in concert to control cellular polyP levels (Section III.B.1). In other prokaryotes in which the activities have been studied, however, their genetic determinants appear to be independently encoded, for example, in *P. aeruginosa* and *Helicobacter pylori*. Bacterial exopolyphosphatases commonly have low activity on short-chain polyP; however a specific tripolyphosphatase (EC 3.6.1.25) has been purified from *Methanobacterium thermoautotrophicum* (van Alebeek *et al.*, 1994). Hydrolysis of tripolyphosphate by a purified exopolyphosphatase from *S. cerevisiae* has also been reported (Kulakovskaya *et al.*, 1999).

The physiologically significant role of a further exopolyphosphatase activity identified in *E. coli* has proved to be that of the dephosphorylation of guanosine pentaphosphate (pppGpp) to produce the tetraphosphate (ppGpp), a regulatory nucleotide involved in the stringent cellular response to amino acid or P deficiency (Keasling *et al.*, 1993). Interestingly it has been demonstrated (Kuroda *et al.*, 1997) that polyP accumulation in *E. coli* is regulated at the enzymatic level through the inhibition of PPX by ppGpp and pppGpp.

No significant similarity has been identified at the genetic level between the exopolyphosphatase gene from *E. coli* and the only eukaryotic homologue cloned to date, that encoding a cytosolic enzyme from *S. cerevisiae*; moreover separate exopolyphosphatases with diverse properties are believed to be present in the yeast cell envelope, cytosol, vacuoles, nuclei, and mitochondrial matrix (Kulaev *et al.*, 1999). This, and the typically much higher exopolyphosphatase-specific activities found in yeast cell extracts, may reflect the fact that at least some bacterial PPKs are able both to synthesize and hydrolyze polyP (Kulaev and Kulakovskaya, 2000). In general, yeast and other lower eukaryotic exopolyphosphatases show K_m values in the low micromolar range and are distinct from their bacterial equivalents in their activity against a broad range of polyP chain lengths and in their dependence on divalent cations.

2. *Endopolyphosphatase (EC 3.6.1.10)*

Unlike exopolyphosphatases, endopolyphosphatases catalyze the internal hydrolytic cleavage of polyP:

$$\text{PolyP}_n + \text{H}_2\text{O} \longrightarrow \text{PolyP}_{n-x} + \text{PolyP}_x \quad (3)$$

Endopolyphosphate activity has been detected in eukaryotic cells but not in prokaryotes (Kulaev and Kulakovskaya, 2000), and the metal-dependent vacuolar enzyme from *S. cerevisiae* has been purified (Kumble and Kornberg, 1996). It cleaves long-chain polyP (~700 residues) to tri-polyP and in addition gives rise to an intermediate product of about 60 residues. Deletion of the corresponding *PPN1* gene has been shown to decrease growth and limit survival of the host strain in minimal medium (Sethuraman *et al.*, 2001).

3. *Polyphosphate:Glucose Phosphotransferase (Polyphosphate Glucokinase; EC 2.7.1.63)*

Glucokinase catalyzes an attack by glucose at the end of the polyP chain:

$$\text{PolyP}_n + \text{Glucose} \longrightarrow \text{PolyP}_{n-1} + \text{Glucose-6-phosphate} \quad (4)$$

The enzyme is specific to glucose and glucosamine and its activity has been identified in a wide variety of bacteria (Wood and Clark, 1988); its purification has been reported from *Mycobacterium tuberculosis*, *Mycobacterium phlei* (Szymona and Ostrowski, 1964; Hsieh *et al.*, 1993, 1996), and from *P. shermanii* (Phillips *et al.*, 1993). The enzyme possesses two active centers, at one of which ATP serves as phosphate donor. In the most phylogenetically ancient organisms the catalytic efficiency of polyP-glucokinase activity is greater than ATP-glucokinase activity, suggesting that the enzyme evolved at a time when polyP played a more prominent role in bioenergetics. In more recent phylogenetic groups the polyP-dependent activity appears to be a ''fossil'' reaction.

4. *Polyphosphate:AMP Phosphotransferase (and Adenylate Kinase)*

This enzyme catalyzes the attack at the end of the polyP chain by adenosine monophosphate (AMP) to produce ADP:

$$\text{PolyP}_n + \text{AMP} \longleftrightarrow \text{PolyP}_{n-1} + \text{ADP} \tag{5}$$

It has been purified from *Acinetobacter* (Bonting *et al.*, 1991) and identified in extracts of both *E. coli* and *Myxococcus xanthus* (Kornberg *et al.*, 1999). The ADP formed can subsequently serve as a substrate for adenylate kinase, which interconverts ADP and ATP:

$$2\text{ADP} \longleftrightarrow \text{AMP} + \text{ATP} \tag{6}$$

Formation of ATP from AMP and polyP can thus occur through the combined action of the two enzymes, even if an electron donor (organic carbon source) or electron acceptor (oxygen) is unavailable to the cell (van Groenestijn *et al.*, 1987, 1989). Ishige and Noguchi (2000) have, however, recently suggested that the polyP:AMP phosphotransferase activity observed in crude extracts of *E. coli* is catalyzed by an enzyme complex that is formed between PPK (polyP:ADP phosphotransferase) and adenylate kinase in the presence of polyP. They propose that adenylate kinase cannot use polyP directly to phosphorylate AMP, but that autophosphorylated PPK protein within the complex (rather than ATP) might provide the donor P group for adenylate kinase.

5. *Polyphosphate:ADP Phosphotransferase (Reverse PPK Activity)*

This enzyme is proposed to catalyze the polyphosphate-dependent phosphorylation of nucleoside diphosphates such as ADP (''reverse'' PPK activity):

$$\text{PolyP}_n + \text{ADP} \longleftrightarrow \text{PolyP}_{n-1} + \text{ATP} \tag{7}$$

Its existence in *P. aeruginosa* as an activity distinct from that of the reverse reaction of PPK has very recently been proposed (Ishige and Noguchi, 2001). Although the enzyme has not been purified it appears to require short-chain polyP (<75) as the phospho-donor and to have an almost 3-fold higher specificity for guauosine diphosphate (GDP) than ADP as phospho-acceptor [it was speculated that large quantities of guanosine triphosphate (GTP) might be required by this pathogenic organism for exopolysaccharide production].

6. *Other Polyphosphate-Degrading Enzymes*

There are in addition literature reports of bacterial transferases that phosphorylate NAD and triose phosphate, respectively, to NADP and 1,3-diphosphoglycerate but neither enzyme has been purified and their status remains uncertain (Wood and Clark, 1988).

IV. The Microorganisms and Polyphosphate-Metabolizing Enzymes of the EBPR Process

A. The Microbial Ecology of the EBPR Process

Traditional microbiological techniques of enrichment and isolation have, for many years, been employed to determine those members of the activated sludge microbial consortia responsible for EBPR (van Loosdrecht *et al.*, 1997; Bond *et al.*, 1999). Such studies have frequently indicated the numerical dominance of members of the *Acinetobacter–Moraxella* group, and other members of the *gamma* subclass of the class *Proteobacteria* in EBPR sludges (Fuhs and Chen, 1975; Deinema *et al.*, 1980; Wentzel *et al.*, 1988; Streichan *et al.*, 1990). Yet despite extensive biochemical characterization the patterns of carbon turnover, phosphate uptake and release, and polyP accumulation in these isolates are not consistent with those observed in EBPR sludges with a high phosphate removal capacity (van Groenestijn *et al.*, 1989; Tandoi *et al.*, 1998); polyP accumulation and release do, however, occur at least to some extent in these organisms (Deinema *et al.*, 1980; Ohtake *et al.*, 1985; Vasiliadis *et al.*, 1990; Beacham *et al.*, 1992).

The study of the microbial ecology of EBPR has now been given new momentum by the development of culture-independent technologies for the *in situ* identification of individual members of complex microbial communities. Microbial community analyses using polyamines, respiratory quinones, fatty acid profiles, fluorescent *in situ* hybridization (FISH), production of cloned 16S rDNA ''libraries,'' and the electrophoretic separation and analysis of PCR-amplified 16S rDNA

fragments are all at present contributing to a radical revision of our understanding of the microbial ecology of the process (Auling *et al.*, 1991; Wagner *et al.*, 1994; Bond *et al.*, 1995; Kampfer *et al.*, 1996; Melasniemi *et al.*, 1998; Hiraishi *et al.*, 1998; Christensson *et al.*, 1998; Hesselmann *et al.*, 1999; Liu *et al.*, 2000; Crocetti *et al.*, 2000; Dabert *et al.*, 2001). These culture-independent approaches have indicated the involvement of a much wider range of organisms in EBPR sludges than had been recognized by traditional methods—at least 30 different phylotypes from major phyla of the domain *Bacteria* (Liu *et al.*, 2000). *Acinetobacter* spp., traditionally assumed to be the predominant EBPR microorganism, have proved to be present in very much lower numbers than would be required to account for the phenomenon (Wagner *et al.*, 1994; Bond *et al.*, 1995; Melasniemi *et al.*, 1998; Hiraishi *et al.*, 1998). Rather those organisms that appear to dominate high-performance EBPR sludges from both laboratory reactors and full-scale wastewater treatment installations are members of the *beta*-2 subclass of the *Proteobacteria* (related to the genera *Rhodocyclus* and *Propionibacter*) and the high GC Gram-positive *Actinobacteria* (Bond *et al.*, 1999; Hesselmann *et al.*, 1999; Crocetti *et al.*, 2000); *Rhodocyclus*-related cells appeared to account for up to 73% of all PAOs in one full-scale EBPR process (Zilles *et al.*, 2002).

B. The Enzymology of Polyphosphate Metabolism in the EBPR Process

Paralleling the ecological complexity of the EBPR process is our lack of knowledge of its biochemical basis, and in particular the identities of those enzymes involved in polyP turnover *in situ*, and the mechanisms of their regulation (Trelstad *et al.*, 1999). Furthermore, enhanced ''biological'' phosphate removal also involves chemical mechanisms such as phosphate precipitation or its adsorption at the outer membranes of microorganisms (Bark *et al.*, 1993); the relative contribution of such processes is difficult to quantify. The majority of work on microbial polyP metabolism has also been carried out on pure cultures obtained by the traditional methods of enrichment and isolation (Section IV.A). As these isolates are now believed to be unlikely to participate in EBPR, our knowledge of the enzymology of the process will remain uncertain until the microorganisms responsible for the phenomenon have been unambiguously identified and methods for their *in vitro* culture have been established.

Polyphosphate kinase (Section III.B.1) is the most extensively studied polyP-synthesizing enzyme and has been detected in a wide range

of prokaryotes—yet even its involvement in the EBPR process is unclear. Significant levels of the enzyme were found in only one of six polyP-accumulating *Acinetobacter* strains isolated from EBPR sludge (van Groenestijn *et al.*, 1989) and in four of 21 similar isolates from two further plants (Bark *et al.*, 1993; Weltin *et al.*, 1996). There thus remains the likelihood of alternative, as-yet-unrecognized routes of microbial polyP biosynthesis (Ogawa *et al.*, 2000) (Section III.B.2).

Of equal importance to those microorganisms involved in the EBPR process is the possession of an efficient energy-generating system from polyP under anaerobic conditions. Again numerous studies have failed to conclusively attribute the degradation of polyP to any specific enzymatic activity. A number of different enzyme systems capable of polyP degradation have, however, been detected. The enzymes polyP:AMP phosphotransferase and adenylate kinase (Section III.C.4) have been shown to form ATP from polyP in a number of *Acinetobacter* strains (van Groenestijn *et al.*, 1987, 1989; Bonting *et al.*, 1991; Kampfer *et al.*, 1992). Indeed a positive correlation exists between the latter activity in cell extracts of a number of activated sludge samples and the percentage of P removed from wastewater by those sludges (van Groenestijn *et al.*, 1989).

By contrast, however, 11 polyP-accumulating activated sludge isolates (none identified as *Acinetobacter* spp.) lacked detectable polyP:AMP phosphotransferase activity, and only three showed (low) levels of PPK. All, however, contained adenylate kinase activity, although in four cases even higher levels of polyP:glucokinase activity were detected (Section III.C.3); three strains contained a glucose-6-phosphate-dependent NAD kinase (Bark *et al.*, 1993). It was speculated that the latter two enzymes in concert could provide an effective polyP-dependent NADP regeneration system. Studies on *Acinetobacter johnsonii* also suggest (van Veen *et al.*, 1994) that phosphate generated from polyP by cellular polyphosphatase activity could contribute to the transmembrane proton gradient and that its efflux might form a mechanism for at least partial conservation of the energy of the polyP phosphoanhydride bond.

The high degree of uncertainty that exists over both the identities of the microorganisms that are central to the EBPR process and the metabolic pathways that are involved in polyP turnover during EBPR demonstrates the need for extensive further study. A final resolution of these issues may come about only with the development of strategies that overcome the perennial difficulty of coupling the *in situ* detection of the polyphosphate-accumulating phenotype with molecular identification of strains that possess it. The possibility of combining

FISH with microautoradiography to identify cells that accumulate radiolabeled phosphate (Lee *et al.*, 1999) appears to offer a particularly promising route to final resolution of the question. Additionally, although it is likely that the maintenance of many such microorganisms under laboratory conditions would be difficult, a number of strategies for their successful cultivation have been suggested (Mino *et al.*, 1998).

Successful isolation of PAOs would allow detailed investigation of the physiology, biochemistry, and genetics of phosphate metabolism in pure culture studies. Many questions still need to be resolved regarding the accumulation of polyP by environmental microorganisms. For polyP formation to occur, phosphate must first be transported into the microbial cell and subsequently converted into ATP before incorporation into the polyP polymer. Much research has been conducted on phosphate transport across the cytoplasmic membrane of bacterial cells and has implicated two major systems (van Veen, 1997):

1. The inorganic phosphate transport system (Pit) is constitutively expressed and has a relatively low specificity for phosphate, with a K_m of about 25 μmol/liter for both phosphate and its analogue, arsenate. Pit does not require a periplasmic phosphate-binding protein and consists of a single transmembrane protein capable of conformational change that permits the alternate exposure of its substrate-binding sites to the inner and outer surfaces of the membrane; phosphate transport is driven by the proton motive force. Pit transports neutral metal phosphates, each in symport with a proton.
2. The phosphate-specific transport system (Pst) is composed of four subunits—a periplasmic substrate-binding protein, two integral proteins within the cytoplasmic membrane that each contains six membrane-spanning helices, and an ATP-binding protein. Unlike Pit, the Pst system transports both $H_2PO_4^-$ and HPO_4^{2-}, but not neutral metal phosphates, and has high substrate affinity with a K_m of less than 1.0 μmol/liter; it is phosphate-starvation inducible (e.g., as part of the Pho regulon in *E. coli*).

Phosphate transport in yeast and other lower eukaryotic cells is also believed to involve both low- and high-affinity uptake systems (Persson *et al.*, 1999). *S. cerevisiae* contains a constitutively expressed low-affinity phosphate transporter with an estimated K_m of 1 mmol/liter. In addition it possesses two derepressible high-affinity systems, each with K_m values for phosphate in the low micromolar range but showing separate pH optima of 4.5 and 9.5; they are thus presumably mechanistically distinct.

Future research on microbial polyP metabolism in the EBPR process must concentrate on producing experimental evidence to indicate the relative roles of these (or other) phosphate transport systems and to investigate the possibility that their activities may be rate limiting for polyP production. It must also clarify the mechanism by which influent phosphate is converted into ATP (or how the high-energy phosphoanhydride bonds of polyP are otherwise produced). The issue of the identities and regulation of the enzymes involved in this process in ''true'' EBPR microorganisms needs particularly to be addressed.

V. Alternative Strategies for the Induction of Polyphosphate Accumulation in Microorganisms

PolyP biosynthesis and accumulation are, of course, not confined to those microorganisms exposed to alternating anaerobic/aerobic environments. Given the prevalence of this phosphate biopolymer throughout the microbial world—which would suggest that it plays a crucial metabolic role—it is becoming clear that the ability to accumulate polyP is triggered by many environmental circumstances other than those artificially imposed by the EBPR regime.

Despite this, however, an exact biological role for polyP remains unknown. The molecule has been demonstrated to serve in microorganisms as a reservoir of energy and phosphate, a chelator of divalent cations, a capsule material, a buffer against alkali, a ''channelling'' agent in the phenomenon of bacterial transformation, and an inhibitor of RNA degradation. Its involvement in the motility of bacterial pathogens has also been demonstrated and it has recently been shown to promote ribosomal protein degradation in association with the Lon protease. (Kornberg *et al.*, 1999; Kornberg and Fraley, 2000; Kuroda *et al.*, 2001).

Of particular significance, in terms of biotechnological exploitation for novel wastewater treatment processes, may be the central regulatory role believed to be played by intracellular polyP in the response of some microbial cells to alterations in either their environment or growth phase (Kornberg *et al.*, 1999). For example, extensive polyP accumulation has been detected in *E. coli* upon exposure to osmotic stress, or to nutritional stress imposed by either nitrogen, amino acid, or phosphate limitation (Ault-Riche *et al.*, 1998; Rao *et al.*, 1998), and in *P. aeruginosa* mucoid strain 8830 during the stationary phase and in response to phosphate and amino acid starvation (Ault-Riche *et al.*, 1998; Kim *et al.*, 1998). The intracellular polyP content of the bacterium *Microlunatus phosphovorus* NM-1 similarly increases upon entry into stationary phase (Kawaharasaki and Nakamura, 1995). The

unicellular alga *Dunaliella salina* and *S. cerevisiae* utilize their intracellular polyP reserves to provide a pH-stat mechanism to counterbalance alkaline stress (Pick *et al.*, 1990; Bental *et al.*, 1991; Pick and Weiss, 1991; Castro *et al.*, 1995), whereas polyP accumulation has been observed in the freshwater sponge *Ephydatia muelleri* upon exposure to various organic pollutants (Imsiecke *et al.*, 1996).

Exposure to acid pH has also been recently shown to result in the induction of polyP accumulation in a variety of environmental microorganisms. This low-pH–stimulated polyP accumulation has been reported in *Candida humicola* G-1 (McGrath and Quinn, 2000) and led to a 10-fold increase in intracellular polyP accumulation in cells grown at pH 5.5 rather than 7.5 (McGrath and Quinn, 2000). Similarly, in *B. cepacia*, maximal phosphate removal and polyP accumulation occurred at pH 5.5 with levels 220% and 330% higher, respectively, than those in cells grown at pH 7.5; intracellular polyP accounted for up to 13.6% of cellular dry weight in acid-grown cells (Mullan *et al.*, 2002a). Additionally polyphosphate kinase activity could be detected only in cells cultured at pH 5.5. Significantly, neither phosphate starvation, nutrient limitation, nor anaerobiosis was required to induce enhanced phosphate uptake and polyP formation. A subsequent survey of the microflora of several municipal-activated sludge plants revealed that some 34% of isolates were capable of enhanced phosphate uptake under acid conditions and that levels of phosphate removal could be increased by 55–124% through adjustment of culture pH to 5.5 (McGrath *et al.*, 2001). The pH optimum for phosphate uptake varied between isolates obtained from these plants but in all cases ranged from pH 5.0 to 6.5 (McGrath *et al.*, 2001).

Strategies that exploit alternative ''triggers'' for the induction of polyP accumulation in microorganisms are potentially of great biotechnological benefit. The imposition of any condition under which polyP biosynthesis and accumulation are necessary for microbial survival (or at least confers a competitive advantage on cells that possess it) should result in enhanced phosphate removal from wastewater. A knowledge of such conditions might then be exploited to provide alternative and possibly superior treatment options for biological P removal from industrial and municipal effluents.

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Biosurfactants: Evolution and Diversity in Bacteria

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I. Introduction

Demand for new specialty chemicals in the agriculture, cosmetic, food, pharmaceutical, and environmental industries is steadily increasing. Because these chemicals must be both effective and environmentally compatible, it is natural to turn to the microbial world to try to meet this demand. Microbes produce a largely unexplored variety of chemicals, such as biosurfactants, that have exciting potential for application in biotechnology and industry. These chemicals are often referred to as natural products. Although biosurfactants are just one class of natural products, they are a representative example of the challenges that exist in discovery, analysis, production, and use of natural products in industry. In this chapter biosurfactants and their potential industrial uses are defined briefly, followed by a discussion of the problems associated with discovery of new biosurfactants even as more DNA sequence information and high throughput processing technology become available.

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A. Biosurfactants Defined

Biosurfactants have a wide range of physical and chemical properties but share certain attributes. All biosurfactants are amphiphilic in nature, containing at least one hydrophilic and one hydrophobic moiety. The hydrophilic moiety can be an ester, hydroxyl, phosphate, carboxyl, or carbohydrate group and is either neutral or negatively charged. There have been no cationic biosurfactants reported, presumably because cationic surfactants in general are quite toxic. The hydrophobic moiety is a fatty acid ranging in size from 8 to 18 carbons. Due to their amphiphilic nature, surfactants tend to accumulate at interfaces (air–water and oil–water) and surfaces. As a result, surfactants reduce the forces of repulsion between unlike phases at interfaces or surfaces and allow the two phases to mix more easily. Specifically, surfactants can reduce surface (liquid–air) and interfacial (liquid–liquid) tension. For example, a proficient biosurfactant can reduce the surface tension between pure water and air from 73 mN/m to less than 30 mN/m. As surfactant monomers are added into solution, the surface or interfacial tension will decrease until the surfactant concentration reaches what is known as the critical micelle concentration (CMC). Above the CMC no further reduction in surface or interfacial tension is observed. At the CMC, surfactant monomers begin to spontaneously associate into structured aggregates such as micelles, vesicles, and lamellae (continuous bilayers) (Fig. 1). These aggregates form as a result of numerous weak chemical interactions between the polar head groups and the nonpolar tail groups, including hydrophobic, van der Waals, and hydrogen bonding. The CMC for any surfactant is dependent on the surfactant structure as well as the pH, ionic strength, and temperature of the solution. Further, the aggregate structure is dictated by the polarity of the solvent in which the surfactant is dissolved. For example, in an aqueous solution, the polar head groups of a micelle will be oriented outward toward the aqueous phase and the hydrophobic tails will associate in the core of the micelle (oil in water micelle). In contrast, in oil the polar head groups will associate in the center of the micelle while the hydrophobic tails will be oriented toward the outside (water in oil micelle).

Although many common cell components are surface active, e.g., fatty acids and phospholipids, a large number of microorganisms produce specific biosurfactant molecules that have unique structures. The earliest interest in biosurfactants arose as a result of their antibiotic activity. The two best-studied biosurfactants, rhamnolipid and surfactin, and their associated antibiotic activities were first described in the

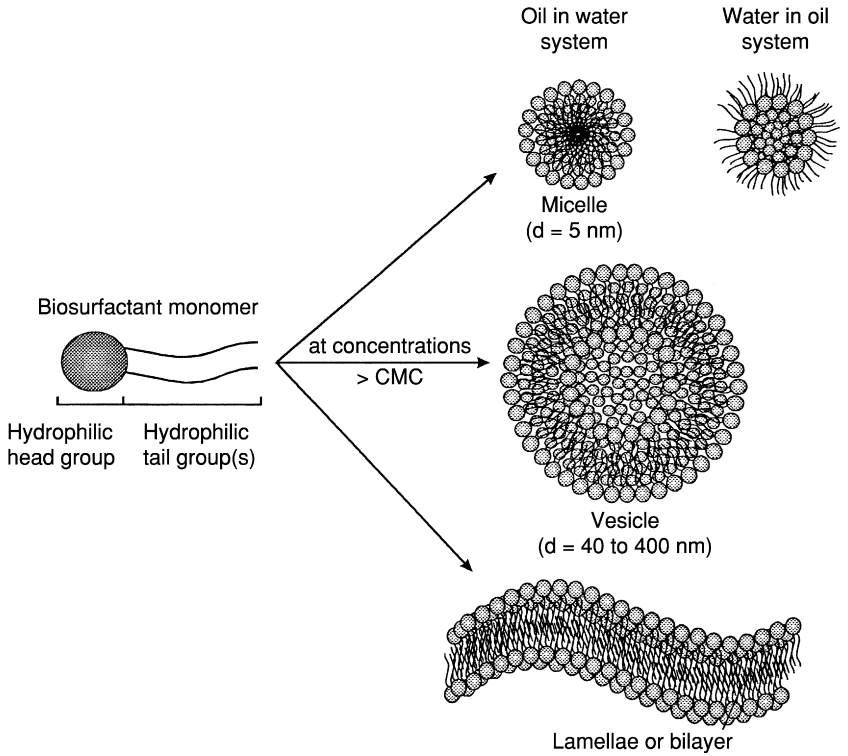

FIG. 1. The types of aggregates formed by biosurfactants.

literature in 1949 (Jarvis and Johnson, 1949) and 1968 (Arima *et al.*, 1968), respectively. Further interest developed from the observation that biosurfactants were produced in response to the presence of hydrophobic substrates, suggesting the possibility for their use in treatment of petroleum wastes (e.g., Itoh *et al.*, 1971) and in petroleum recovery (e.g., Donaldson *et al.*, 1989). Interest in biosurfactants remains strong because they are considered environmentally compatible.

B. BIOSURFACTANT-PRODUCING MICROORGANISMS

Biosurfactants are synthesized by a variety of microorganisms. A search of the literature indicates that the ability to produce biosurfactants is widespread in the bacterial and archaeal domains (Fig. 2). Some of the bacterial genera reported to produce surfactants include

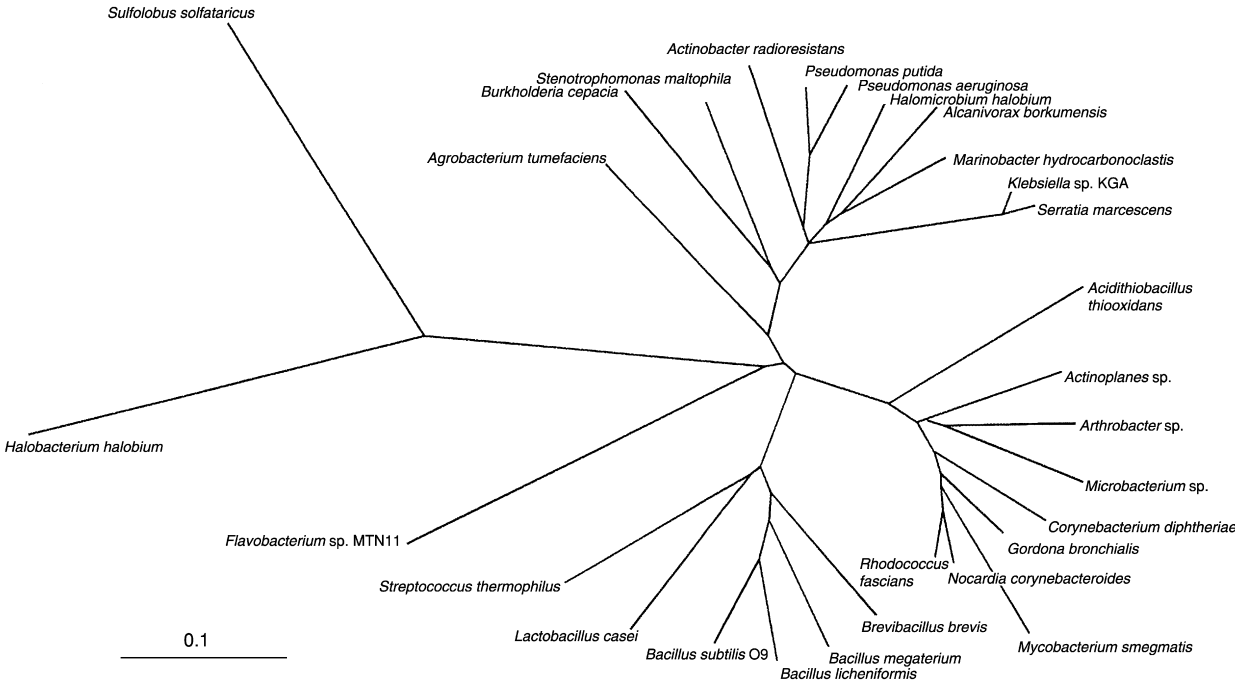

FIG. 2. A phylogenetic tree based on the 16 S rDNA sequences from genera representing microorganisms that produce biosurfactants (Bodour and Maier, 2003). The unrooted tree was created using the neighbor joining method.

Pseudomonas (Maier and Soberón-Chávez, 2000), *Rhodococcus* (Bryant, 1990), *Mycobacterium* (Wong *et al.*, 1979), *Nocardia* (Ioneda *et al.*, 1970), *Flavobacterium* (Bodour and Maier, 2003), *Corynebacterium* (Cooper *et al.*, 1982), *Clostridium* (Cooper *et al.*, 1980), *Acinetobacter* (Käppeli and Finnerty, 1979), *Thiobacillus* (Beebe and Umbreit, 1971), *Bacillus* (Yakimov *et al.*, 1995), *Serratia* (Matsuyama *et al.*, 1985), *Arthrobacter* (Morikawa *et al.*, 1993), and *Alcanivorax* (Yakimov *et al.*, 1998). Although the ability to produce biosurfactants is widespread, the type of biosurfactant produced is genus and sometimes even species specific. For example, *Pseudomonas aeruginosa* produces rhamnolipid, a glycolipid, and *Pseudomonas fluorescens* produces viscosin, a lipopeptide biosurfactant.

Biosurfactant-producing organisms have been isolated from a wide diversity of environments including soil, seawater, marine sediments, and oil fields (deep subsurface environments). A study of polyaromatic hydrocarbon (PAH)-degrading isolates obtained from contaminated soil sites showed that 67% of the isolates produced surfactants (Willumsen and Karlson, 1997). A recent study in my laboratory involved screening 21 uncontaminated, hydrocarbon-contaminated, metal-contaminated, and hydrocarbon–metal cocontaminated soils for biosurfactant producers. Twenty of the 21 soils were found to contain at least one biosurfactant-producing isolate even using a very limited screening methodology (Bodour and Maier, 2003).

C. *In Situ* Biosurfactant Production

Several pieces of evidence indicate that biosurfactants are produced, sometimes at high levels, in the environment. First, is that biosurfactants are produced in ocean oil spill areas resulting in visible foaming and emulsification. One study demonstrated biosurfactant production (trehalose lipid was identified) in soil in response to addition of hydrocarbons (Oberbremer and Müller-Hurtig, 1989). Similar results have been observed recently in my laboratory in systems from which rhamnolipid- and trehaloselipid-producing microbes were isolated and identified (Curtis, 1999). Finally, there has been much interest in using indigenous biosurfactant producers to facilitate tertiary oil recovery. Yakimov *et al.* (1997) demonstrated that *Bacillus licheniformis* produced biosurfactants in an oil reservoir under aerobic or anaerobic conditions at temperatures up to 55°C and salinities of up to 12% NaCl. These observations clearly demonstrate that biosurfactants are produced *in situ*. However, there exists very little fundamental information about the conditions under which biosurfactants are produced.

II. Types of Biosurfactants

Biosurfactants are classified broadly based on their major structural features and the organism that produces them. The major groups include (1) glycolipids, (2) lipoproteins, (3) phospholipids, neutral lipids, and fatty acids, and (4) polymeric biosurfactants. Biosurfactants within each major group are generally produced as a mixture of closely related compounds. For example, rhamnolipids are generally produced in mixtures of rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, commonly known as monorhamnolipid, and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, commonly known as dirhamnolipid. Further, there may be up to four different mono- and dirhamnolipid homologs produced that vary in the number of carbons in the fatty acid moieties (Fig. 3). For example, Rendell *et al.* (1990) identified a mixture of mono- and dirhamnolipids from a clinical *P. aeruginosa* isolate that included C_{10}–C_{10} (β-hydroxydecanoyl-β-hydroxydecanoate, the major component), C_8–C_{10}, C_{10}–C_8, C_{10}–C_{12}, C_{12}–C_{10}, and C_{10}–$C_{12:1}$ homologs.

The surfactant mixture produced is dependent on the producing isolate, as will be discussed in a following section, and also on the carbon source available for growth. For example, Robert *et al.* (1989) showed that when grown on dodecane, succinate, citrate, or olive oil, *P. aeruginosa* 44T1 produced a mixture of the mono- and dirhamnolipids such as those described above. In contrast, when grown on glucose or mannitol, 44T1 produced a more complex mixture that included a mono- and dirhamnolipid with only one fatty acid moiety in addition to the normal mono- and dirhamnolipids. The fact that different surfactant mixtures are produced by closely related isolates and that the carbon source influences the mixture produced is important because the physical–chemical properties of these mixtures can vary considerably and thus, may be suitable for quite different applications.

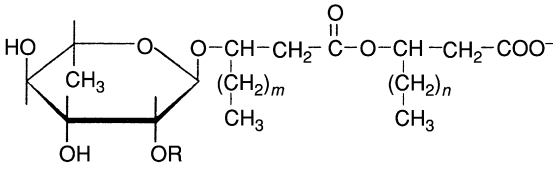

FIG. 3. The structure of monorhamnolipid where R = H or rhamnose, and m and $n = 4$ to 8.

A. Glycolipids

The most widely studied biosurfactants are the glycolipids, which are produced by a wide variety of microorganisms. Glycolipids have one or more carbohydrate groups attached to one or more long-chain aliphatic acid or hydroxy-aliphatic acid groups, and range in molecular weight from 500 to 1500. The best studied glycolipids include rhamnolipids (Jarvis and Johnson, 1949), trehalose lipids (Suzuki *et al.*, 1969), sophorolipids (Gorin *et al.*, 1961), and a new group mannosylerythritol lipids (Kitamoto *et al.*, 1990). Other types of glycolipids have been reported in the literature such as cellobiose lipid (Spoeckner *et al.*, 1999), glucose lipid (Ishigami *et al.*, 1994), glycoglycerolipid (Nakata, 2000), sugar-based bioemulsifiers (Kim *et al.*, 1996, 2000; Van Hoogmoed *et al.*, 2000), and many different hexose lipids (Cairns *et al.*, 1982; Fiebig *et al.*, 1997; Golyshin *et al.*, 1999; Ha *et al.*, 1991).

B. Lipoproteins

Lipoproteins have a protein moiety attached to a fatty acid. The protein moiety can be neutral or anionic and the amino acids are often arranged in a cyclic structure. As a group, the lipoprotein biosurfactants are perhaps best known for their antibiotic activities. For example, gramicidin S is produced by *Bacillus brevis* (Marahiel *et al.*, 1977) and the polymyxins are produced by *Bacillus polmyxa* (Suzuki *et al.*, 1965). Both are cyclic lipopeptide antibiotics that have notable surface activity. The best characterized lipoprotein biosurfactants are those produced by *Bacillus* sp., including surfactin, iturin, fengycin, and lichenysin. These have molecular weights ranging from 1000 to 1500.

C. Phospholipids, Neutral Lipids, and Acidic Lipids

There are several lipid-containing molecules integral to cell structure that are also surface active and have activities normally associated with biosurfactants. These include phospholipids, acidic lipids such as fatty acids, and neutral lipids such as triacylglycerol. For example, Beebe and Umbreit (1971) isolated a mixture of phospholipids and neutral lipids from *Thiobacillus thiooxidans* that was capable of wetting elemental sulfur. Phospholipid-rich and lipopolysaccharide (LPS)-rich vesicles, produced by *Acinetobacter* sp. HO1-N during growth on hexadecane, were shown to function in enhancing the solubility of hexadecane (Käppeli and Finnerty, 1979). *Arthrobacter paraffineus* KY 4303 is reported to produce a primary fatty alcohol when

grown on paraffin that coats the paraffin in the medium (Suzuki and Ogawa, 1972). A neutral lipid is produced by *Nocardia erythropolis* ATCC 4277 during growth on 4% hydrocarbon (MacDonald *et al.*, 1981). One lipid that is not integral to the cell structure is spiculisporic acid, a tricarboxylic acid produced by *Penicillium*. Spiculisporic acid is produced during growth on glucose and yields of up to 110 g/liter have been achieved (Ishigami *et al.*, 2000). Although the surface activity of spiculisporic acid is low, derivatives of this molecule have good surface activity. For example, a disubstituted *n*-hexylamine salt of the S-form reduced surface tension to 27 mN/m (Ishigami *et al.*, 1987).

D. Polymeric Biosurfactants

The polymeric biosurfactants are characterized by their high molecular weight, ranging from 50,000 to greater than 1,000,000. These polymers can be carbohydrate or protein based and in addition usually contain lipids. In some cases the polymer may be a mixture of carbohydrate, protein, and lipid. These compounds, noted for their emulsification abilities, are produced by a number of bacteria, Archaea, and yeast. Of great interest commercially is the fact that each polymeric biosurfactant has different hydrocarbon specificity with respect to degree of emulsification. In general, the polymeric biosurfactants do not lower surface or interfacial tension significantly. These polymers are quite heterogeneous and therefore have been difficult to categorize as precisely as the low-molecular-weight biosurfactants. The best characterized of the polymeric biosurfactants is emulsan, produced by *Acinetobacter calcoaceticus* RAG1 (Rosenberg *et al.*, 1979). Emulsan is a heteropolysaccharide linked to fatty acids through *o*-ester and amide bonds. There is also a protein associated with this polymer that is required for emulsification activity.

III. Biosurfactant Function

A. Natural Role of Biosurfactants

The physiological function of biosurfactants is still unknown, although there has been much speculation in the literature and there may exist multiple functions for these unique and fascinating molecules. One suggested physiological role of biosurfactants is to facilitate growth of microorganisms on water-immiscible substrates by reducing the interfacial tension, and thus enhancing the bioavailability

of the substrate. Other suggested functions include enhancement of adhesion of cells to insoluble substrates (Neu, 1996), antibiotic activity, and a possible role in pathogenesis of cystic fibrosis and burn wounds (Rumbaugh *et al.*, 1999). It was also discovered that some biosurfactants strongly complex metals (Tan *et al.*, 1994), suggesting a role for biosurfactants in the interaction of microorganisms with metals in their environment.

B. Industrial Applications for Biosurfactants

Currently, the surfactant industry, which exceeds $9 billion per year, markets predominantly synthetic surfactants. However, some biosurfactants, e.g., rhamnolipid, sophorolipid, and spiculosporic acid, can be produced commercially at levels nearing and even exceeding 100 g/liter (Ishigami *et al.*, 2000; Maier and Soberón-Chávez, 2000; Rau *et al.*, 1996). At this level, the cost of producing biosurfactants becomes competitive with the cost of producing synthetic surfactants. As the production cost becomes competitive and as the commercial availability of biosurfactants increases, the commercial use of biosurfactants can be expected to grow. Important to note is that only two biosurfactants have been studied extensively. However, the analytical and molecular tools now available make the tasks of identifying new biosurfactants, and understanding how and why biosurfactants are produced, more feasible and rapid.

The following applications presently use biosurfactants or have been suggested. They are mentioned only briefly here but have been reviewed extensively by Bodour and Maier (2002). Potentials for application of biosurfactants in the field of medicine include use as antibiotics for protection of cell cultures in medical research (Vollenbroich *et al.*, 1997a), as antivirals (Kracht *et al.*, 1999; Vollenbroich *et al.*, 1997b), as antiinflammatory agents (Kim *et al.*, 1998), in the prevention of biofilm formation (Busscher *et al.*, 1997), and as antitumor agents (Isoda *et al.*, 1997).

In addition to these clinical applications, a number of industrial applications for biosurfactants have been investigated. Suggested applications are quite varied. One interesting example is their use to enhance the heterogeneous enzymatic hydrolysis of cellulose and steam-exploded wood (Helle *et al.*, 1993). Biosurfactants have long been suggested for use as additives for microbially enhanced oil recovery (MEOR) and for the cleaning of oily sludges from storage tanks (Banat, 1994). For these applications isolates from extreme environments (temperature, salinity, or pH) are of special interest (Gurjar

et al., 1995; Trebbau de Acevedo and McInerney, 1996). More recently it has been suggested that biosurfactants may aid in preventing fouling of industrial surfaces by preventing biofilm formation (Busscher *et al.*, 1996).

Biosurfactants and chemically modified biosurfactants are being investigated for use in the field of high-value products. For example, Ishigami and Suzuki (1997) report the development of a technique that utilizes biosurfactants for monitoring the micropolarity and microfluidity of surfaces. In this case the use of a biosurfactant is an alternative to the synthetic chemical normally used, which has much higher environmental toxicity. A second example is production of stereospecific L-rhamnose. Rhamnolipids have been suggested as an economical source of L-rhamnose, which is used commercially in the production of high-quality flavor compounds and as a starting material for synthesis of some organic compounds (Linhardt *et al.*, 1989). Surfactants and emulsifiers are routinely used in the food industry. For example, Torabizadeh *et al.* (1996) found the mannoprotein emulsifier produced by *Saccharomyces cerevisiae* could be used as a mayonnaise additive. This emulsifier is found in the cell wall and can be obtained cheaply and in high yield [80–100 g/kg (g/g wet cells)]. In addition it is stable from pH 3 to 11, and the by-products can be used as animal feed supplements.

The cosmetic and health care industries use large amounts of surfactants for a wide variety of products, including insect repellents, antacids, acne pads, antidandruff products, contact lens solutions, hair color and care products, deodorants, nail care products, lipstick, eyeshadow, mascara, toothpaste, denture cleaners, antiperspirants, lubricated condoms, baby products, foot care products, antiseptics, shaving and depilatory products, and moisturizers (Kleckner and Kosaric, 1993). Biosurfactants in general are considered to have some advantages over synthetic surfactants, the predominant type of surfactant used in this industry. These advantages are low irritancy or antiirritating effects and compatibility with skin.

There are several reports on the efficacy of biosurfactants in biological control. Rhamnolipids have been shown to have activity against zoosporic plant pathogens at very low concentrations and were successful at control in a near-commercial hydroponic recirculating cultural system (Stanghellini and Miller, 1997). Similarly, surfactin and a similar lipopeptide, iturin A, produced by *Bacillus subtilis* RB14, have been identified as playing a role in the suppression of damping-off disease of tomato seedlings caused by *Rhizoctonia solani* (Asaka and Shoda, 1996).

Finally, biosurfactants have been explored for their bioremediation potential, and show promise for application to sites impacted by both organic and metal contaminants. Specific applications include enhancing contaminant biodegradation in sites that are contaminated with organics alone (Bregnard *et al.*, 1998; Herman *et al.*, 1997; Jain *et al.*, 1992; Oberbremer *et al.*, 1990) or that are cocontaminated with metals and organics (Maslin and Maier, 2000; Sandrin *et al.*, 2000). Biosurfactants have also shown potential for use as additives to aid in cleaning or flushing organic (Bai *et al.*, 1998; Ivshina *et al.*, 1998; Van Dyke *et al.*, 1993;) or metal (Mulligan *et al.*, 1999; Ochoa-Loza *et al.*, 2001; Torrens *et al.*, 1998; Zosim *et al.*, 1983) contaminants out of tanks or soils.

IV. The Search for New Biosurfactants

A. Discovery of New Biosurfactants

As outlined above, the potential for industrial application of biosurfactants is largely unexplored. Even though biosurfactant production is widespread, relatively few biosurfactant structures have been completely elucidated and even fewer have been well characterized in terms of their biosynthesis, regulation, and encoded genetic information. The reason that few biosurfactants have been structurally elucidated is that the process required to extract, purify, and analyze the surfactant discovered is both tedious and difficult. This is further complicated by the fact that purification and structure elucidation are really chemistry problems that few microbiology laboratories are well equipped to carry out.

The elucidation of surfactant biosynthetic pathways (including structural and regulatory genes) is equally complicated. This is well demonstrated by the work recently completed for the biosurfactant rhamnolipid (Brint and Ohman, 1995; Campos-García *et al.*, 1998; Ochsner *et al.*, 1994a,b; Ochsner and Reiser, 1995; Olvera *et al.*, 1999; Pearson *et al.*, 1997; Pesci *et al.*, 1997, 1999; Rahim *et al.*, 2001; Van Deldon *et al.*, 1998; Wild *et al.*, 1997). This work has revealed a complex organization of both regulatory and structural genes. An analysis of the *P. aeruginosa* PAO1 gene sequence shows that the rhamnolipid genes are located disparately on the chromosome (Stover *et al.*, 2000). Specifically, assuming that dTDP-L-rhamnose is available in the cell, there are at least three disparate locations for the structural genes required for the synthesis of the remainder of the rhamnolipid molecule (Fig. 4). This includes a cluster of four genes beginning at 3893007 that

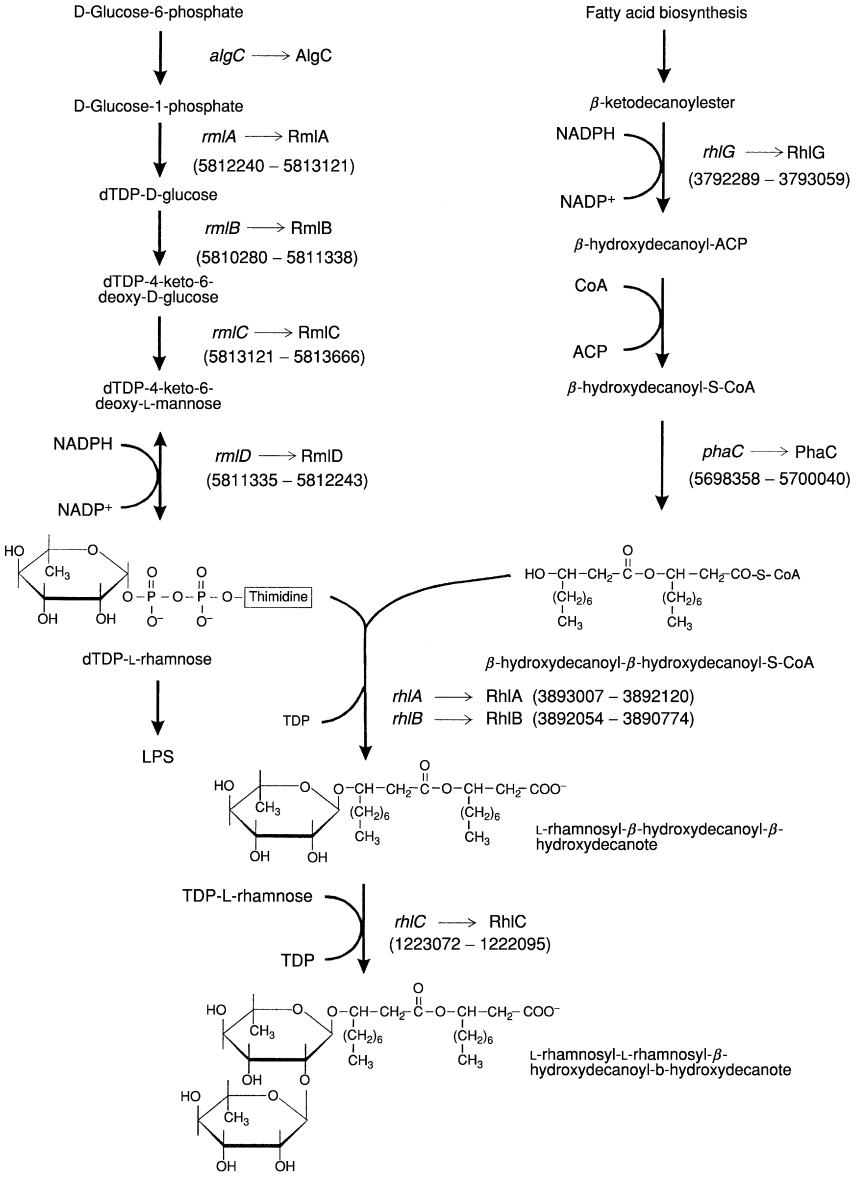

FIG. 4. The biosynthetic pathway for rhamnolipid showing the genes and proteins involved in each step. The numbers in parentheses next to the gene notations indicate the chromosomal location of the gene in *Pseudomonas aeruginosa* PAO1 (Stover *et al.*, 2000).

codes for two structural genes, *rhlA* (rhamnosyl transferase I) and *rhlB* (rhamnosyl transferase I), and two regulatory genes, *rhlI* (autoinducer synthesis protein) and *rhlR* (transcription regulator). In contrast, the structural gene *rhlG* encoding a β-ketoacyl reductase, begins at 3792289, and the structural gene *rhlC* encoding rhamnosyl transferase II begins at 1223072. Similarly, there are two locations for the regulatory genes, which include two transcriptional regulators, the *rhlR* gene mentioned previously and *lasR* (beginning at 1558171).

In rhamnolipid biosynthesis, the *rhlAB* genes are needed to combine dTDP-L-rhamnose with β-hydroxydecanoyl-β-hydroxydecanoate to make monorhamnolipid. That these genes are located in a separate operon from the *rhlC* gene, which is needed to combine dTDP-L-rhamnose with rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate to make dirhamnolipid, is somewhat unusual but allows for the possibility of regulating the relative amounts of mono- and dirhamnolipid produced. It has been shown for the isolate studied (*P. aeruginosa* PAO1) that *rhlAB* and *rhlC* are coordinately regulated. Each has a σ^{54}-type promoter and similar ''lux box'' sequences that match in 16 out of 19 bases (Rahim *et al.*, 2001). These data suggest that for PAO1, transcriptional regulation of mono- and dirhamnolipid is similar. However, it is still unclear whether there are additional levels of regulation that can allow an individual isolate like PAO1 to regulate the relative amounts of mono- and dirhamnolipid it makes in response to a change in environmental conditions.

As a side note, it seems worth mentioning that the complexity of rhamnolipid biosynthesis suggests that it will be difficult to create overproducers in a heterologous host system. Production of rhamnolipid in heterologous hosts has been reported only once. Ochsner *et al.* (1995) achieved successful expression of rhamnolipid in *P. putida* and *P. fluorescens* although at relatively low levels. The same group failed to achieve rhamnolipid production in *Escherichia coli*, although the strain did synthesize active rhamnosyl transferase I. It is likely that similar complexities will exist for other biosurfactants, making scaled-up production of these materials in heterologous hosts problematic. That said, as mentioned earlier, biosurfactants have been produced in very high yields, exceeding 100 g/liter, without the use of heterologous hosts.

From the information gained in studying rhamnolipid, it has become clear that surfactant genes are not conserved among different genera. For example, a set of *rhlB* primers constructed in my laboratory is very specific for *P. aeruginosa* (unpublished data). These primers do not detect any other surfactant-producing genus. Further, they do not

detect any surfactant-producing *Pseudomonas* species other than *aeruginosa*. A direct comparison of the *P. aeruginosa rhl* genes with the *srf* genes that encode for surfactin biosynthesis in *Bacillus* shows that they are completely different. It is somewhat surprising that surfactant genes are not conserved across the many different biosurfactant-producing populations. Biosurfactant production seems to be a rather common ability and must confer some kind of competitive advantage to the producing microorganism, yet it appears that this ability has evolved across the bacterial and archaeal domains in a parallel and nonconvergent fashion with respect to biosynthesis and regulation.

In more practical terms, considering biosurfactants as natural products with commercial potential, if surfactant genes are not conserved among different populations this makes it impossible to construct a molecular screening tool to search for new surfactants. Even searching for the presence of genes for known biosurfactants is problematic since so few have been genetically characterized. How then can samples be screened to enable the discovery process? For now, we must rely on more traditional chemical screening approaches that are based on either reduction of surface or interfacial tension or on emulsification ability. The drop collapse assay is one such rapid screening method (Jain *et al.*, 1991) that was later modified (Bodour and Miller-Maier, 1998). One advantage of this method is that it requires very small sample volumes (5 μl) and a large number of samples can be screened at once.

B. Discovery of New Biosurfactant Homologs

As mentioned earlier in this discussion, biosurfactants are generally produced as mixtures. The example given was rhamnolipid for which two major forms are produced, mono- and dirhamnolipid. An examination of the physical–chemical properties of these two surfactants shows that they are quite different. In contrast to dirhamnolipid, monorhamnolipid complexes cadmium 10 times more strongly (unpublished data), is a more powerful solubilizing agent (Zhang *et al.*, 1997), has lower water solubility, and sorbs to surfaces more strongly. As a second example, a novel nonionic rhamnolipid, rhamnosyl-(rhamnosyl)-β-hydroxydecanoyl-β-hydroxydecanoic acid methyl ester has been reported with attendent unique properties (Hirayma and Kato, 1982). A comparison of dirhamnolipid with dirhamnolipid methyl ester indicates that the methyl ester is able to reduce the interfacial tension between hexadecane and water to less than 0.1 dyn/cm, whereas dirhamnolipid reduces interfacial tension to 5 dyn/cm. A similar comparison of surface tension shows a reduction to 31 dyn/cm for the

methyl ester and to 36 dyn/cm for dirhamnolipid (Zhang and Miller, 1995). Finally, the ability of the methyl ester to enhance biodegradation of slightly soluble hydrocarbons was much greater than for dirhamnolipid (Zhang and Miller, 1995).

In general, *P. aeruginosa* isolates, either clinical or environmental, seem to make mixtures of mono- and dirhamnolipid. But it should be noted that there are exceptions. For example, *P. aeruginosa* ATCC 9027 produces only monorhamnolipid (Zhang and Miller, 1992), and *P. aeruginosa* 158 was reported to produce rhamnolipid methyl esters (Hirayama and Kato, 1982). This suggests that it is important to look at multiple isolates from a biosurfactant-producing species in order to discover the various structural homologs possible within a biosurfactant type. It is critical to note that care must be taken when screening closely related strains using molecular techniques. For example, *P. aeruginosa* isolates ATCC 9027 and IGB83 have identical 16 S rDNA sequences and yet produce quite different surfactant mixtures (monorhamnolipid only vs. a mono- and dirhamnolipid mix). Similarly, a group of four environmental *B. subtilis* strains all with nearly identical 16 S rDNA sequences and all matching most closely (99%) to Accession Number AB018484 produced surfactants that caused different reductions in surface tension (33.3, 33.9, 39.2, and 49.4 dyn/cm) (Bodour and Maier, 2002a). In both cases, it was found that a REP (repetitive extragenic palindromic) analysis was adequate to distinguish unique biosurfactant-producing isolates, but 16 S rDNA polymerase chain reaction (PCR) followed by sequencing was not.

V. Summary

In summary, biosurfactants are an example of a class of microbial natural products that has coevolved among many genera. But whereas the biosurfactants produced in the bacterial and archaeal domains are convergent in function (suggesting that they are very important), they have developed in parallel with respect to genotype and phenotype (the surfactants are not related genetically or in terms of molecular structure). Because of this parallel evolution, currently available molecular screening techniques are of little use for the discovery of new biosurfactants. Development of such techniques will continue to be problematic because there is no relationship between the surfactants produced by different microbial genera and even species. Yet, the potential for application of biosurfactants and other natural products is great due to growing demand for biodegradable and environmentally friendly analogues for synthetic chemicals.

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Comparative Biology of Mesophilic and Thermophilic Nitrile Hydratases

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I. Occurrence of Nitrile-Degrading Enzyme Systems

In nature, two distinct pathways for the conversion of nitriles to carboxylic acids exist (Fig. 1). The nitrilase (NTase; EC 3.5.5.1) pathway catalyzes the direct conversion of a nitrile to its corresponding carboxylic acid, whereas the nitrile hydratase (NHase; EC 4.2.1.84) pathway catalyzes the conversion of a nitrile to its corresponding amide, which is then converted to a carboxylic acid by an amidase. Until recently the role of these enzymes in microorganisms was unclear. However, it has been shown that some microbial strains metabolize aldoximes [utilized by many plant families as intermediates in the biosynthesis of plant natural products such as cyanogenic glucosides and glucosinates (Kato *et al.*, 1998)] via nitriles to the corresponding carboxylic acids by a combination of aldoxime dehydratase and nitrile-hydrolyzing enzymes (Kato *et al.*, 1998, 2000a). In an examination of 975 microorganisms, including 45 genera of bacteria and 11 genera of actinomyces, all 19 nitrile-degrading microorganisms (13 species, 7 genera) were found to exhibit aldoxime dehydratase activity (Kato *et al.*, 2000a). The cloning

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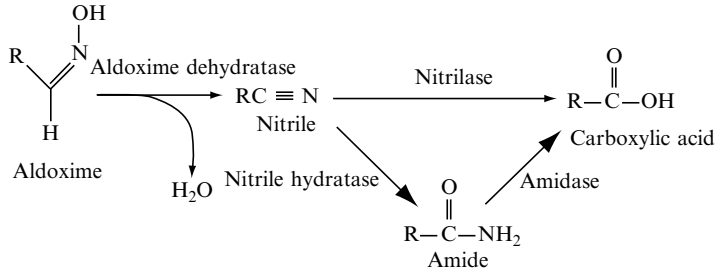

FIG. 1. Pathways for the microbial metabolism of aldoximes via nitriles (redrawn from Kato *et al.*, 2000a).

of a gene cluster containing both aldoxime dehydratase and nitrilase genes from *Bacillus* sp. strain OxB-1 has further corroborated these findings (Kato *et al.*, 2000b).

Since the initial isolation of a bacterial NHase from *Rhodococcus rhodochrous* (formerly *Arthrobacter*) J-1 (Asano *et al.*, 1980), a large number of NHases have been detected in a variety of microorganisms (Table I), isolated from diverse ecosystems including deep sea trenches (Heald *et al.*, 2001), nitrile-contaminated soils (Padmakumar and Oriel, 1999), and thermal lake sediments (Pereira *et al.*, 1998). However, despite this diversity in origin NHases have been detected in only a limited range of bacterial families (Fig. 2). Arguably, the genus *Rhodococcus* dominates the collective of known mesophilic NHase-producing microorganisms.

To the best of our knowledge, only five thermophilic NHase-producing organisms have thus far been described, four of which belong to the genus *Bacillus* (Pereira *et al.*, 1998; Takashima *et al.*, 1998; Cramp and Cowan, 1999; Padmakumar and Oriel, 1999) while the fifth is designated as *Pseudonocardia thermophila* (Yamaki *et al.*, 1997). This little-known organism belongs to the same order (Actinomycetales) as the genus *Rhodococcus*. All of these organisms grow in the 45–65°C temperature range of the moderate thermophiles (Cowan, 1992). To the authors' knowledge, no NHase has yet been isolated from an extremely thermophilic or hyperthermophilic microorganism, nor has this class of enzyme been identified in any Archaeal isolate or genome sequence.

A. Isolation Strategies

To date, nitrile-metabolizing organisms have typically been isolated by ''classic'' trophic selection strategies, where nitriles are offered as sole carbon and/or nitrogen sources to support the growth of

TABLE I

STRUCTURAL AND FUNCTIONAL PROPERTIES OF NHASES FROM VARIOUS BACTERIA

Microorganism	Subunit MW (kDa)	Cofactor	T_{opt} (T_{stab}) (°C)	pH_{opt}	Substrate preference	Production	Gene organization	Reference
A. tumefaciens	α27 β27	ND	40	7.0	Aromatic, cylic, enantioselective	Inducible	ND	Bauer *et al.* (1994)
Bacillus sp. BR449[a]	α25 β27	Co^{III}	55 (60)	7.5	ND	Constitutive	β–α	Kim and Oriel (2000)
Bacillus sp. RAPc8[a]	α28 β29	Co^{III}	60 (50)	7.0	Aliphatic, cyclic aliphatic, dinitriles	Constitutive	β–α	Pereira *et al.* (1998)
B. pallidus DAC 521[a]	α27 β29	? (Co^{III})	50 (45)	7.0–7.5	Aliphatic	Constitutive	ND	Cramp and Cowan (1999)
B. smithii[a]	α26 β29	Co^{III}	40 (50)	7.2	Aliphatic	Constitutive	β–α	Takashima *et al.* (1998)
Ps. thermophila[a]	α29 β32	Co^{III}	55 (50)	ND	ND	ND	β–α	Yamaki *et al.* (1997)
P. putida	α23 β24	Co^{III}	30 (35)	7.2	Aliphatic, dinitriles, enantioselective	Constitutive	α–β	Payne *et al.* (1997)
Rhodococcus sp. YH3-3	α27 β34.5	Co^{III}	40 (40)	7.0	Aliphatic, aromatic	Inducible	ND	Kato *et al.* (1999)
R. rhodochrous J1 H-NHase	α23 β26	Co^{III}	35 (50)	6.5–6.8	Aliphatic, aromatic	Inducible	β–α	Komeda *et al.* (1996b)
R. rhodochrous J1 L-NHase	α23 β25	Co^{III}	40 (30)	8.8	Aliphatic, aromatic	Inducible	β–α	Komeda *et al.* (1996a)
R. erythropolis	α23 β24	Fe^{III}	ND	ND	Aliphatic, aromatic	Constitutive	α–β	Duran *et al.* (1993)
Rhodococcus sp. R312	α23 β24	Fe^{III}	35 (20)	7–8.5	Aliphatic, cyclic	Constitutive	α–β	Nagasawa *et al.* (1986)
P. chlororaphis	α22 β25	Fe^{III}	20 (20)	6.0–7.5	Aliphatic	Inducible	α–β	Nagasawa and Yamada (1987)

[a] Themophilic organisms.

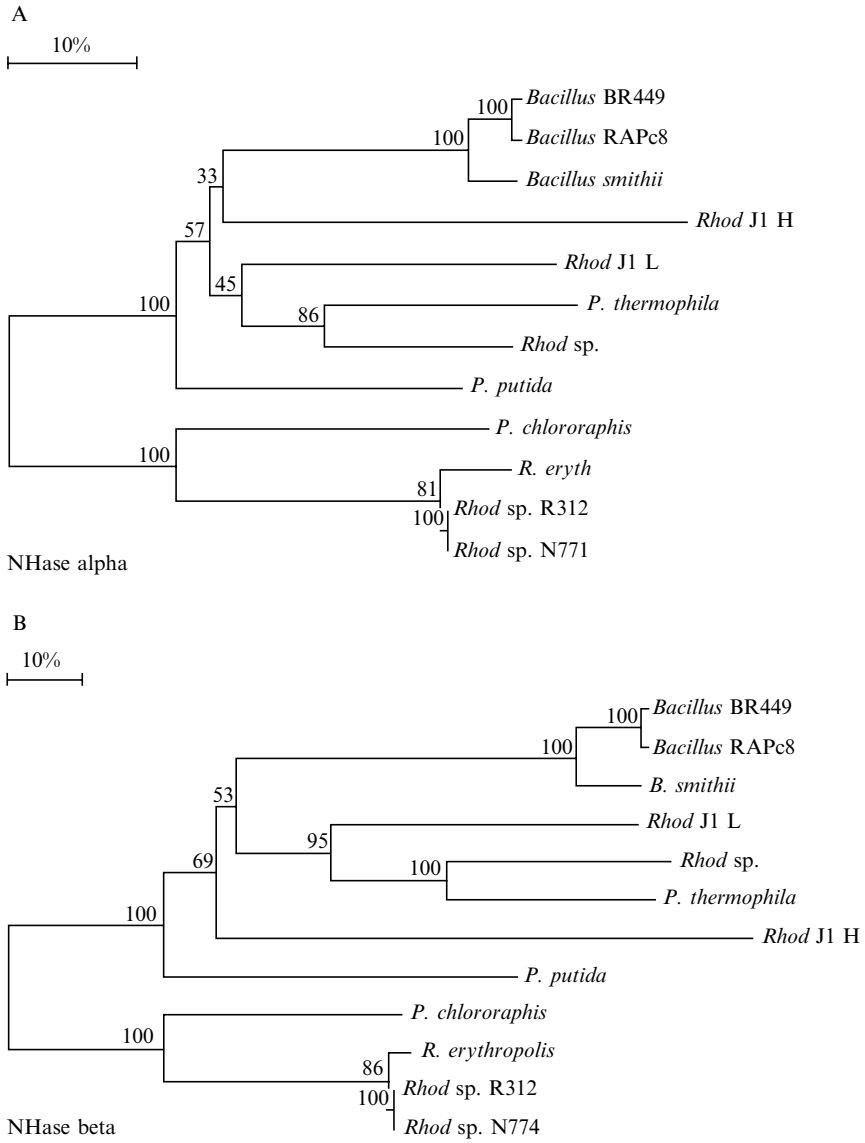

FIG. 2. Phylogenetic trees showing the relationship of large fragments of (A) α-subunits and (B) β-subunits of nitrile of nitrile hydratases from various microorganisms. Trees were constructed using CLUSTALW and Treecon packages. One hundred bootstrap operations were performed; values are marked at junctions. Bar = 10% difference in amino acid sequence identity.

''nitrilase''-positive organisms (Cowan *et al.*, 1998). Although this method has been highly effective, it polls a very low proportion of the true genomic diversity, and skews positive data in favor of rapidly growing, heterotrophic, nonfastidious, usually aerobic microorganisms. The enzyme distributions shown in Table I are entirely consistent with this view.

There is a widely held view that the prokaryote species so far cultured constitute only a small fraction of the actual microbial population observed in nature: phylogenetic studies over the past decade have indicated that up to 99% of the microorganisms existing in nature cannot be cultivated by standard techniques (Ward *et al.*, 1990; Hugenholtz and Pace, 1996). The genomes of the total microbiota found in nature are therefore considered to contain much more genetic information than the culturable subset. Thus, an alternative approach to conventional enzyme screening is to access this extensive microbial molecular diversity by isolating DNA without culturing the organisms present. Routine molecular biological techniques can then be applied to the environmental DNA in order to access and functionally characterize the target molecules.

Recently, this approach has been applied in two separate polymerase chain reaction (PCR)-based studies aimed at the isolation of NHase genes directly from environmental samples. The first study targeted NHase genes from a variety of environmental samples using primers corresponding exactly to the NHases operons of *R. erythropolis* or *R. rhodochrous* J1 (Precigou *et al.*, 2001). Full-length NHase gene sequences were successfully amplified, cloned, and expressed in *Escherichia coli* (although the activities observed were low). Although NHase sequences were amplified from all samples tested in the study, suggesting that NHase-producing organisms are widely distributed throughout microbial communities, the deduced amino acid sequences showed high identity (90–99%) with the previously described NHases from *Pseudomonas putida* (Fallon *et al.*, 1997) and several *Rhodococcus* species (Kobayashi *et al.*, 1991).

The second study, conducted in the authors' laboratory in association with Mitsubishi-Rayon Chemical Co. Ltd. (Cameron and Cowan, unpublished results), was an investigation of NHase diversity in thermal environments. PCR amplification using degenerate primers based on NHase α-subunit sequence (Co-type and Fe-type) alignments generated only sequences with high identity (89–93% at DNA level) to previously described thermostable *Bacillus* NHases, supporting the view that enzymes from *Bacillus* species dominate the thermostable NHase representatives.

Thus, although the rather uneven distributions observed through traditional screening methods may simply reflect the fact that many groups of organisms have not been screened for NHase activity, these PCR-based strategies for the detection of NHase genes in environmental samples apparently corroborate those distribution frequencies. However, it should be stressed that the technique is not truly culture independent, as the design of effective primers requires prior knowledge of NHase sequences, derived from the cloning of NHase genes from cultured organisms. In this way, it is likely that the results were strongly biased toward the known NHase-producing clades.

II. Enzymology of Mesophilic and Thermophilic NHases

A. Structural Characteristics

Despite significant differences in origin, stability, cofactor requirements, and catalytic characteristics between the various NHase subgroups (Table I), there is a high degree of similarity between these enzymes in terms of size and protein sequence (see Fig. 3). It is believed therefore that all enzymes in the NHase family have very similar structures and catalytic mechanisms (Mascharak, 2002). Although there is limited information concerning the structures of the thermostable NHases, we believe, given the observations of high protein sequence identity with the mesophilic enzymes, that this group follows the same trend.

1. *Size*

Typically, NHases are heteromultimers composed of two distinct subunits, designated α and β. The subunits typically have molecular weights ranging from 22 to 28 kDa; by convention the β-subunit is designated the larger. It is commonly assumed that although the minimal functional unit is an $\alpha\beta$ dimer, the NHases of both mesophiles and thermophiles are usually heterotetrameric in structure—$(\alpha\beta)_2$. *R. rhodochrous* J1 produces two NHases: L-NHase, which is tetrameric, and H-NHase, which forms multimers of up to 11 $\alpha\beta$ dimers (Nagasawa *et al.*, 1991). The molecular weight of *Rhodococcus* sp. N-771 in solution appears to be in equilibrium between dimer and tetramer; in the concentration range from 0.01 to 1 mg/ml the tetramer predominates (Nakasako *et al.*, 1999). In the three NHase crystal structures reported to date, the asymmetric unit consists of two dimers (Huang *et al.*, 1997; Nagashima *et al.*, 1998; Miyanaga *et al.*, 2001). As NHase concentration in the cell is often high (e.g., up to 10% of total protein in

Rhodococcus sp. N-771 cells), these results suggest that the natural state *in vivo* is the heterotetramer (Nakasako *et al.*, 1999).

2. *Cofactors*

Early studies of NHases reported that pyrroloquinoline quinone (PQQ) was a cofactor for *Rhodococcus* sp. R312 NHase (Nagasawa and Yamada, 1987), based primarily on the spectral properties of the solution enzyme. However, this hypothesis was definitively disproved with the resolution of the enzyme's crystal structure (Huang *et al.*, 1997). The crystal structures described to date (Huang *et al.*, 1997; Nagashima *et al.*, 1998; Miyanaga *et al.*, 2001), together with numerous spectroscopic studies, have formed the basis of a subdivision into two groups: (1) the Fe-type NHases (e.g., *Rhodococcus* sp. R312 NHase) and (2) the Co-type NHases (e.g., *R. rhodochrous* J1 H-NHase and L-NHase). The Fe-type enzymes are characterized by the presence of a single low-spin nonheme Fe^{III} ion per $\alpha\beta$ dimer and characteristic absorption peaks at 700 and 420 nm (Artaud *et al.*, 1999; Mascharak, 2002). The Co-type NHases contain a single noncorronoid Co^{III} ion per $\alpha\beta$ dimer and have a maximum absorption at 410 nm (Kobayashi and Shimizu, 1999). The Co-type NHases appear to have a wider substrate specificity and a higher degree of molecular stability than their Fe-type counterparts. All reported thermophilic NHases belong to the Co-type group.

3. *Photoactivation*

The NHases from *Rhodococcus* sp. R312 (Duran *et al.*, 1992), *Rhodococcus* sp. N-771, *Rhodococcus* sp. N-774 (Nakajima *et al.*, 1987) (which share identical amino acid sequences), *Rhodococcus erythropolis* (Cummings *et al.*, 1995), and *Commamonas testosteroni* NI1 (Bonnet *et al.*, 1997), form a subgroup within the Fe-type enzymes. All have been shown to display a unique photoreactivity, both *in vivo* and *in vitro* (Nakajima *et al.*, 1987; Nagamune *et al.*, 1990; Duran *et al.*, 1992; Cummings *et al.*, 1995; Bonnet *et al.*, 1997). The enzymes are inactive in the dark but activated upon light irradiation. Incubation in the dark inactivates the active form. Spectroscopic studies of the enzyme of *Rhodococcus* sp. N-771 indicated that the photoreactive site was an iron complex located on the α-subunit (Tsujimura *et al.*, 1996, 1997). The inactivation was found to be due to a nitric oxide (NO) molecule bound directly to the nonheme iron (Tsujimura *et al.*, 1997), which dissociates upon light irradiation causing a local conformational change of the iron center α-subunit residues and subsequent activation of the enzyme (Nagashima *et al.*, 1998). This was the first known example of

NO regulation of enzyme activity in bacteria, though at present its physiological significance remains cryptic. In keeping with the assignment of the thermophilic NHases to the Co-type enzyme group, none has shown photoactivation properties.

A

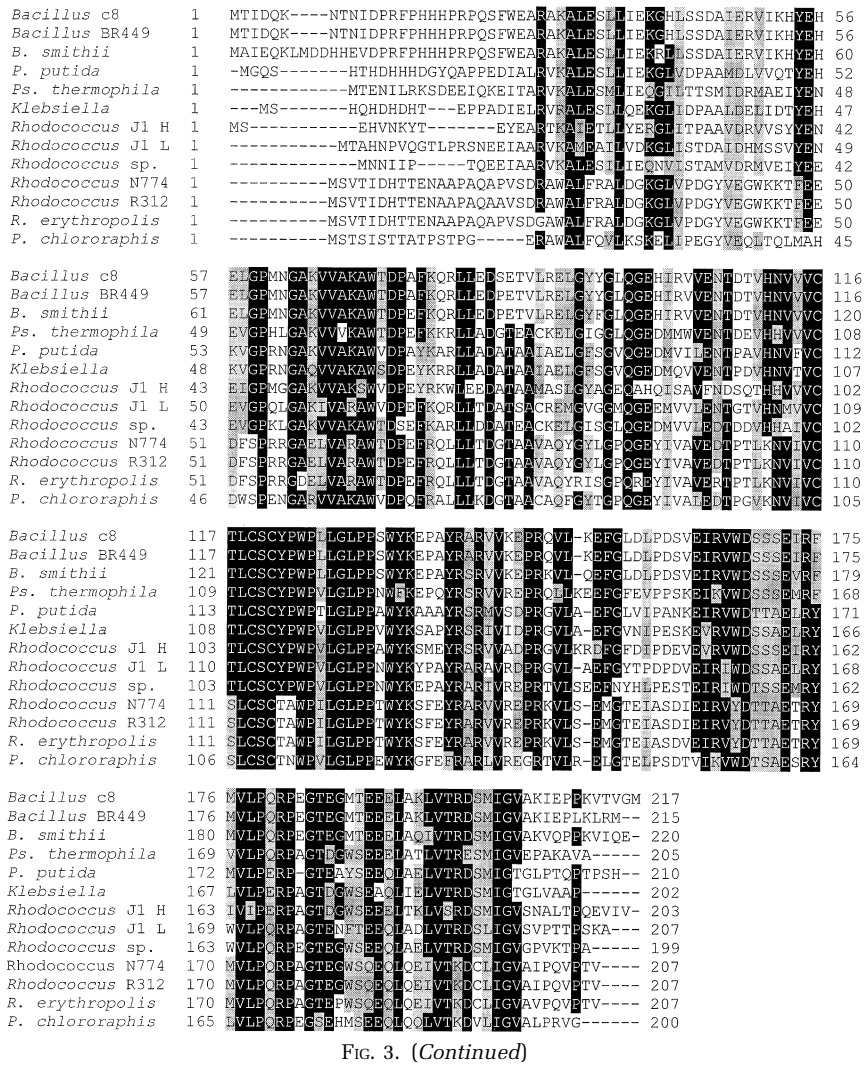

FIG. 3. (*Continued*)

B

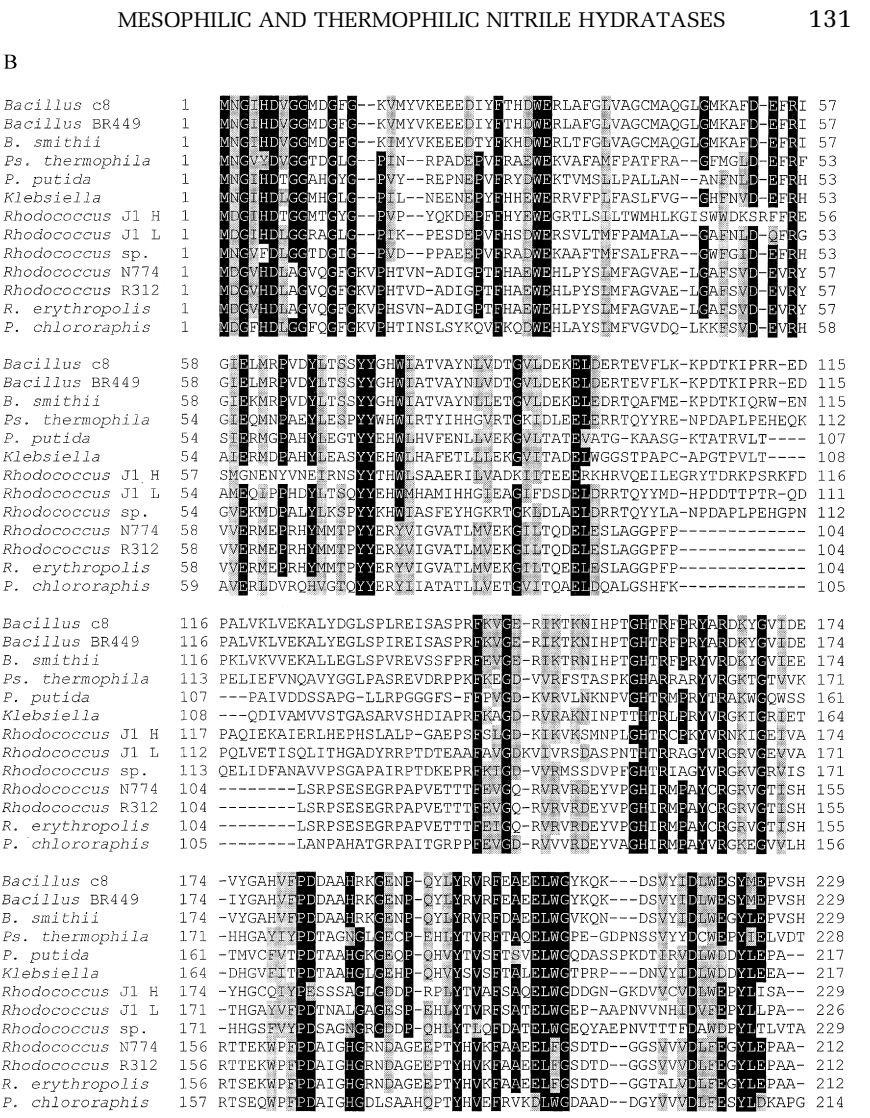

FIG. 3. Alignments of the amino acid sequences of (A) α-subunits and (B) β-subunits of nitrile hydratases from various microorganisms. The first nine sequences listed in the alignment are of Co-type enzymes, followed by the iron-containing enzymes of *Rhodococcus* N774, *Rhodococcus* R312, *R. erythropolis*, and *P. chlororaphis*. Highlighted at 60% conservation, identical residues are in black, similar residues in gray.

4. *Structure*

a. Fe-type Nhase. The structural characterization of the NHase family has, to date, been chiefly focused on the iron-NHases, particularly that of *Rhodococcus* sp. R312 NHase (Nelson *et al.*, 1991; Jin *et al.*, 1993a; Brennan *et al.*, 1996a; Scarrow *et al.*, 1996). Initial studies involving a variety of spectroscopic techniques indicated that the active site contained a six-coordinate, low-spin Fe^{3+} ion with a single oxygen, two sulfur, and three nitrogen ligands. The nitrogen ligands were concluded to be the imidazole nitrogens of histidine residues and the oxygen was thought to be an hydroxide ion (Nelson *et al.*, 1991; Jin *et al.*, 1993a).

These data have been reevaluated following the determination of the crystal structures of photoactivated *Rhodococcus* sp. R312 NHase (Huang *et al.*, 1997) and nitrosylated NHase from *Rhodococcus* sp. N-771 (Nagashima *et al.*, 1998) to 2.3 and 1.7 Å, respectively. The two structures are very similar, indicating that the conformation is conserved between active and inactive states (Nagashima, *et al.*, 1998). In each case, the crystallographic asymmetric unit is made up of two $(\alpha\beta)_2$ heterodimers. The two subunits form a tight dimer, stabilized by the N-terminal loops of each subunit that wrap around the opposite subunit; these interactions suggest that dimer formation is a dynamic rather than simple docking process (Nakasako *et al.*, 1999). The electrostatic properties of the interfacial surfaces of each subunit are quite different; Nakasako *et al.* (1999) proposed that up to 50 hydration water molecules that are densely distributed over the negatively charged β-subunit surface may aid in dimer stabilization by moderating this charge and hence decreasing the free energy in heterodimer association.

The iron center is located in a central cavity formed by the subunit interface. All protein ligands to the iron are provided by the α-subunit. In accordance with the earlier spectroscopic data (Nelson *et al.*, 1991; Jin *et al.*, 1993b; Brennan *et al.*, 1996a; Scarrow *et al.*, 1996) the ligands were found on five vertices of an octahedron. However, ligands identified from the crystal analysis were not consistent with the 2S, 3N, and O prediction (Nelson *et al.*, 1991; Jin *et al.*, 1993a) and no histidine residues were found within the cavity. In fact, the ligands came from three cysteine thiolates (αC110, αC113, and αC115) and two main-chain nitrogen atoms (αS114 and αC115). Huang *et al.* (1997) reported that the sixth position was apparently unoccupied. This is possibly attributable to the low resolution of the structure, as ENDOR analysis of the R312 enzyme has indicated that the ligand could be an hydroxide ion

(Mascharak, 2002), whereas Nagashima *et al.* (1998) reported an NO ligand at this position (reflecting the difference between the active and inactive states). The residues αC112 and αC114 of *Rhodococcus* sp. N-771 are posttranslationally oxidized to cysteine sulfinic (–SO_2H) and sulfenic acids (–SOH), respectively, in the NO inactivated enzyme. The photoactivated N-771 enzyme has also been recently crystallized (Odaka *et al.*, 2001) and interestingly αC114 existed as cysteine sulfinic acid, supposedly due to the instability of cysteine sulfenic acid. The activity of the enzyme in the crystal was reduced to only 0.5% of that of the native enzyme, indicating that this residue is involved in the catalytic mechanism of the enzyme (Kobayashi and Shimizu, 1998). Despite having an identical amino acid sequence and photoreactive behavior, the crystal structure of *Rhodococcus* sp. R312 NHase does not display such modifications. This difference has been attributed to the lower structural resolution achieved in this study rather than structural differences with the enzyme from *Rhodococcus* sp. N-771 (Nagashima *et al.*, 1998). The NO molecule is thought to be stabilized by the oxygen atoms of the three cysteines, which protrude into the iron center in a format termed a ''claw setting'' (Nagashima *et al.*, 1998). Light irradiation is thought to break the Fe–N (NO) bond and induce a structural change in the claw setting. αC113 and αC115 form hydrogen bonds with βR56 and βR141, which are conserved through all known NHases (Huang *et al.*, 1997; Nagashima *et al.*, 1998). Mutation of these residues results in loss of activity, suggesting that they are involved in the stability of the claw setting and that this setting is important for NHase activity (Piersma *et al.*, 2000). A channel from the bulk solvent to the active center is formed by the subunit interface, involving five residues from the α-subunit and four from the β-subunit. A cluster of 10 aromatic residues is found around this region and is believed to stabilize the channel (Nagashima *et al.*, 1998). The entrance of the inactive N-771 enzyme, 10 Å from the center and only 4 Å wide, is too narrow for substrates to enter. It is suggested therefore that dynamic structural changes, as are observed during light activation, are required to open and close the channel during the catalytic cycle.

b. Co-type Nhase. Until recently, little structural information was available concerning Co-type NHases. Electron paramagnetic resonance (EPR) and extended X-ray absorption fine structure (EXAFS) spectroscopy on the H-NHase of *R. rhodochrous* J1 indicated that the ligand environment was very similar to the Fe-type NHases (Brennan *et al.*, 1996b). The enzyme was found to contain a noncorrin Co^{3+} ion

with a mixed S and N octahedral ligand field. However, these studies ruled out the possibility of NO coordination to the cobalt ion. The –V–C–(T/S)–L–C–S–C– sequence that makes up the cofactor liganding part of the active site is highly conserved among all NHases, the single difference being that Co-type NHases have threonine in the third position, whereas the Fe-type NHases have serine. It is possible, therefore, that the residue found in this position is the major determinant of cofactor type (Kobayashi and Shimizu, 1998). The crystal structure of *Ps. thermophila* NHase, a thermostable Co-type NHase, was published very recently (Miyanaga *et al.*, 2001). Resolved to 1.8 Å, the structure exhibited a very high similarity to the Fe-type NHase. The structure around the active center is almost identical, and the cofactor ligands αC111 and αC113 were posttranslationally modified to cysteine sulfinic and sulfenic acids to form a claw setting similar to that of the Fe-type NHase. The posttranslational modification of the second cysteine ligand to sulfinic acid has recently been reported to also be conserved in *R. rhodochrous* J1 H-NHase, although no data are yet available concerning the third cysteine ligand (Hashimoto *et al.*, 2002).

In the Co-type NHases, the major deviation from the Fe-type structures involves the residues between β95 and β138. The central portion of this region corresponds in alignments (Fig. 3A) to an insertion in the Co-type NHases (cf. the Fe type) of between 12 and 21 residues (20 residues in the *Ps. thermophila* enzyme). The *Ps. thermophila* NHase structure demonstrates that this region comprises an α-helix (residues β111–β125), which interacts with a second α-helix on the external surface of the α-subunit. It has been proposed that this interaction contributes to the thermostability of the *Ps. thermophila* enzyme (Miyanaga *et al.*, 2001). However, it should be noted that similar sequences are also found in the Co-type NHases of *Rhodococcus* sp. (Mayaux *et al.*, 1991) and both *R. rhodochrous* J1 NHases (Kobayashi *et al.*, 1991), which are not particularly thermostable enzymes (Table II). It is also noticeable that an active site residue (βW72) of the *Ps. thermophila* enzyme (conserved in the Co-type NHases) is oriented in a different direction to βY76 of *Rhodococcus* sp. N-771 (conserved in Fe-type NHases) (Nagashima *et al.*, 1998). This difference in orientation leads to a larger space in the substrate-binding site, which may account for the general differences in substrate specificities of the two groups (Miyanaga *et al.*, 2001).

Recent studies with the NHase of *Rhodococcus* sp. N-771 have revealed that the enzyme showed activity when the iron cofactor was substituted with cobalt ions, though oxidation of both the cobalt(II) to

TABLE II

THERMAL STABILITY OF VARIOUS NHASES[a]

Microorganism	Temperature (°C)	Incubation time	% Activity remaining[b]	Reference
Bacillus sp. BR449[c]	60	2 h	100	Padmakumar and Oriel (1999)
Bacillus sp. RAPc8[c]	50	2.5 h	50	Pereira *et al.* (1998)
	60	16 min	50	
B. pallidus DAC521[c]	50	51 min	50	Cramp and Cowan (1999)
	60	7 min	50	
Bacillus smithii[c]	55	1.5 h	50	Takashima *et al.* (1998)
Ps. thermophila[c,d]	50	2 h	100	Yamaki *et al.* (1997)
	60	2 h	90	
P. chlororaphis[e]	20	10 min	100	Nagasawa and Yamada (1987)
	35	10 min	53	
P. putida[f]	50	10 min	0	Fallon *et al.* (1997)
P. putida[g]	50	20 min	60	Payne *et al.* (1997)
Rhodococcus sp. N-774	30	30 min	100	Nagasawa and Yamada (1995)
R. rhodochrous J1 H NHase[h]	50	30 min	100	Nagasawa *et al.* (1991)
	60	1 h	50	Kobayashi *et al.* (1992)
R. rhodochrous J1 L-NHase	30	30 min	100	Wieser *et al.* (1998)

[a] Stability is defined by remaining activity following incubation for a specific time period at a specific temperature.
[b] Where 100% is given as remaining activity, authors reported that activity levels dropped dramatically above those temperatures.
[c] Denotes thermophilic organisms.
[d] Stabilized in 34 m*M* *n*-butyric acid.
[e] Stabilized in 22 m*M* *n*-butyric acid.
[f] Whole-cell assay.
[g] Stabilized in 40 m*M* Na-butyrate.
[h] Stabilized in 44 m*M* *n*-butyric acid.

Co^{3+} and αC113 to the cysteine sulfinic acid was essential for this activity (Nojiri *et al.*, 2000). This study supports the hypothesis that Co- and Fe-type NHases share very similar structures and, presumably, reaction mechanisms.

B. Reaction Mechanism

A number of mechanisms have been proposed for the hydrolysis of nitriles by NHases (Nagasawa *et al.*, 1986; Huang *et al.*, 1997; Kobayashi, 1997; Odaka *et al.*, 2001). Based on the effects of addition of substrate or competitive inhibitors on the EPR and resonance Raman spectra, it is thought that the metal ion is involved as a Lewis acid in catalysis (Huang *et al.*, 1997).

Three reaction models involving the metal ion as a Lewis acid have been proposed (Huang *et al.*, 1997). (1) The nitrile substrate binds directly to the metal ion, thereby activating it for hydration. The nitrile carbon atom is then subjected to nucleophilic attack by a water

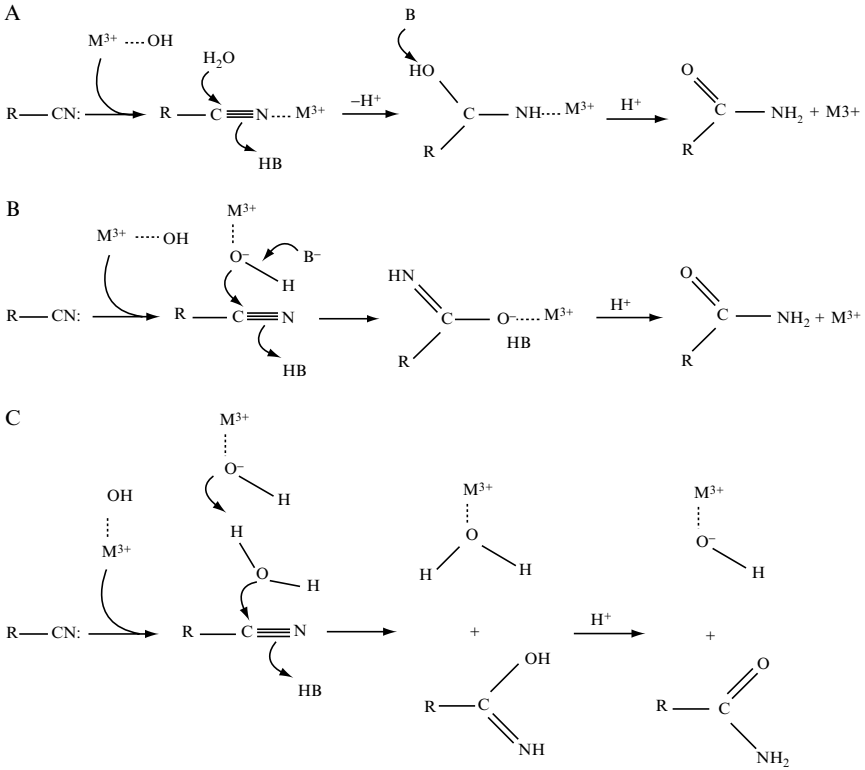

Fig. 4. Three putative reaction mechanisms of NHase: (A) direct association of nitrile to metal cofactor (inner sphere model), (B) nucleophilic attack by a metal-bound hydroxide ion (second inner sphere model), and (C) nucleophilic attack by an activated water molecule (outer sphere model) (redrawn from Huang *et al.*, 1997).

molecule held in place by a proximal residue (Tyr-72 has been indicated) (Fig. 4A). (2) The nitrile carbon atom of the substrate is subjected to nucleophilic attack by a metal-bound hydroxide ion (Fig. 4B). (3) The final mechanism does not involve an enzyme-bound intermediate, thus avoiding ligand exchange. A metal-bound hydroxide activates a water molecule, which then acts as the nucleophile, attacking the nitrile carbon atom (Fig. 4C).

Early hypotheses suggested that one of the conserved cysteine thiolates acted as a nucleophile, followed by hydrolysis of the resulting thioimidate (Kopf *et al.*, 1996). These have largely been discarded following the observation that those residues are coordinated to the metal ion (Huang *et al.*, 1997; Nagashima *et al.*, 1998; Miyanaga *et al.*, 2001). Due to the slow rates of ligand exchange for trivalent metals, in particular for Co^{III}, it has been argued that mechanism (3) is the most likely since this does not involve ligand exchange (Huang *et al.*, 1997). Furthermore, although the exchange kinetics of low-spin Fe^{III} and Co^{III} are very different, the observation that the rates of hydration of certain nitriles are very similar between some Co-type and Fe-type NHase has been interpreted as an indication that mechanism (3) is used (Mascharak, 2002) (although there is currently no empirical evidence for this mechanism).

However, in the *Rhodococcus* sp. R312 NHase crystal structure (Huang *et al.*, 1997), the substrate analogue iodoacetonitrile is directly associated with the metal ion, replacing the solvent derived hydroxide ligand (Doan *et al.*, 1999). Additionally, rapid ligand displacement has been demonstrated in synthetic models of both Co^{III} (Shearer *et al.*, 2001) and Fe^{III} (Schweitzer *et al.*, 2002) NHase. The enhanced ligand dissociation rates have been attributed to a strong labilizing effect of *trans* thiolates in the synthetic model (Shearer *et al.*, 2001): NHase has a cysteinate *trans* to the substrate cavity, which may facilitate catalysis through a similar mechanism. Very recently, it was demonstrated with a synthetic model of Fe^{III} NHase with similar spectroscopic properties to Fe-type NHase that Fe^{III} in an NHase-type ligand environment could reversibly bind nitriles (Shearer *et al.*, 2002). Thus, the data amassed to date suggest that the direct association model is the most likely mechanism for NHase catalysis.

The post-translational modifications of the active site cysteine ligands to cysteine-sulphinic ($-SO_2H$) and sulphenic acids (–SOH) have also been suggested to be important for catalysis. Murakami *et al.* (2000) reconstituted unmodified recombinant *Rhodococcus* sp. N-771 α and β NHase subunits under argon. Although the resultant enzyme initially displayed no NHase activity, subsequent aerobic incubation

was found to result in the induction of the αC112-SO_2H and αC114–SOH modifications and recovery of activity (Murakami *et al.*, 2000). Furthermore, the crystal structure of the same enzyme with a substrate analogue, cyclohexyl isocyanide has also been recently determined (Nojiri *et al.*, 2001). In this structure, it was found that the analogue bound directly to the iron cofactor, and that the hydroxyl group of αC114–SOH apparently disappeared. However, it is not yet clear if the group was actually removed from or disordered around the sulfur atom of αC114.

C. Specificity

The general trend for substrate specificity among the NHases characterized to date seems to be almost exclusively aliphatic. However, some enzymes with activity on aromatic nitriles have been described (Tables I and III). Although it has been claimed that the iron-containing NHases are specific for aliphatic nitriles, whereas the cobalt-containing NHases preferentially hydrate aromatic nitriles (Kobayashi and Shimizu, 1998; Miyanaga *et al.*, 2001), this is not supported by a review of specificity (Table III). The Fe-type NHase of *R. erythropolis* showed broad substrate specificity, and was capable of hydrating aliphatic, aromatic, and heterocyclic nitriles (Duran *et al.*, 1993), whereas the Co-type enzymes of *Bacillus smithii, Bacillus pallidus* DAC521, *Bacillus* sp. RAPc8, and *P. putida* demonstrate no significant activity on (homo)aromatic nitriles (Payne *et al.*, 1997; Pereira *et al.*, 1998; Takashima *et al.*, 1998; Cramp and Cowan, 1999).

It has been suggested that the lack of aromatic specificity in Fe-type NHases is due to the narrow entrance channel from the bulk solvent, which enables only small, aliphatic molecules to enter the catalytic site (Nakasako *et al.*, 1999). A second hypothesis involving the spatial arrangement of the catalytic cavity suggests that a larger cavity caused by the displacement by a leucine residue and a phenylalanine residue of βW72 of *Ps. thermophila* NHase (in comparison to the corresponding βY76 of *Rhodococcus* sp. N-771) may be responsible for the differences in substrate preferences between Co-type and Fe-type NHases.

Analysis of the substrate specificity of *Bacillus* sp. RAPc8 NHase showed that although the enzyme demonstrated no activity on homoaromatic nitriles such as benzonitrile and benzyl cyanide (Pereira *et al.*, 1998), the similarly bulky heteroaromatic nitrile, 3-cyanopyridine, was accepted as a substrate (Cameron *et al.*, 2003). These results indicate that for this enzyme, lack of activity toward the homoaromatic

TABLE III

COMPARISON OF SUBSTRATE SPECIFICITIES OF VARIOUS NHASES

Substrate	Relative activity[a] for *Bacillus* sp. RAPc8	*B. pallidus* DAC521[b]	*R. rhodochrous B. smithii*	*R. rhodochrous* J1 H-NHase	*R. rhodochrous* J1 L-NHase	*Rhodococcus* sp. YH3-3	*R. erythropolis*	*P. chlororaphis*
Acetonitrile	100	28	540	605			5	2
Chloroacetonitrile	43	–	–	592	100		20	31
Acrylonitrile	67	37	390	476	15	156	45	81
Propionitrile	32	19	100	432	22	278	15	100
Methacrylonitrile	64	37	–	86	21	322	–	15
Butyronitrile	69	26	290	350	54	449	100	77
Isobutyronitrile	59	18	23	1	2	56.3	11	0.1
Valeronitrile	108	23	240	3	–	654	–	3
Isovaleronitrile	56	0	–	0	–		–	0
cis, *trans*-Crotonitrile	50	38	23	78	5	81.4	–	0
	32	56						
Benzyl cyanide	0	–	–	13	–		–	0
Benzonitrile	0	0	1	68	17	226	46	0
3-Cyanopyridine	53	–	4	100	11	100	55	–
Malononitrile	7	0	–	–	–		–	–
Glutaronitrile	32	0	–	–	–		–	–
Adiponitrile	29	0	7	–	–		–	–

[a] Data from Pereira *et al.* (1998), Cramp and Cowan (1999), Takashima *et al.* (1998), Nagasawa *et al.* (1991), Wieser *et al.* (1998), Kato *et al.* (1999), Duran *et al.* (1993), and Nagasawa and Yamada (1987).

[b] Specificity of *B. pallidus* DAC521 NHase expressed as percentage substrate (50 m*M*) initial concentration utilized over a 30-min hydrolysis period.

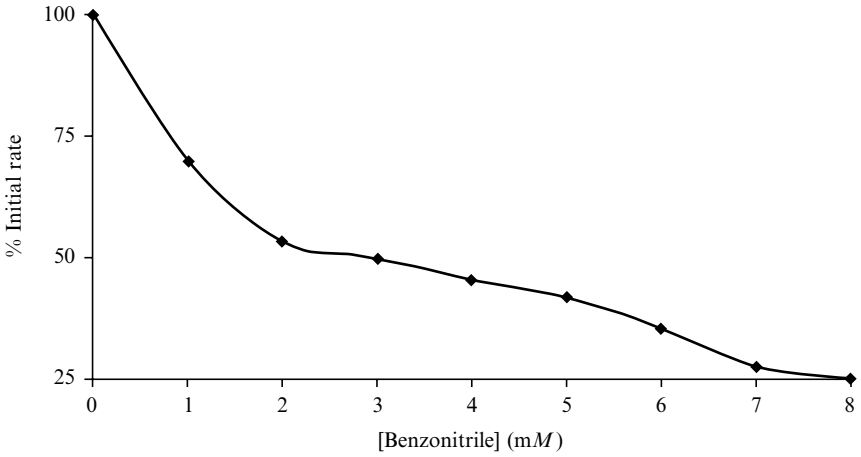

FIG. 5. Benzonitrile inhibition of acetonitrile hydrolysis by semipure recombinant *Bacillus* sp. RAPc8 NHase.

nitriles is probably not associated with catalytic cavity volume. Furthermore, we have also observed that acetonitrile-degrading activity of RAPc8 NHase, as with *Bacillus* DAC521 NHase (Cramp and Cowan, 1999), is competitively inhibited by benzonitrile (Fig. 5). Based on these observations, our working hypothesis is that the lack of activity on aromatic substrate analogues was due to aromatic interaction between the substrate and aromatic residues within the active site cleft. We are currently investigating this hypothesis through site-specific replacement of key active-site aromatic residues.

Several NHases with very broad substrate ranges have been described. *Bacillus* sp. RAPc8 demonstrated activity only on aliphatic nitriles but catalyzed the hydration of branched, linear, cyclic-aliphatic, and dinitriles. The highest affinity recorded was for acetonitrile, with a K_m value of 8.8 m*M* (Pereira *et al.*, 1998). Both the L-NHase and H-NHase of *R. rhodochrous* J1 have broad substrate ranges, with similar K_m values for the aliphatic nitriles (e.g., for acrylonitrile, K_m, values of 2.67 and 1.89 m*M* and specific activities of 828 and 1760 U/mg for L-NHase and H-NHase, respectively). However, the affinity and activity of L-NHase for aromatic nitriles was significantly higher than H-NHase (Wieser *et al.*, 1998).

In contrast to the general consensus that NHases lack enantioselectivity (which was often found in the coexpressed amidase), there have been several reports of NHases with enantioselectivity. *Agrobacterium*

tumefaciens d3 NHase displayed activity on aromatic and heterocyclic nitriles and produces (*S*)-amides enantioselectively (>90% enantiomeric excess) from a number of racemic phenylpropionitriles (Bauer *et al.*, 1994). *P. putida* NHase also produced (*S*)-amides from racemic nitriles. For example, racemic 2-(4-chlorophenyl)-3-methylbutyronitrile was converted to the (*S*)-amide at >90% enantiomeric excess (Fallon *et al.*, 1997).

D. Thermostability

Most NHases are particularly thermolabile, although it has been demonstrated that *n*-butyric acid acts as a potent stabilizing agent for some NHases by preventing oxidation of the modified cysteine sulfenic acid residue to cysteine sulfinic acid (Nagasawa and Yamada, 1987; Odaka *et al.*, 2001). There appears to be a correlation between stability and cofactor, with Co-type NHases being generally more stable than Fe-type (Table II). In the past 5 years, at least five NHases from moderate thermophiles have been characterized, four of which are thermophilic *Bacillus* isolates, the fifth being a *Ps. thermophila* strain. With the exception of *B. pallidus* DAC521 NHase (for which the cofactor is not known), all are Co-type enzymes.

Due to the different approaches of various researchers in determining and reporting thermal stability, a direct comparison between the enzymes is not straightforward. Nevertheless, Table II outlines the stability of some of the better characterized enzymes.

The remarkable similarity in structural and functional characteristics between the NHases, particularly among the Co-type enzymes, is preserved within the thermostable representatives of the family (Cowan *et al.*, 1998). As yet, there are no definitive determinants of the increased stability of the thermostable NHases. It has been suggested that an interaction between two α-helices that is present in the crystal structure of *Ps. thermophila* NHase but not in those of the two Fe-type NHase structures (Huang *et al.*, 1997; Nakasako *et al.*, 1999) may contribute to the thermostability of this enzyme (Miyanaga *et al.*, 2001). However, very similar sequences corresponding to this region are found in the mesophilic Co-type enzymes, indicating that this may be a characteristic of only this subgroup of enzymes. Furthermore, comparisons of the sequences of thermostable NHases with those of the mesophiles do not highlight significant differences in amino acid distributions (Kumar *et al.*, 2000), nor could any such differences be readily attributed to differences in thermal stability. Given that the thermostable NHases described to date are only moderately more stable

than their mesophilic homologues, it is likely that this additional stability may be accounted for by only a few additional intramolecular interactions.

III. Molecular Biology of NHases

A. Gene Structure

Over the past 13 years, the genes for many of the NHases described in the literature have been cloned. In addition, the recent completion of several microbial genomes has revealed probable NHase genes in two legume symbionts: *Mesorhizobium loti* (NCBI Microbial Genomes Annotation Project) and *Sinorhizobium meliloti* (Capela *et al.*, 2001). In each case the α- and β-subunits were coded within two separate, adjacent open reading frames (ORF) (Fig. 6), separated by sequences of between 16 and 29 bases (Duran *et al.*, 1993; Kim and Oriel, 2000), except in *Ps. thermophila*, whose the genes overlapped by 4 bp (Yamaki *et al.*, 1997). Typically, the α-subunit ORF was located upstream of the β-subunit ORF. However, in *R. rhodochrous* J1, *Ps. thermophila*, *Bacillus* sp. BR449, *Bacillus* sp. RAPc8, and *B. smithii*, the β-subunit gene precedes the α-subunit gene.

The α- and β-subunit genes are of similar size and range from 609 to 660 bp for the α-subunit and from 636 to 706 bp for the β-subunit. With the exception of the *R. rhodochrous* J1 genes, all NHases characterized to date are positioned approximately 100 bp downstream of an amidase gene. No amidase sequence has been found close to the H-NHase genes of *R. rhodochrous* J1, whereas an amidase gene was found 1.9 kb downstream of the α-subunit gene of the L-NHase gene (Kobayashi *et al.*, 1992). In the case of the H-NHase operon, an insertion sequence (IS1164) was found upstream of the α- and β-subunit genes instead of an amidase gene (Kobayashi *et al.*, 1997a; Komeda *et al.*, 1996b). It is possible that the unusual structure of this operon is due to a rearrangement of the gene cluster by IS1164 and that this insertion sequence is responsible for the occurrence of two NHase operons in *R. rhodochrous* J1 through duplication of one of the gene clusters.

With respect to the gene structures of the thermophilic NHase-producing organisms, other than the arrangement of the α- and β-subunit genes, little information is available regarding the layout of the NHase operons of *B. smithii* (Takashima *et al.*, 1996) or *Ps. thermophila* (Yamaki *et al.*, 1997). The cloned NHase operon sections of *Bacillus* sp. BR449 (Kim and Oriel, 2000) and *Bacillus* sp. RAPc8

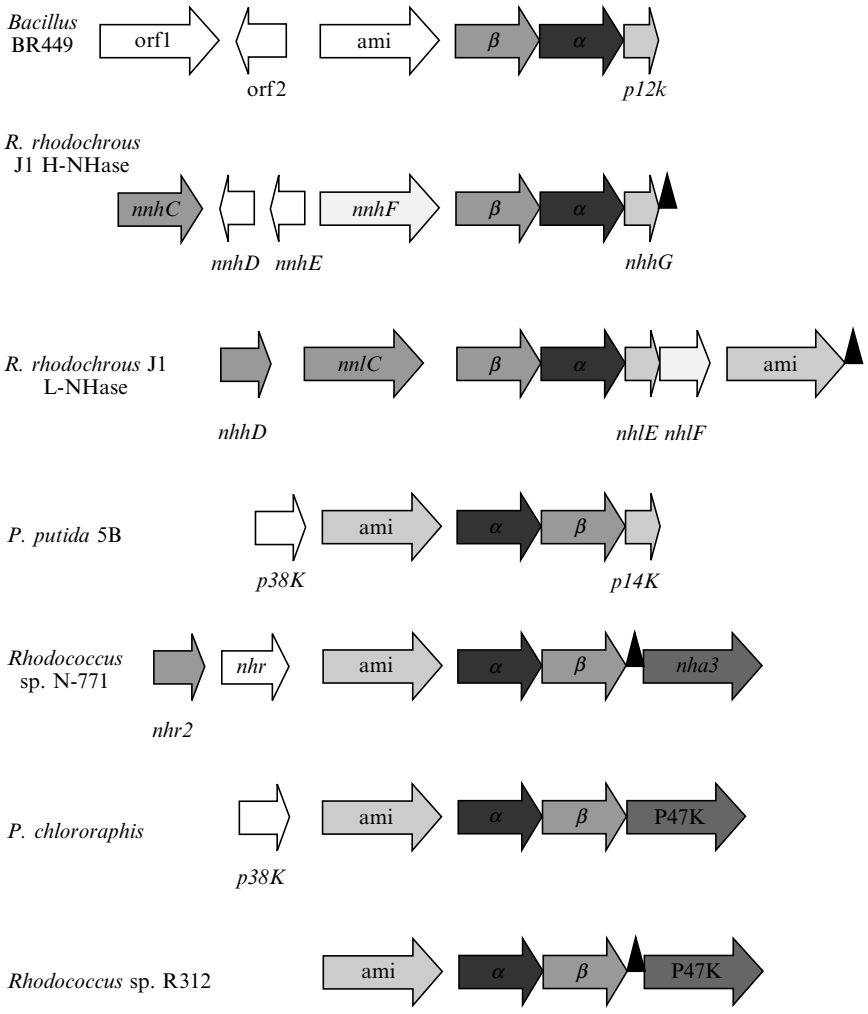

FIG. 6. Arrangement of the gene clusters from various NHase-producing organisms. The direction and (approximate) sizes of the genes are indicated by arrows; homologous genes are indicated by the same color shading. Stem-loop structures that serve as potential transcriptional terminators are shown as black triangles.

(Cameron and Cowan, 2003) span opposing sections of the locus. Nevertheless, a comparison of the thermophile NHase genes reveals consistencies in their structure and organization: all are arranged in β–α order and all contain the VCTLCSCY motif in the α-subunit,

characteristic of Co-type NHases. This second observation would seem to support the proposal that the Co-type enzymes are generally more thermostable than their Fe-type counterparts (Kobayashi and Shimizu, 1998).

Interestingly, the NHase-associated amidases of the thermophilic organisms *Bacillus* sp. BR449 and *Bacillus* sp. RAPc8, unlike their mesophilic counterparts, belong to the nitrilase-related amidase family despite the structural similarities of the NHase operons (Fig. 6) with those of the mesophiles (to the authors' knowledge, there are currently no publicly available sequences for the NHase-associated amidases of *B. smithii* and *Ps. thermophila*). Bacterial aliphatic amidases (broadly classed as acylamide amidohydrolase, EC 3.5.1.4) are made up of two types (Chebrou *et al.*, 1996; Fournand and Arnaud, 2001): the nitrilase-related amidase family and the enantioselective amidases (which show an evolutionary relationship with aspartic proteinases) (Kobayashi *et al.*, 1997b). With the exception of those of *Bacillus* sp. BR449 and *Bacillus* sp. RAPc8, all NHase-coupled amidases belong to the second family. Hence the anomaly presented by the amidases of these two *Bacillus* species would seem to suggest that in this case, the NHase operon was assembled from genes preexisting within the organism's genome rather than being the result of horizontal gene transfer as has previously been suggested (Chebrou *et al.*, 1996; Pereira *et al.*, 1998).

B. Gene Regulation and Expression

1. *Inducibility of NHase Production*

The control of production of active NHase in the native organisms varies considerably in different organisms. Some NHases are produced constitutively, irrespective of the presence or absence of nitriles or amides, whereas others have been shown to be inducible. There appears to be no correlation between the mode of production and cofactor type (Table I). Early studies showed that the inducible NHases, as with their associated amidases, are generally induced by their amide reaction products rather than by nitriles (Kobayashi and Shimizu, 1998) [although *Corynebacterium pseudodiphteriticum* ZBB-41 and *Rhodococcus* sp. YH 3-3 NHases are induced by both nitriles and amides (Li *et al.*, 1992; Kato *et al.*, 1999)]. However, the recent large-scale investigation of the distribution of aldoxime dehydratases has demonstrated that aldoximes are good inducers for nitrile-hydrolyzing enzymes as well as aldoxime dehydratase (Kato *et al.*, 2000a).

Nevertheless, it is not yet clear whether aldoxime dehydratase and the NHase/amidase systems are coregulated and therefore if this is a direct induction, or if the nitrile reaction product of aldoxime dehydratase causes the induction of the nitrile-degrading system. These observations, together with the close location of the structural genes and the absence of transcriptional terminators between them, indicate that amidases and NHases are under the control of the same regulatory elements (Bigey *et al.*, 1999).

The regulation of the *R. rhodochrous* J1 genes has been particularly well characterized (reviewed in Kobayashi and Shimizu, 1998). Both H-NHase and L-NHase are induced by crotonamide. However, urea or cyclohexanecarboxamide induces either H-NHase or L-NHase genes, respectively (Yamada and Kobayashi, 1996). The H-NHase operon contains two regulatory genes, *nhhC* and *nhhD*, both located upstream of the NHase genes. NhhC, homologous to the negative regulator AmiC of the *Pseudomonas aeruginosa* aliphatic amidase, exhibits a positive regulation, in the presence of amide, of NhhD production. The presence of NhhD then induces H-NHase expression (Kobayashi and Shimizu, 1998). The 5′ upstream region of the L-NHase operon is also required for L-NHase expression; two genes, *nhlD* and *nhlC*, serve positive and negative regulatory roles, respectively. In the presence of amide, NhlC (also an AmiC homologue) inhibits NhlD repression of L-NHase production (Komeda *et al.*, 1996a).

The formation of the thermostable NHases appears to be constitutive, although no data are available on the control of production of *Ps. thermophila* NHase, and although expression of *B. smithii* NHase is not induced by nitriles or amides, it is inhibited by ammonia (Takashima *et al.*, 2000). Similarly the constitutive production of *B. pallidus* NHase is apparently repressed on benzonitrile induction of the nitrilase (Cramp, 1997).

2. *Transcript Production*

The colocation of the NHase and amidase genes in nitrile-metabolizing organisms supports the hypothesis that these enzymes are involved in a two-step reaction in the nitrile degradation pathway. No definitive transcription terminators have been found between the amidase, α or β genes in any of the NHase gene clusters described so far: stem-loop structures that may act as transcription terminators have been identified immediately downstream of the β-subunit genes of *Pseudomonas chlororaphis* (Nishiyama *et al.*, 1991) and *Rhodococcus* sp. R312 (Bigey *et al.*, 1999). These data indicate that the amidase and nitrile hydratase genes are coexpressed from a single polycistronic mRNA, often

including additional flanking genes. This hypothesis has been confirmed for the NHase operon of *Rhodococcus* sp. R312 through transcriptional analysis of the operon of a mutant derivative, *Rhodococcus* sp. ACV2 (Bigey *et al.*, 1999). However, transcript analysis of *R. rhodochrous* M8 revealed that the NHase mRNA was only ~2 kb in size—precluding the possibility of expression of NHase and amidase as a single polycistronic mRNA (Pogorelova *et al.*, 1996). Thus it appears that there are significant differences in the production and regulatory mechanisms used by different NHase-producing organisms, reflecting the diversity of these microorganisms and supporting the premise that NHase operons are assembled from preexisting genes within the organism's genome. Following the molecular characterization of the *Bacillus* sp. BR449 NHase operon, Kim and Oriel (2000) hypothesized that the spacing between the NHase and amidase genes, and the significant heterologous NHase expression in reverse orientation to the vector promoter, implicated an unidentified promoter upstream to the NHase genes. Such a promoter could facilitate expression of the NHase genes independently of the amidase. Although no promoter elements were detected within the cloned NHase locus of *Bacillus* sp. RAPc8 (Cameron and Cowan, unpublished results), the validity of either hypothesis can be irrefutably demonstrated only by transcript analysis.

3. *Cofactor Requirements*

There are conflicting results concerning the effect on NHase production of supplementation of growth media with cofactor ions. Although no information appears to be available on the effect of supplementing the growth media of Fe-type NHase producers with Fe^{2+} ions, experiments have shown that the specific activities of *Bacillus* sp. BR449, *B. smithii*, and *R. rhodochrous* J1 NHases are significantly enhanced by addition of 0.1–0.5 m*M* $CoCl_2$ to the growth media (Kobayashi *et al.*, 1991; Takashima *et al.*, 1998; Padmakumar and Oriel, 1999). Investigations with recombinant *R. rhodochrous* J1 H-NHase have shown that cobalt is required for activity rather than protein expression (Kobayashi *et al.*, 1991; Komeda *et al.*, 1996a). Similarly, recombinant *Bacillus* sp. BR449 NHase is expressed in a largely inactive form in the absence of cobalt ions but can be activated upon incubation in 5 μ*M* $CoCl_2$ at 50°C (Kim and Oriel, 2000; Kim *et al.*, 2001). These findings suggest that cobalt is involved in enhancing protein folding rather than induction of NHase expression (Kobayashi *et al.*, 1991, 1992; Cowan *et al.*, 1998). Conversely, northern blot analysis of *R. rhodochrous* M8 RNA has shown that supplementation of the

growth medium with cobalt ions significantly increases the level (by at least one order of magnitude) of mRNA for NHase in comparison with cells grown in cobalt-limiting conditions, although it is not know whether this increase is due to an up-regulation of transcript production or to rapid degradation of NHase mRNA (Pogorelova *et al.*, 1996).

4. *Recombinant Expression and Activator Proteins*

Many early attempts to express cloned NHase genes were ineffective, often resulting in the production of insoluble and inactive inclusion body protein (Ikehata *et al.*, 1989; Mizunashi *et al.*, 1998). Successful heterologous expression was achieved through the development of host-vector systems in *R. rhodochrous* strains for the expression of recombinant *Rhodococcus* NHases (Hashimoto *et al.*, 1992) and the discovery that overexpression of active protein occurred when additional ''activator proteins'' were coexpressed from genes flanking the NHase genes (Nishiyama *et al.*, 1991; Hashimoto *et al.*, 1994).

Overproduction of *P. putida* NHase was found to require coexpression of P14K, a 14-kDa protein with homology to the N-terminal region of the NHase β-subunit, the gene for which is found immediately downstream of the NHase β-subunit (Wu *et al.*, 1997). Similar proteins, also with β-subunit homology, have been found downstream of the Co-type NHase genes of *Bacillus* sp. BR449 and *R. rhodochrous* J1 (Komeda *et al.*, 1996a,b; Kim and Oriel, 2000). Efficient production of recombinant expression of Fe-type NHases of *P. chlororaphis*, *Rhodococcus* sp. N-771, and *Rhodococcus* sp. R312 has been found to require the coexpression of genes encoding homologous 47-kDa proteins, located downstream of the NHase genes (Nishiyama *et al.*, 1991; Bigey *et al.*, 1999; Nojiri *et al.*, 1999). It is noteworthy that the β-homologue proteins are associated with the Co-type NHases, whereas the P47K proteins are Fe-type associated.

Although the precise function of these proteins is not known, it has been suggested that they are involved in incorporation of the cofactor ion into the active site (Bunch, 1998). The observation that their presence is required for the expression of constitutively produced enzymes (Bigey *et al.*, 1999; Nojiri *et al.*, 1999) indicates that they are not involved in regulation of expression. Studies of the *P. putida* P14K protein have shown that it is expressed at a very low level, leading Wu *et al.* (1997) to postulate that it serves as a chaperone or acts in a catalytic capacity. Furthermore, it has been shown that cobalt is required for activity rather than for expression or correct folding and that its incorporation into the enzyme is not passive. The chaperone/catalyst

hypothesis might therefore indicate that the β homologues are actively involved in the integration of cobalt into the active site.

Although genes coding for β-subunit homologues are found immediately downstream of the NHase genes of the thermophiles *Bacillus* sp. BR449 (Kim and Oriel, 2000) and *Bacillus* sp. RAPc8 (Cameron and Cowan, unpublished results), it was shown in each case that functional expression of NHase in *E. coli* required only the structural genes and the inclusion of cobalt ions in the growth medium. Expression of the P12K protein of *Bacillus* sp. BR449 was not detected when the *p12k* gene was included in expression constructs, neither did the gene seem to affect NHase expression or activity (Kim and Oriel, 2000). These results may indicate that the original function of the β homologues of the two *Bacilli* has been lost and is no longer required for active NHase expression.

IV. NHase Applications

A. Biotransformations

The capacity of enzyme systems to convert a cyano functionality to either an amide or to an acid is potentially valuable in the synthesis of numerous commodity and speciality chemicals. However, there are additional advantages in the use of biocatalysis, including the performance of reactions under mild conditions, the avoidance of deleterious reactions to other sensitive functional groups, and the potential for both regioselectivity and stereoselectivity.

The enzymatic conversion of acrylonitrile to acrylamide is one of the more successful applications of biotechnology in commodity chemical production and has unarguably demonstrated the commercial viability of NHase (Kobayashi *et al.*, 1992; Cowan *et al.*, 1998). Acrylamide is widely used industrially in coagulators and soil conditioners, for paper treatment and paper sizing, and for adhesives, paints, and petroleum recovery agents (Yamada and Kobayashi, 1996). Prior to the introduction of the enzymatic process, a conventional chemical synthesis, using copper salts as a catalyst for the hydration of acrylonitrile, was used. However, this process was expensive and inefficient, providing the incentive to search for superior catalysts (Kobayashi *et al.*, 1992). The industrial bioconversion of acrylonitrile to acrylamide started in 1985 using the Fe-type NHase of *Rhodococcus* sp. N-774, the first-generation strain employed by the Nitto Chemical Industry, Tokyo, Japan (Nagasawa and Yamada, 1989). The catalyst for this process has been upgraded twice since that date, first, in 1988, with the Fe-type

NHase of *P. chlororaphis* B23, then again, in 1991, with the Co-type H-NHase of *R. rhodochrous* J1 (Nagasawa and Yamada, 1995). Currently, Mitsubishi Rayon Co., Ltd (Yokohama, Japan) (with whom Nitto Chemical Industry merged in 1998) produces in excess of 20,000 metric tons of acrylamide per year. The process uses a series of fixed-bed reactors containing the immobilized *R. rhodochrous* J1 cells, producing acrylamide at ~99.99% yield. Overall, productivity has increased by over 50% since initiation of the process, and costs have been reduced dramatically (Thomas *et al.*, 2002).

In 1999, Lonza Guangzhou Fine Chemicals (China) initiated production of nicotinamide (niancinamide, vitamin B_3) in a process developed by Lonza (Switzerland) (Thomas *et al.*, 2002). The process involves a four-stage chemoenzymatic synthesis, where the starting material 3-methyl-1,5-diaminopentane (a nylon 6,6 by-product) is catalytically converted into 3-picoline, which in turn is ammoxidated into 3-cyanopyridine, and then biocatalytically hydrolyzed to nicotinamide using immobilized *R. rhodochrous* J1 cells induced to produce L-NHase (Heveling *et al.*, 1998). The advantages of this process over the original chemical process include low energy usage, a low rate of emission, as well as stoichiometric conversion of high concentrations of the substrate to the nicotinamide product at a purity of over 99.5% (Heveling *et al.*, 1998; Shimizu, 2001).

A further example of an NHase-catalyzed synthesis currently in commercial operation is the production of 5-cyanovaleramide (5-CVAM) from adiponitrile (ADN), a starting material for the synthesis of a DuPont herbicide, azafenidin (Thomas *et al.*, 2002). This synthesis was also originally operated using a chemical catalyst. However, problems of low conversion rates (~25%) and significant production (~20% of converted ADN) of the undesirable by-product adipamide (ADAM), the need for solvent extraction, and unacceptable catalyst deactivation led to the development of a biocatalytic process. Immobilized *P. chlororaphis* B23 cells are now used in the hydration of adiponitrile to 5-CVAM, with 96% selectivity at a yield of 93%. The production of significant volumes of catalyst waste was an additional limitation of the chemical catalysis process. In contrast, the *P. chlororaphis* biocatalytic process produces only 0.006 kg of catalyst waste per kg of 5-CVAM product (by weight) (Hann *et al.*, 1999).

Although the three processes described above are the only commercially successful applications of nitrile-metabolizing enzyme systems, there are numerous recent publications describing other biotransformations that have potential for future exploitation. NHases showing novel stereoselectivity, regiospecificity, or substrate specificity may be

developed to produce amides that are components of pharmaceutical compounds (Kobayashi and Shimizu, 1998).

Such potential developments include the production, in conjunction with amidase, either as whole-cell systems or purified enzymic systems, of optically pure amino acids, hydroxy acids, and keto acids (Nagasawa and Yamada, 1989). For example, *Rhodococcus* sp. R312 has been shown to hydrolyze lactonitrile to DL-lactic acid (Thompson *et al.*, 1988) and the stereospecific conversion of aminopropionitrile to L-alanine by whole cells of an *Acinetobacter* sp. has also been demonstrated (Macadam and Knowles, 1985).

D-Phenylglycine amide is an intermediate in the industrial synthesis of β-lactam antibiotics. Wegman *et al.* (2001) isolated a new *Rhodococcus* sp. (closely related to the type strain of *R. globerulus*) that could successfully convert a racemic mixture of phenylglycine nitrile to D-phenylglycine amide and L-phenylglycine.

This organism harbored a nitrile-metabolizing system with a nonstereoselective nitrile hydratase and a highly L-selective amidase, capable of high levels of activity at high substrate concentrations (Wegman *et al.*, 2001). Organisms capable of aliphatic and aromatic nitrile biotransformations with similar stereochemistry have been reported previously (see, for example, Sugai *et al.*, 1997).

2-Arylpropanoic acids are an important class of antiinflammatory pharmaceutical compounds (naproxen and ibuprofen are commercially important examples). The (*S*)-enantiomer of these agents has been shown to be much more active than the (*R*)-enantiomer. Chemical methods for their synthesis involving the resolution of isomers by physical means can be costly and thus commercially undesirable. One of the strategies being developed for the preparation of optically active 2-arylpropanoic acids is the enantioselective enzymatic hydrolysis of the corresponding nitriles. The enantioselective hydrolysis of racemic (*R/S*)-naproxen nitrile to (*S*)-naproxen using *Rhodococcus* sp. C3II whole cells has been successfully demonstrated (Effenberger and Bohme, 1994). This strain constitutively expressed (*S*)-enantiomer-specific nitrile hydratase and amidase. Both *Rhodococcus* sp. C311 and *R. erythropolis* MP50 have been shown to hydrolyze a variety of other substrates in the synthesis of various 2-arylpropanoic acids (Effenberger and Graef, 1998). The two strains have complementary activities with respect to regioselective biotransformation of dinitriles and diamides, where *Rhodococcus* sp. C3II selectively produces mononitrile and monoamide derivatives whereas *R. erythropolis* MP50 preferentially forms mononitrile, monoacid, and monoamide derivatives (Effenberger and Graef, 1998). Enzymatic production of (*S*)-ibuprofen

has also been demonstrated using *Rhodococcus* sp. AJ270 (Snell and Colby, 1998).

Since its isolation, *Rhodococcus* sp. AJ270 has proved to be a versatile biocatalyst, exhibiting a very broad substrate specificity that includes aromatic, aliphatic, and heterocyclic nitriles, and has been shown to be effective in a number of useful biotransformations. These include the conversion of racemic 2-arylcyclopropane carbonitriles to enantiopure preparations of various pharmaceutically important cyclopropyl compounds (Wang and Feng, 2000) and the biotransformation of various aryl glycine nitriles to aryl glycines (Wang and Lin, 2001).

Nitrile-metabolizing enzyme systems have been reported to act on bulky hydrophobic substrates such as steroids (Kaufmann, 1999, #3044). The progestin dienogest (17α-cyanomethyl-17β-hydroxyestra-4, 9-dien-3-one) is a hormonal contraceptive derived from nortestosterone. Transformation of the cyanomethyl group at position 17α to the amide or carboxylic acid derivative has been targeted as a means of developing this compound for other indications and for generating higher potency. However, in short fermentations (2–24 h) the dominant reaction using *R. erythropolis* as the whole-cell catalyst was the aromatization of ring A, and the amide derivatives were evident only after prolonged fermentation periods (2–27 days).

Kimura *et al.* (2002) have recently reported an integrated chemoenzymatic synthesis of (*R*)-2-naphthylmethoxyacetic acid, a valuable chiral NMR reagent used in resolution of the absolute configuration of secondary alcohols. This *R. rhodochrous* strain IFO 15564-catalyzed process substantially truncates the typical synthetic pathway, where the racemate is synthesized chemically and the enantiomers separated chromatographically.

B. Bioremediation and Biodegradation

There is a significant release of synthetic nitriles into the environment, particularly via industrial wastewaters (Jallegas *et al.*, 1980; Mascharak, 2002). Because conventional methods for reducing the nitrile burden in these effluents adds significant costs to manufacturing processes, novel approaches involving low-cost bioremediation have recently been undertaken (Kobayashi and Shimizu, 1998). Two different approaches have been reported for remediation of acrylonitrile in industrial waste streams. The first describes the enzymic degradation of acrylonitrile (using either NHase or Nase) in the aqueous polymer emulsions used in raw rubber and plastic manufacture (Battistel *et al.*,

1997). The second cites the development of a stable activated sludge system, consisting of a consortium of microbial isolates (including NHase-containing bacteria), for the treatment of the toxic waste stream produced from the large-scale manufacture of acrylonitrile (Thompson *et al.*, 1988). The authors are not aware if either process has been implemented on an industrial scale.

Environmental accumulation of synthetic polymers that are largely resistant to microbial degradation is a growing cause for concern. Incorporation of natural polymers in plastics will alleviate this problem as it helps with the disintegration of plastics. Plastics synthesized by the copolymerization of acrylonitrile and starch have been successfully degraded by *Bacillus cereus*, exploiting the intracellular nitrile hydratase and oxidative pathways (Saroja *et al.*, 2000).

The authors note that there is little published evidence that thermophilic nitrile-metabolizing enzyme systems have been exploited in the development of innovative biotransformation or bioremediation processes. Given the known level of industrial interest in thermophilic enzymes in general and thermostable nitrile-metabolizing enzymes in particular, this state appears anomalous. This apparent dichotomy may be attributed to several factors: (1) the effectiveness of the ''third-generation'' *Rhodococcus* catalysts in the acrylamide and nicotinamide processes has resulted in a very high ''entry'' level for any replacement biocatalyst in these processes; (2) as has been demonstrated with numerous other enzyme systems, thermostability per see is insufficient for guaranteed commercial implementation, and other factors, including specificity, turnover, and bulk availability of the enzyme are all key requirements; and (3) the availability of thermophilic nitrile-metabolizing enzymes is relatively recent (1995) (Cramp *et al.*, 1997) and these enzymes have not yet been widely disseminated around the scientific community. These ''limitations'' notwithstanding, it is the authors' view that in the inevitable growth of successful nitrile biotransformation processes, thermostable nitrile hydratases derived from thermophilic microorganisms will play an important part.

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From Enzyme Adaptation to Gene Regulation

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From its inception, microbiology has been an ''applied'' discipline. The relationships between microbes and disease, between microbes and traditional food and agricultural practices, and between microbes and industrial processes have been the underlying motivations for much of the interest in microbiology from its inception to the present day. The diversity of the microbial world, both in terms of ecology and in terms of physiology, makes microbes exceedingly useful biological organisms. Early study of microbes in the nineteenth century, which employed the newly developed tools of chemistry and concepts of physiology, showed that microbes seemed to possess an astounding variety and plasticity in terms of their biological properties. Some microbial ''by-products'' such as alcohol and carbon dioxide had been well known for centuries; others such as specific amino acids, pigments, and later, antibiotics, were being discovered less than a century ago. The advances in the study of animal metabolism and in the chemistry of living organisms were often based on studies of the metabolic processes of microbes. It is well-known, of course, that the first cell-free conversion of sugar to ethanol was demonstrated in extracts of yeast cells in 1897 by Eduard Buchner (1897), and the importance of this demonstration for biochemistry, in general, is widely recognized (Fruton, 1999). Buchner's work showed that the inner workings of organisms might be amenable to study and analysis in the chemistry laboratory through the study of individual chemical reactions. The study of microbial biochemistry was to become a field of intense research in the 1930s, and the fundamental work leading to later successes in what has come to be called microbial genetics was part of this research program in microbial biochemistry. This chapter will briefly survey some of the main pathways of investigation in these related fields that led from bacterial nutrition to physiological genetics.

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The distinction between the biology of an *individual* microbe, be it yeast, bacterium, or protozoon, and the biology of a large *population* of microbes has evolved and clarified since the early days of microbiology. Often the entire mass culture was considered as an organism, per se, and its properties were examined in detail. Thus, the ''phases of growth'' of a culture was of intense interest (indeed, it is still a topic in beginning microbiology courses). On one hand, this research led to a now discredited belief that bacteria undergo life cycles that might include different morphological and biochemical forms: a bacillus could exhibit the morphology of a coccus depending on its growth phase, for example. This concept (cyclogeny) undermined efforts in the 1920s and 1930s to develop a reliable classification system based on fixed properties of bacterial species (Enderlein, 1925). On the other hand, the growth requirements of culture were a convenient way to study the nutritional habits of individual cells. This research on bacterial nutrition was more fruitful because it generated questions, further research, and new explanations about the physiology of bacterial cells.

One such phenomenon that grew out of the study of bacterial nutrition was called ''enzyme adaptation.'' This concept linked studies on bacterial nutrition to the eventual detailed understanding of the mechanism of environmental regulation of bacterial gene expression. This chapter will briefly review this conceptual evolution.

Turbidimetric and other methods of analysis of the biomass in liquid cultures were widely employed in the early 1900s to monitor bacterial growth. ''Growth curves'' were the stock in trade of many microbiologists. The Klett–Summerson nephelomenter (a device for measuring turbidity) was a rugged and simple instrument and was widely used in both microbiological and biochemical laboratories; indeed, experimental results were often reported directly in ''Klett units,'' which were the voltmeter readings from this instrument. With simple and reliable methods to measure growth in mass culture, it was soon discovered that bacteria had highly specific nutritional requirements: for example, some sugars supported growth, some did not; some bacteria required complex organic media, and others could be grown on a medium with only inorganic salts with a single pure organic carbon source.

Early views of bacterial metabolism embraced the diversity of microbial chemical reactions, but were inclined to view the bacterial cell ''as a system capable of performing a variety of chemical reactions in virtue of possessing a number of catalysts or enzymes characteristic of a species and invariably present'' (Stephenson, 1939, p. 301). This diversity was exploited both scientifically and industrially to use microbes to obtain a wide variety of end products as well as metabolic intermediates, e.g.,

to produce indole from tryptophan, to reduce nitrate to nitrite, and to interconvert various carbohydrates. Upon further study, however, ''the constancy of the enzymic makeup of many bacterial species is shown to be an illusion, and at least two types of variation can be disclosed'' (Stephenson, 1939, p. 301).

As early as the 1920s, the ability of bacterial cells to undergo infrequent abrupt and permanent changes in characteristics was interpreted as a manifestation of the phenomenon of mutation as had been described in higher organisms (Summers, 1991). The relation of these mutations to the growth conditions where they could be observed, was, however, unclear. In the 1930s this question was confronted directly by I. M. Lewis (1934), who studied the mutation of a lactose-negative strain of *Escherichia coli* (*Bacillus coli mutabile*) to lactose-utilizing proficiency. Lewis laboriously isolated colonies and found that even in the absence of growth in lactose, the ability to ferment this sugar arose spontaneously in about one cell in 10^5. This work was the beginning of a long line of investigations that quite conclusively showed that mutation is (almost always) independent of selection.

The second kind of adaptation, that ''due to chemical environment,'' is of special historical interest. As early as 1900, Frédéric Dienert (1900) found that yeasts that were grown for some time in galactose-containing medium became adapted to this medium and would grow rapidly without a lag when subcultured into fresh galactose medium, but that this ''adaptation'' was lost after a period of growth in glucose-containing medium. By 1930 Hennig Karström in Helsinki had found several instances of such adaptation (Karström, 1930). For example, he found that a strain of *Bacillus aerogenes* could grow on (''ferment'' to use the older term) xylose if ''adapted'' to do so, but that this strain could ferment glucose ''constitutively'' without the need for adaptation. When he examined the enzyme content of these adapted and unadapted cells, he found that there were some enzymes that were ''constitutive'' and some that were ''adaptive.'' Thus, the metabolic properties of the culture mirrored the intracellular chemistry. By experiments in which the medium was changed in various ways, Karström and others showed that metabolic adaptation could sometimes take place even without measurable increase in cell numbers in the culture.

Marjory Stephenson, a leading bacterial physiologist in the middle of the twentieth century, described these variations in her influential book, *Bacterial Metabolism* (Stephenson, 1939, 1949), as ''Adaptation by Natural Selection'' and ''Adaptation due to Chemical Environment.'' The former included the phenomenon that is now termed mutation. The latter mode of adaptation is the main focus of this essay.

Stephenson was educated in biochemistry and came under the influence of Fredrick Gowland Hopkins at Cambridge University in the 1920s. Hopkins conceived of a program of ''general biochemistry'' that ranged from microbes to man. For complex reasons, both intellectual and practical, Stephenson embarked on a research program in bacterial biochemistry and soon established her laboratory as a leading place for this kind of science (Kohler, 1985). Stephenson's taste tended toward study of complex natural systems rather than model chemical reactions in vogue in the 1920s and she soon became enmeshed in the complex enzyme reactions of microbial metabolism.

Between 1931 and the start of World War II, Stephenson and her students John Yudkin and Ernest Gale investigated bacterial metabolic variation in detail, often exploiting the lactose-fermenting system in enteric bacteria to study it. The mechanism of chemical adaptation, however, eluded them. Yudkin proposed a mechanism linking the presence of the small molecules in the media to the synthesis of the metabolic machinery of the cell (''mass action theory of enzyme formation''). He suggested that there is an intracellular chemical equilibrium between an enzyme and its precursor. This equilibrium might be altered if the concentration of the substrate of the enzyme (in this case the substance to which the culture is being adapted) was suddenly increased. The combination of the chemical substrate with the intracellular enzyme would ''pull'' the equilibrium in favor of the formation of more enzyme from its immediate precursor (Stephenson, 1939, p. 310, 1949, p. 299). Stephenson, however, was more cautious and seemed content to consider enzyme adaptation as an unsolved problem. The final paragraph of her monograph expressed her belief in the importance of the study of bacterial metabolism: ''It [the bacterial cell] is immensely tolerant of experimental meddling and offers material for the study of processes of growth, variation and development of enzymes without parallel in any other biological material'' (Stephenson, 1949, pp. 311–312).

In 1934 another research group on ''bacterial chemistry'' consisting of Paul Fildes and B. C. J. G. Knight was established at Middlesex Hospital in London (Fildes, 1971). Fildes and Knight investigated bacterial nutrition and established vitamin B_1 (thiamine) as a growth factor for *Staphylococcus aureus*. Their work on bacterial growth factors suggested a unity of metabolic biochemistry at the cellular level, and they investigated the variations in grow factor requirements. One recurrent theme in their early work was the finding that they could ''train'' bacteria to grow on media deficient in some essential metabolite.

For example, they could train *Bact. typhosum* to grow on medium without tryptophan or without indole. Fildes noted that ''during this time little attention was given to the mechanism of the training process, but it was certainly supposed that the enzyme make-up of the bacteria became altered as a result of a stimulus produced by the deficiency of the metabolite'' (Fildes and Whitaker, 1948).

By the mid-1940s, however, Fildes and his colleagues undertook a study of the mechanism of this ubiquitous ''training.'' Was it another example of enzyme adaptation or was it something else? Using only simple growth curves, viable colony counts on agar plates, and ingenious experimental designs, they concluded ''that 'training' bacteria to dispense with certain nutritive substances normally essential may be looked upon as a cumbersome method for selecting genetic mutants'' (Fildes and Whitaker, 1948). Little by little, the underlying mechanisms of the different kinds of biochemical variations seen in bacteria were becoming clear, and little by little, genetics was joining biochemistry as a powerful approach to study bacterial physiology.

The mechanisms and reactions involved in protein synthesis were completely unknown at that time, and extrapolation of basic chemical concepts to this process was both logical and frequent. In addition to Yudkin's model of ''mass action,'' other proposals based on known chemical principles were put forth. For example, the Nobel Prizewinner Sir Cyril Hinshelwood applied his skills as a chemical kineticist to the problems of bacterial physiology and in two lengthy monographs (Hinshelwood, 1946; Dean and Hinshelwood, 1966), the second one coauthored with A. C. R. Dean, developed a detailed theory of intracellular multiple equilibria that he hoped would explain every aspect of bacterial physiology from mutations to enzyme adaption. Hinshelwood's approach, which was exemplary of a group of scientists who believed the time was ripe for the mathematization of biology, was often called a biometric approach. Although this field has been eclipsed by other developments, it flourished for several decades at mid century. Journals such as Rashevsky's *Bulletin of Mathematical Biology, Biometrics*, and *Growth* published papers on mathematical analyses of bacterial growth and metabolism. Hinshelwood started from the belief that exponential growth curves point to an underlying biological ''law'' of growth: ''This exponential law is a straightforward expression of experimental fact. Every component of a cell gives the appearance of being autocatalytically formed'' (Dean and Hinshelwood, 1966, p. 27). He attempted to derive the necessary mathematics to explain this apparent autocatalyic formation in terms of the inputs, i.e., nutrients, and outputs, i.e., cell growth.

Long-term stable changes such as mutations were at first explained by this biometric approach as representing highly stable, energetically favored kinetic states, into which cells became ''trapped.'' By 1966, however, Hinshelwood was forced to acknowledge that these mutations were the consequence of hereditary changes in the genetic constitution of the cell represented not by states of equilibria but by chemical structures. Adaptive responses, however, were still an intriguing challenge, and he wrote: ''The whole picture [of enzyme adaptation], so far from suggesting a mere mechanical printing off of replicas, suggests an elaborately geared process of control and response, and it is hard to resist the conclusion that the variations in composition and enzyme activity are closely linked with the regulation of the whole cell function'' (Dean and Hinshelwood, 1966, p. 86).

Thus, although many of the enthusiasms of the biometricians have been consigned to the dustbin of history, it is important to realize that they were taken seriously by many mainstream biologists and considered by many scientists as providing a crucial pathway of research to understand the inner workings of the cell.

Against this background of the biometric approach to bacterial physiology, we turn to the research program at the Pasteur Institute in Paris in the group of André Lwoff. Lwoff started his scientific work at the Pasteur Institute in 1919 studying the nutrition of protozoa and in 1944, after almost three decades of research in microbial nutrition, he published his influential monograph on the evolution of microbial physiology (Lwoff, 1944). This book summarized the role of vitamins, originally discovered as essential for mammals, in the nutrition of microbes. Lwoff analyzed the importance of nutrients in microbial cellular metabolism, an analysis that led to better understanding of the relationships between vitamins and coenzymes. Comparative studies on different microbes also suggested evolutionary relationships that could explain the wide diversity in both growth requirements and metabolic capacities of microbes.

At this time a young student, Jaques Monod, working at the Sorbonne under the Vichy French government in 1940, had taken up the study of bacterial growth kinetics. In an approach strongly influenced by Georges Teissier, a biometics enthusiast, Monod investigated the growth of bacterial cultures in the presence of different carbohydrates. In one variation of this experiment, he tried combinations of two sugars at once. Certain mixtures of sugars promoted ''normal'' kinetics (i.e., exponential growth curves), but other mixtures resulted in biphasic curves with ''two complete growth cycles'' (Monod, 1972). Monod showed his growth curves to Lwoff as the local expert in microbial

growth studies; Lwoff immediately recognized the relevance of Stephenson's work on enzyme adaptation to Monod's biphasic growth curves and invited Monod to continue with this work in his laboratory at the Pasteur Institute.

The relationship between variation because of mutation and variation because of enzyme adaptation was still of interest to Monod, and he sought to discover ''the still quite obscure relations between this phenomenon (adaptation) and the one Massini, Lewis, and others had discovered: the appearance and selection of 'spontaneous' mutants.'' (Monod, 1972). Monod then obtained a strain of *E. coli mutabile*, a lac^- strain isolated from Lwoff's gastrointestinal track (following Massini: lactose-negative strains that produced lactose-positive variants at a reasonably high frequency were called ''mutabile''). In addition to finding mutations (now known to be ''revertants'') that were lac^+, he and Alice Audureau showed that the original lactose-negative and the lactose-positive variant did not differ with regard to the presence of an enzyme system to utilize lactose (neither strain had it under normal growth conditions), but that the strains differed in their ability to make the enzyme system in the presence of lactose. This result provided a linkage between a genetic property, a metabolic reaction, and a nutritional requirement. Monod became fascinated by this relationship and sought out collaborators in both enzymology and genetics to further develop his study of these phenomena. Through contacts with the geneticist Boris Ephrussi in France and T. H. Morgan at Cal Tech, he learned of the power of genetic approaches to analyze physiological processes in both fungi and fruit flies and Monod was ''convinced that one day these ideas would be applied to bacteria'' (Monod, 1972).

As Monod and others have chronicled, the productive interaction of genetics and bacterial physiology was an important event in the development of our current understanding of how genes are regulated and expressed on one hand, and how cells respond and adapt to their ever-changing environment on the other.

An almost forgotten consequence of the elegant studies of Monod and his school on the phenomena of enzyme adaptation has been the unification of two contending views of genetics. As Jan Sapp (1987) has demonstrated, prior to mid-century, there were two distinct sorts of genetics: genetics as the study of the transmission of characters from generation to generation, i.e., transmission genetics, or the ''nuclear monopoly'' of Morgan and his school of American genetics, and then there was genetics as the study of how genes controlled cell function, for example, development or metabolism. This view of genetics

often involved cytoplasmic factors and was mostly associated with Continental scientists. It was the beauty and genius of the work on physiological genetics in the 1960s that these two views merged as just different ways of looking at the same biological system. This unification was helped, too, by the postwar cybernetic view of the gene in terms of information theory: units of information that required both copying and decoding.

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Acid Resistance in *Escherichia coli*

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I. Introduction

Enteric organisms, such as *Escherichia coli*, colonize and cause disease in the human intestinal tract. Intestinal pathogens arrive at their human hosts usually via contaminated food or water, travel through the host's upper digestive tract, which includes the stomach, before reaching the bowel where pathogenesis occurs. The normal human stomach averages a pH of 2 with an emptying time of approximately 2 h (Texter *et al.*, 1968). Consequently, *E. coli* must survive an acid challenge of less than 3 and as low as 2 for a considerable period of time in order to gain entrance into the less acidic environment of the intestinal tract (Texter *et al.*, 1968; Gorden and Small, 1993). An organism's capacity to survive this challenge may be directly correlated with infectious dose (ID_{50}). For example, *Vibrio cholerae*, an acid-sensitive organism, has an infectious dose in the range of 10^6–10^{11} organisms, whereas *E. coli* and *Shigella*, which are more acid-resistant organisms, have very low infectious doses (as low as 10 organisms) (Boyd, 1995). This correlation underscores the importance of acid resistance in the pathogenesis of enteric organisms.

Although an effective system to provide resistance to pH 2 would seem a requirement for such a low ID_{50}, *E. coli* grown to exponential phase in the laboratory is quite acid sensitive. However, *E. coli* becomes acid resistant upon entry into stationary phase. Research from several laboratories has shown that stationary phase triggers at least three

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genetically and physiologically distinct acid resistance systems (Shi and Bennett, 1994; Lin *et al.*, 1995, 1996; Stim-Herndon *et al.*, 1996; Castanie-Cornet *et al.*, 1999; Tramonti *et al.*, 2002). The one characteristic common to all three systems is that each protects stationary phase cells from acid stress, albeit under different conditions.

Acid resistance system 1 (AR1) is a stationary phase, acid-induced, glucose-repressed system that requires the alternative sigma factor RpoS to be expressed. The structural components of AR1 as well as the mechanism(s) by which it protects are still unknown. Acid resistance system 2 (AR2) is a stationary phase–induced and glutamate-dependent system. This system requires glutamate decarboxylase and a putative glutamate: γ-aminobutyric acid (GABA) antiporter, as well as exogenous glutamate to function at pH 2. Acid resistance system 3 (AR3) is acid induced under anaerobic conditions. It is arginine-dependent, and requires the presence of an acid-inducible arginine decarboxylase (AdiA) to function. Analogous to AR2, AR3 will protect cells from extreme acid only if arginine is present extracellularly. It is postulated that both AR2 and AR3 confer acid resistance by consuming intracellular protons. Proton consumption may produce a less acidic internal pH that allows cells to survive in extremely acidic environments.

All of these acid resistance systems provide different levels of protection. The glutamate-dependent system (AR2) provides the highest level of protection, functioning at pH 2 or less. The arginine-dependent (AR3) and the oxidative systems (AR1) provide a lower level of protection, functioning at pH 2.5 or higher (Audia *et al.*, 2001). These three stationary phase–induced systems are the main constituents of *E. coli* acid resistance and the primary focus of this review.

In addition to these acid resistance systems, other less effective acid tolerance/habituation systems have been reported for *E. coli* (Goodson and Rowbury, 1989a). Acid tolerance in exponential phase has been described for various enteric organisms, and the exponential phase induction of acid tolerance has been reported in *E. coli* (Foster and Hall, 1991; Lin *et al.*, 1995), although the mechanisms are not well characterized. The net result of these different stationary and exponential phase systems is an overlapping protection against pH stresses ranging from pH 4 to 1.5.

II. Stationary Phase Acid Resistance

A. Acid Resistance System 1

Acid resistance system 1, sometimes referred to as the oxidative or glucose-repressed system, is distinct from the other stationary phase

systems in that there is no requirement for exogenous amino acid substrates during acid challenge. Activation of this system occurs when cells are grown in mildly acidic (pH 5.5) complex media (LB) in the absence of glucose. Cultured under these conditions, cells will survive a pH 2.5 challenge in minimal glucose medium (Castanie-Cornet *et al.*, 1999). Key regulators of this system include the alternative sigma factor σ^s, cyclic AMP (cAMP), and cAMP receptor protein (CRP). Mutations in any of these genes block the action of AR1 (Castanie-Cornet *et al.*, 1999). The alternative sigma factor σ^s is important for gene expression during transitions from log phase to stationary phase and is critically important for survival during various environmental stresses including acid challenge (Hengge-Aronis, 1993; Cheville *et al.*, 1996). It is not known whether regulation of AR1 by RpoS or CRP is direct or indirect (Castanie-Cornet *et al.*, 1999). The specific AR1 target genes controlled by these regulators are also unknown.

As noted above, the activity of AR1 is acid induced. The acid regulation of AR1 activity is evident when comparing cells grown to stationary phase in an alkaline-buffered complex media to those grown in acid-buffered complex media. Cells cultured in alkaline-buffered media are sensitive to pH 2.5 in minimal-salts glucose media, whereas cells grown in acid-buffered media are resistant. The system components, however, appear to be present in stationary phase cells regardless of growth pH. The apparent acid induction of this system is due to an undefined inhibitor present in alkaline-grown (pH 8.0) but not acid-grown (pH 5.5) LB cultures. The effect of this inhibitor can be abrogated by washing cells or by the addition of exogenous glutamate or glutamine (Castanie-Cornet *et al.*, 1999). The mechanism by which glutamate/glutamine rescues cells is not known, but neither amino acid will rescue AR1 in a *gadC* mutant defective in AR2, suggesting a possible connection between protection provided by AR1 and AR2 (Castanie-Cornet *et al.*, 1999).

As discussed earlier, acid resistance provided by AR1 differs from AR2 and AR3 in several respects. One difference is that, unlike AR2 and AR3, the structural components of AR1 have not been identified. Though it provides the least level of protection of the three inducible stationary phase acid resistance systems, AR1 has been shown to be a key element in *E. coli* survivability during acid stress above pH 3 (Audia *et al.*, 2001; S. Price *et al.*, unpublished observations). Recent evidence linking the F_0/F_1 proton translocating ATPase with AR1 indicates this ATPase could act as a proton pump during acid challenge, extruding protons out of the cell concomitant to ATP hydrolysis (H. Richard and J. W. Foster, unpublished observations). Although

certainly an inefficient process, proton pumping may help maintain an intracellular pH conductive to cell survival in extreme acid conditions.

In addition to possible proton pumping by the F_0/F_1 ATPase, differences in membrane cyclopropane fatty acid (CFA) content may be an important factor in decreasing proton permeability during acid stress. CFA synthesis occurs during transition from log phase to stationary phase and, like AR1, is dependent on RpoS (Wang and Cronan, 1994; Castanie-Cornet *et al.*, 1999). Studies have shown that the absence of *cfa* (which codes for CFA synthetase) produces cells more sensitive to pH 3 (Chang and Cronan, 1999). The conversion of unsaturated fatty acids (UFA) to CFA by CFA synthetase may decrease proton permeability, which would then in turn increase acid resistance. Although CFA synthesis is not the only factor involved in survival at pH 3, it likely plays a role in the protection provided by AR1 as well as by AR2 and AR3.

Another protential component of AR1 is HdeA. *hdeA* is located in an operon with *hdeB*, which encodes a structural homolog of HdeA (Gajiwala and Burley, 2000). Exposure to short-chain fatty acids, such as acetate, induces extreme acid resistance in *E. coli*. HdeA is part of the RpoS regulon induced by acetate (Arnold *et al.*, 2001; Kirkpatrick *et al.*, 2001). Given that RpoS is an essential component of AR1 (Castanie-Cornet *et al.*, 1999) and *hdeA* mutation results in extreme acid sensitivity (Gajiwala and Burley, 2000), it is likely that HdeA is an important component of AR1 resistance. The exact mechanism by which HdeA protects the cell is not known. One model suggests that upon acidification of the periplasm, HdeA homodimers disassociate to form monomers, which then bind to acid-denatured proteins in the periplasmic space. This interaction prevents irreversible aggregation of these proteins until reaching the less acidic environment of the intestine where the proteins can refold properly (Gajiwala and Burley, 2000). This ''chaperone'' function of HdeA may therefore aid in the protection provided by all three stationary phase acid resistance systems.

B. Acid Resistance System 2

1. *Biochemistry and Physiology*

Of all of the stationary phase acid resistance systems, system 2 has been studied most extensively. AR2 is a glutamate-dependent system induced during stationary phase. Activation of the system occurs when cells are cultured in complex media (LB) containing glucose

(LBG). Growth in glucose represses AR1, which allows the direct study of AR2. Under these conditions, cells will survive a pH 2 challenge in minimal-salts glucose medium when exogenous glutamate is present. The structural components of this system include the glutamate decarboxylase isozymes GadA and GadB, and a putative glutamate: GABA antiporter encoded by *gadC* (Hersh *et al.*, 1996). The genes encoding GadA and GadB are found at different sites in the chromosome, 78 and 33 min, respectively. The *gadC* gene forms an operon with *gadB* (*gadBC*; Fig. 1A). The transcription of *gadC* can proceed from either the *gadB* promoter or an internal *gadC* promoter (De Biase *et al.*, 1999). Mutations in the putative antiporter (*gadC*) or glutamate decarboxylase (*gadA gadB* double mutant) block glutamate-dependent acid resistance. Due to redundancy of the decarboxylases, a mutation in one of the two decarboxylase genes will not seriously affect resistance (Castanie-Cornet *et al.*, 1999).

The two glutamate decarboxylases encoded by *gadA* and *gadB* are isozymes with pH optima of 3.8 (Boeker and Snell, 1972; Smith *et al.*, 1992). Glutamate decarboxylase is a pyridoxal phosphate (PLP)-dependent enzyme that catalyzes the conversion of glutamate to GABA, and in the process consumes one proton and releases CO_2 (Boeker and Snell, 1972). At pH values above the optimum, a substituted aldimine forms via nucleophillic attack by an active site residue of GadA/B on the hydrogen-bonded aldimine, thus blocking this site and rendering the enzyme inactive (Fig. 2). At an optimal pH, nucleophillic attack does not occur, and the enzyme remains active (Walsh, 1977).

Little is known about the physiology of the putative antiporter, GadC. It is postulated that the GABA produced by the decarboxylation of intracellular glutamate is transported out of the cell via GadC with the concomitant uptake of exogenous glutamate (Foster, 1993) (Fig. 3). At present it is not thought that the export process itself contributes directly to acid resistance.

There are several hypotheses describing how this glutamate-dependent acid resistance system may protect the cell from acid stress. The most favored of these suggests that the consumption of intracellular protons during decarboxylation maintains the internal pH in a range conducive to cell survival (Foster, 2001) (Fig. 3). However, when considering the p*K* values of the three ionizable groups of glutamate (pK_{a1} 2.3, pK_{a2} 9.67, pK_{a3} 4.28), concerns are raised as to the efficacy of this model (Dawson *et al.*, 1979). For example, if the pH of the challenge media is 2.0–2.5, then extracellular glutamate will have two fully protonated groups [amino (pK_{a2}) and R groups (pK_{a3})] and one half-protonated group [α-carboxyl group (pK_{a1})] (Fig. 3). Once inside

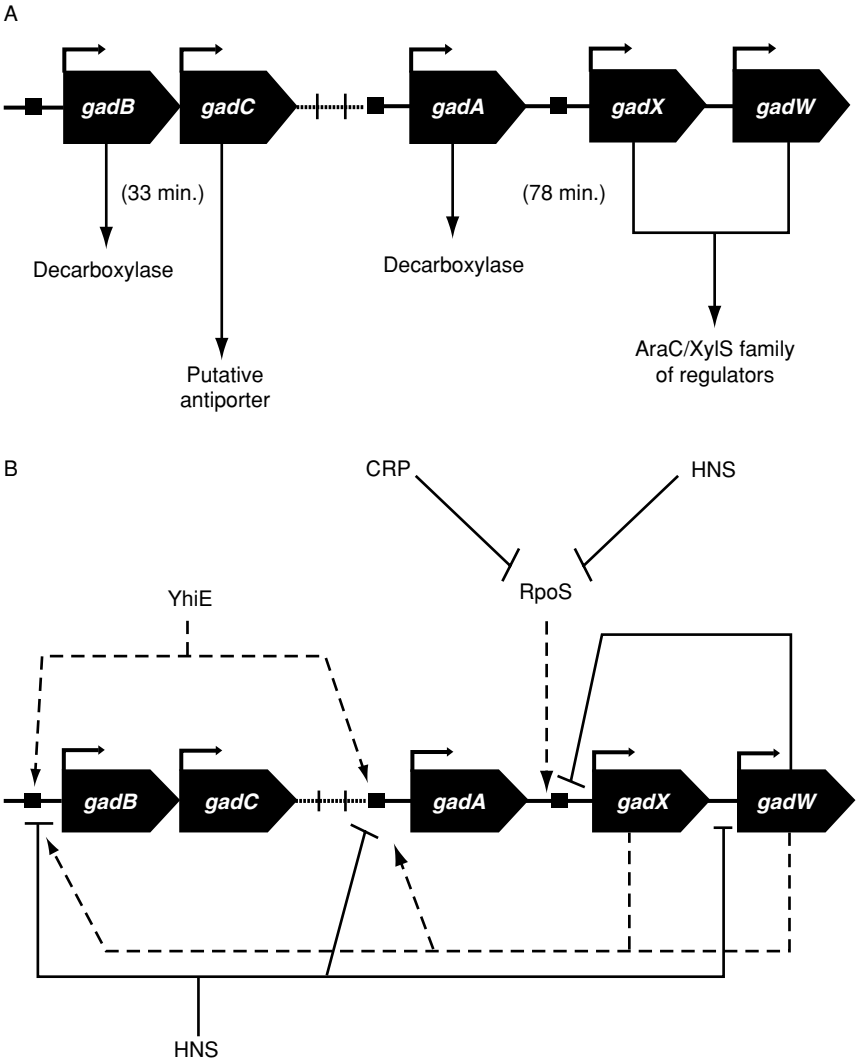

FIG. 1. (A) Genetic organization of acid resistance system 2. The *gadBC* operon is located at 33 min, whereas *gadA*, *gadX*, and *gadW* are found at 78 min. The 20-bp regulatory region (Gad box) is similar in sequence and is found upstream (−52 to −72) of the transcriptional start sites of both the *gadA* and the *gadBC* loci. (B) Genetic regulatory network of acid resistance system 2. The same effect by each regulator is seen on the expression of both *gadA* and *gadBC*. The site at which all of the regulators exert their effect is not known. Bent arrows indicate promoters. Solid black boxes represent the 20-bp regulatory region (Gad Box) required for transcriptional regulation. Positive regulators are represented by a dashed line, and negative regulators are represented by a solid line.

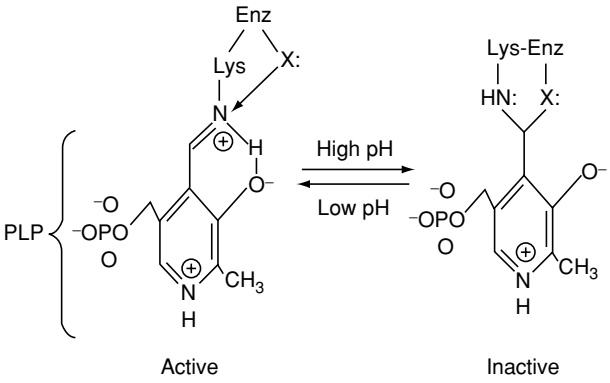

FIG. 2. Model of pH regulation of glutamate (GadA/B) and arginine decarboxylase (AdiA). The transition between active and inactive forms of the enzymes is regulated by pH. The arrow from X represents the nucleophilic attack on the acitive site.

the cell, where pH may range from 4 to 5 (H. Richard and J. W. Foster, unpublished observations), the protonated α-carboxyl and R groups would be expected to deprotonate before decarboxylation.

This would add to proton stress inside the cell and limit the net proton consumption by this reaction. However, it is predicted that the pH of the antiporter channel will be similar to the intracellular pH (pH 4–5). Therefore, any protonated groups (α-carboxyl or R) on glutamate should deprotonate *before* entering the cytoplasm. Furthermore, recent evidence suggests that AR2 does help the cell maintain a less acidic internal pH supporting the efficacy of this model in describing glutamate-dependent acid resistance (H. Richard and J. W. Foster, unpublished observations).

In addition to proton consumption via decarboxylation, it is hypothesized that *E. coli*, as was originally proposed for *Helicobacter pylori* (Toledo *et al.*, 2002; Stingl *et al.*, 2002), may buffer the surrounding environment leading to a localized rise in external pH. In *E. coli*, GABA, the end product of glutamate decarboxylation, may act as a proton acceptor upon release into the periplasm or external environment, thus potentially leading to localized buffering of the surrounding acidic environment (Fig. 3).

Maintenance of a less acidic internal pH via amino acid decarboxylation could be important to survival during acid stress simply because a higher internal pH will mitigate cell damage. However, AR2 may also contribute to electrical potential ($\Delta\psi$) in cells subjected to acid challenge. The electrical potential across the cell membrane ($\Delta\psi$) is

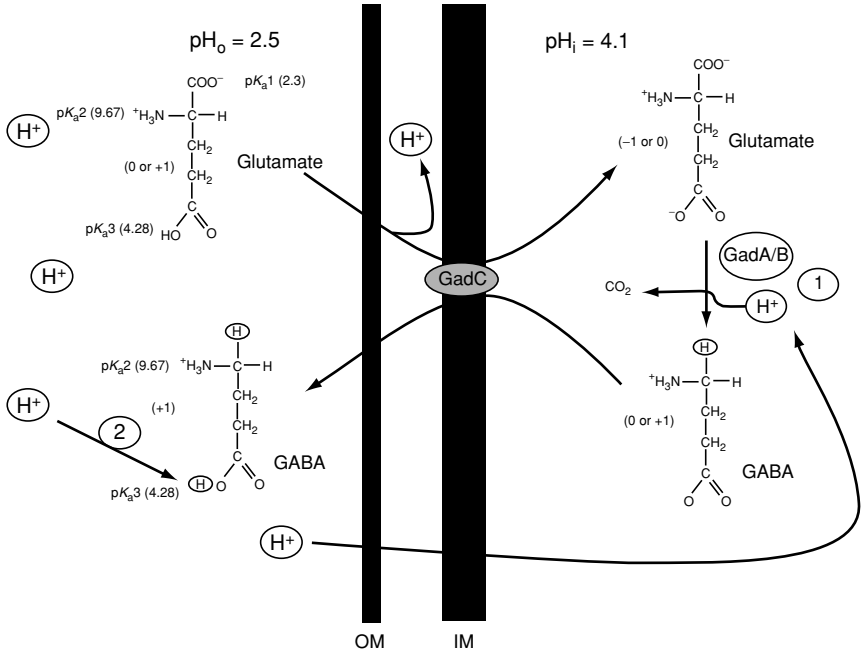

FIG. 3. Proposed model of protection by acid resistance system 2. At an acidic pH (2.5) protons move into the cell and acidify the cytoplasm. When this happens glutamate is transported by GadC, decarboxylated by GadA/B, into γ-aminobutyric acid (GABA), with the consumption of a proton and the release of CO_2. GABA is then transported out of the cell with the concomitant uptake of glutamate. It is predicted that protons remaining on the ionizable group of glutamate will be released in the periplasm prior to passage through GadC or during passage through the channel. There are two potential points where protons can be consumed. 1 represents the consumption of a proton in the cytoplasm, and 2 represents the consumption of a proton in the external environment by GABA. The pK_aS of the ionizable groups of both glutamate and GABA are distinguished by a group name (pK_a 1...etc.), and the net charge of the molecules is given in parentheses.

a major component of the proton motive force necessary for energy production and cell survival. The antiport of glutamate and GABA by GadC is electrogenic with a net charge of +1 transported across the membrane at an acidic external pH. Glutamate molecules, after entering the acid stressed cell, (pH$_{in}$ 4.1; H. Richard and J. W. Foster, unpublished observations) will be either neutral or −1 depending on the protonation state of the ionizable groups. GABA, after exiting the cell through the antiporter, will have a +1 charge, thus contributing to the overall electrical potential (negative inside) across the cell membrane (Fig. 3).

The potential contribution of glutamate decarboxylation and antiporter activity to proton motive force suggests that AR2 is coupled to energy production via the proton translocating ATPase (F_0/F_1). An example of this type of energy coupling can be seen in *Lactobacillus* spps. where the decarboxylation of glutamate to GABA and the concomitant transport by GadC contribute to both ΔpH and $\Delta\psi$ producing the energy required for ATP synthesis by the proton translocating ATPase (Higuchi *et al.*, 1997; Harold and Maloney, 1996). Although this particular example of energy coupling in *Lactobacillus* is not directly related to survival during extreme acid challenge, it raises the question as to whether energy production via the ATPase may be significant to the survival of *E. coli* during acid stress. Contrary to this prediction, we have recently obtained evidence that the proton-translocating ATPase is not required for the acid resistance provided by this system (H. Richard and J. W. Foster, unpublished observations).

2. *Regulation of AR2*

The regulatory controls governing the expression of AR2 are quite complex. There are at least two regulatory circuits controlling *gadA/BC*. One system, involving the *yhiE* gene product, appears essential for expression under all conditions (Z. Ma, H. Richard, and J. W. Foster, unpublished observations). A second system encompassing cAMP, CRP, RpoS, and GadX(YhiX) oversees control in rich media (LB).

The *yhiE* gene, which encodes a 20.6-kDa potential regulatory protein, was recently discovered to be acid induced as part of a major gene array study (Tucker *et al.*, 2002). Evidence from our laboratory has demonstrated that YhiE is required for *gadA/BC* expression under all conditions tested (H. Richard, Z. Ma, and J. W. Foster, unpublished observations), suggesting a potential global regulatory role for YhiE in *gadA/BC* expression.

The second regulatory system was exposed when comparing the expression of *gadA/BC* in minimal-salts media versus rich media. When cells are grown in minimal salts, *gadA/BC* transcription is acid induced in both exponential and stationary phase cells. However, little to no expression occurs in exponential phase cells grown in rich media (Castanie-Cornet and Foster, 2001). Expression of *gadA/BC* becomes evident in rich media only when cells are grown to stationary phase (Castanie-Cornet and Foster, 2001). Evidence suggests the following model of *gadA/BC* transcriptional regulation (Fig. 1B). In rich media where cAMP levels are high, CRP–cAMP represses the expression of the RpoS sigma factor. As cells enter stationary phase, RpoS levels begin to rise and trigger expression of many genes, including *gadX*,

whose product directly induces expression of *gadA/BC* (see below). Whereas YhiE is required for expression in either rich or minimal media, RpoS and GadX are needed only for induction in rich media (Castanie-Cornet and Foster, 2001; Z. Ma *et al.*, 2002).

GadX and GadW belong to the AraC/XylS-like family of transcriptional regulators. The genes encoding these regulators are found downstream of *gadA* at 78 min (Fig. 1A). A separate promoter drives each gene; however, *gadX* can also be transcribed as an operon from the *gadA* promoter. As noted above, GadX is a positive regulator of *gadA/BC* expression acting directly at the *gadA/BC* promoter regions (Tramonti *et al.*, 2002). A 20-bp *cis*-acting region located 53 bp upstream of both the *gadA* and *gadBC* promoters is essential for this transcriptional regulation (Castanie-Cornet and Foster, 2001). Both DNA gel shift and DNase I footprinting assays suggest that GadX binds in and around this region at multiple sites, although the pattern and number of interactions between GadX and the upstream region of *gadA* differ from that of the upstream region of *gadBC* (Tramonti *et al.*, 2002).

GadW has also been shown to both directly and indirectly affect *gadA/BC* expression in rich media. Like GadX, DNA gel shift data indicate GadW directly binds to both promoters leading to direct regulation of *gadA/BC* expression (Z. Ma *et al.*, unpublished observations). Indirect regulation of *gadA/BC* by GadW occurs by direct repression of *gadX* expression, which will in turn suppress *gadA/BC* transcription.

Another regulator of *gadA/BC* is the nucleoid protein H-NS, which is a global regulator of gene expression shown by gene array to affect numerous genes involved in cell survival during environmental stresses (Hommais *et al.*, 2001). H-NS has a pleotropic effect on AR2 by acting as a negative regulator of *gadX*, *gadW*, *rpoS*, and *gadA/BC* expression (De Biase *et al.*, 1999; Hommais *et al.*, 2001; Tramonti *et al.*, 2002). In some strains, at least, H-NS exerts its effects only in exponential phase cells grown in complex media (LB) (Z. Ma *et al.*, 2002). Once in stationary phase, it is thought that H-NS repression is overcome by RpoS (Hengge-Aronis, 1993).

C. Acid Resistance System 3

1. *Biochemistry and Physiology*

Acid resistance system 3 is arginine-dependent and has structural components similar to those of AR2. AR3 requires arginine decarboxylase (encoded by *adiA*) and a recently identified arginine:agmatine

antiporter (encoded by *yjdE*) to function (S. Gong, H. Richard, and J. W. Foster, in press). *E. coli* possesses two arginine decarboxylase genes, *speA* and *adiA*; however, only the acid-inducible AdiA functions in arginine-dependent acid resistance. SpeA is a biosynthetic enzyme important for polyamine biosynthesis, whereas AdiA is an acid-inducible biodegradative enzyme, not involved in polyamine synthesis but critical for AR3 function (Glansdorff, 1996). The pH optima of these two enzymes, pH 8.4 and pH 5.2, respectively, are consistent with their roles in unstressed and acid-stressed cells (Boeker and Snell, 1972). Under acid stress conditions (pH 2.5) cells have an internal pH of 4–5 (H. Richard and J. W. Foster, unpublished observations) allowing only AdiA to function. AdiA is a PLP-dependent enzyme that catalyzes the conversion of arginine to agmatine and, in the process, consumes one proton and releases CO_2 (Fig. 5). Acid regulation of AdiA activity occurs via the same mechanism described previously for glutamate decarboxylase (Section II.A.1) (Walsh, 1977).

Until recently, the only structural component known for this system was AdiA. *yjdE*, a gene downstream of *adiA*, has recently been identified as another essential structural component of this system (Fig. 4). The product of *yjdE* is most likely the arginine:agmatine antiporter.

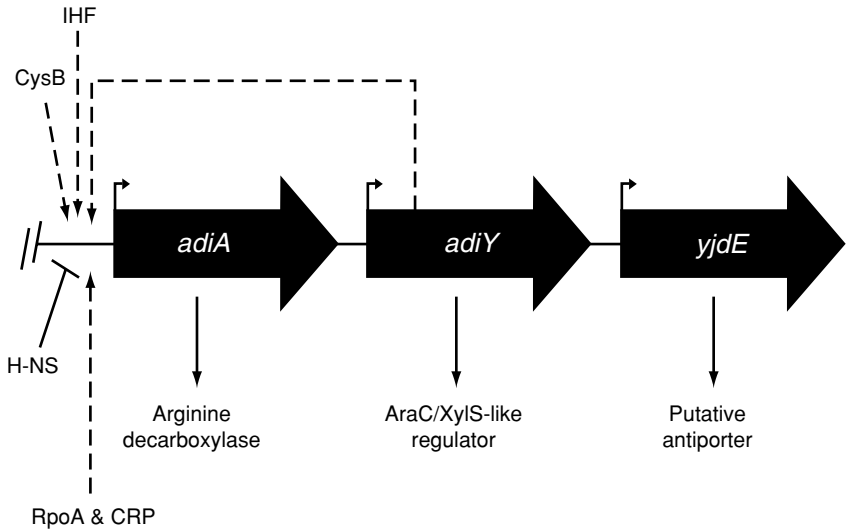

FIG. 4. Genetic organization and regulation of acid resistance system 3. Both *adiA* and *adiY* expression are driven off of distinct promoters (bent arrows represent the promoters). Positive regulators are represented by a dashed line and negative regulators are represented by a solid line. The site of regulation by these proteins is unknown.

Mutants defective in this gene selectively lose AR3 (AR1 and 2 remain functional) and fail to exchange extracellular arginine for agmatine (S. Gong, H. Richard, and J. W. Foster, in press).

Given the similarities of the structural components in both glutamate and arginine-dependent acid resistance, the mechanisms by which they protect are also thought to be similar. As with AR2, the consumption of a proton in the decarboxylation reaction is thought to maintain internal pH in a physiological range conducive to cell survival (Foster, 2001) (Fig. 5). Unlike GABA, however, the product of arginine decarboxylation (agmatine) cannot act as a proton acceptor when moving from an internal pH of 4–4.5 to an external pH of 2.5. For localized external buffering to occur, as seen with GABA in AR2, an available

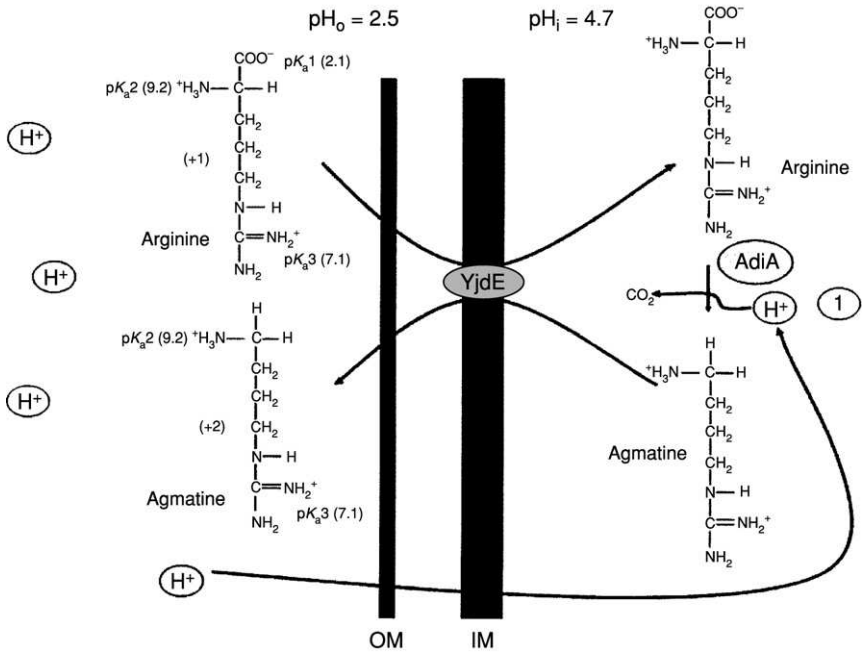

FIG. 5. Proposed model of protection by acid resistance system 3. At an acidic pH (2.5) protons move into the cell and acidify the cytoplasm. When this happens arginine is transported by YjdE and decarboxylated by AdiA into agmatine, with the consumption of a proton and the release of CO_2. Agmatine is then transported out of the cell with the concomitant uptake of arginine. 1 represents the consumption of a proton in the cytoplasm; no external buffering appears to occur for this system due to the relative pK_as of agmatine. The pK_as of the ionizable groups of both arginine and agmatine are distinguished by a group name (pK_a 1 . . . etc.), and the net charge of the molecule is given in parentheses.

ionizable group is required upon exit of agmatine from the cell. Given the p*K* values of agmatine (pK_{a2} 9.2 and pK_{a3} 7.1), this molecule will be fully protonated at a pH 4 (Dawson *et al.*, 1979). Thus, agmatine secretion cannot help neutralize the acidic external environment (Fig. 5).

As with AR2, AR3 also has the potential to contribute to $\Delta\psi$. The antiport of arginine and agmatine is electrogenic at an acidic pH. At an external pH of 2.5, arginine has a net charge of +1 as it enters the cell and agmatine is +2 as it is transported out of the cell. Thus, the antiport of arginine and agmatine may directly contribute to the proton motive force (PMF) across the membrane (Fig. 5). The contribution of arginine decarboxylation and electrogenic transport to PMF suggests that AR3 may also be coupled to energy production by the proton translocating ATPase (F_0/F_1) (Anantharam *et al.*, 1989; Harold and Maloney, 1989; Higuchi *et al.*, 1997). However, recent data suggest that as with AR2, the proton-translocating ATPase is not required for the protection provided by this system (H. Richard and J. W. Foster, unpublished observations).

2. *Regulation of AR3*

Important players known to affect the transcriptional regulation of AR3 are CysB, RpoA, CRP, AdiY, H-NS, and IHF (Fig. 4). CysB is a member of the LysR family of regulatory proteins and has been shown by mutational analysis to be required for acid induction of *adiA* expression (Shi and Bennett, 1994). Acid sensitivity conferred as a consequence of a *cysB* mutation confirms the pivitol role of this protein in AR3 function (Shi and Bennett, 1994). A significant decrease in *adiA* transcription can also be seen in an *rpoA* mutant (Shi and Bennett, 1994) indicating that the RNA polymerase α-subunit plays a crucial role in *adiA* transcription as well. *In vitro* studies suggest that RpoA may interact with CRP in an upstream region to activate transcription. Possible binding sites for both RpoA and CRP are found upstream of the *adiA* transcriptional start site, further suggesting an interaction between these proteins is important in the transcriptional regulation of *adiA* (Shi and Bennett, 1994).

AdiY belongs to the AraC/XylS family of transcriptional regulators and is found downstream of *adiA* at 93 min (Berlyn, 1998). When cells are grown at an alkaline pH (8.0), the presence of AdiY in multicopy increases *adiA* transcription (Stim-Herndon *et al.*, 1996). It is thought that AdiY functions in AR3 by relieving the H-NS repression of *adiA* transcription. However, there is still a moderate increase in *adiA* transcription in an *hns* mutant suggesting AdiY may have additional roles in this system (Stim-Herndon *et al.*, 1996). In concert

with AdiY, integration host factor (IHF) is thought to positively regulate *adiA* transcription (Stim-Herndon *et al.*, 1996). IHF is a protein involved with DNA bending and may act at a predicted binding site upstream of the *adiA* promoter. When this IHF binding site was modified, the effect of AdiY on *adiA* transcription was negated. This suggests a cooperative interaction between AdiY and IHF in *adiA* transcriptional regulation (Stim-Herndon *et al.*, 1996).

III. Log Phase Acid Tolerance

Log phase acid tolerance systems have been described for various enteric organisms. For example, *Salmonella* spp. have an acid-inducible acid tolerance response (ATR) that protects cells at a pH as low as 3.3 after adaptation/habituation at a mildly acidic pH (pH 4.5) (Goodson and Rowbury, 1989a; Foster and Hall, 1990). Similarly, when certain strains of *E. coli* are adapted to acidic pH (5.8 or 4.3) conditions during exponential phase growth, cells can survive an acid challenge as low as pH 3 (Lin *et al.*, 1995). Exposure to these sublethal levels of acid in complex or minimal-salts media can habituate the organisms allowing them to withstand lethal levels of acid (Goodson and Rowbury, 1989a). Acid adaptation may increase protection against extreme acid challenge by decreasing membrane proton permeability through the production of CFA in the cell membrane (see also Section II.A Brown *et al.*, 1997). In addition to CFA synthesis, the production of certain extracellular factors has been implicated in the increased resistance to acid stress of habituated cultures (Rowbury and Goodson, 1999; Rowbury, 2001).

There are several observations that distinguish this acid-inducible log phase acid tolerance from the stationary phase acid resistance systems. Perhaps most importantly, log phase acid tolerance does not protect cells below a pH of 3. Additionally, there is no requirement in log phase acid tolerance for the presence of exogenous amino acids as seen in stationary phase AR2 and AR3. Furthermore, the level of resistance provided by log phase acid tolerance at a pH of 3 is significantly less than that provided by the stationary phase acid resistance systems at lower pHs (Lin *et al.*, 1995).

Evidence suggests that log phase ATR/habituation may protect cells in the presence of short-chain fatty acid stress (Goodson and Rowbury, 1989b). Although this protection is effective within a shorter timeframe than in stationary phase acid resistance, this finding may be an important step in defining the role of log phase acid tolerance/habituation systems in the overall capacity of *E. coli* to withstand an acid challenge (Goodson and Rowbury, 1989b).

IV. Potential Role of Acid Resistance in Food Safety and Pathogenesis

It could be predicted that acid resistance contributes to the low infectious dose of commensal and pathogenic strains of *E. coli*, but a question often asked is when is *E. coli* acid resistance induced in the real world; before or after ingestion? All available evidence, much of which comes from studies using cattle, suggests that induction occurs prior to ingestion.

Cattle serve as important reservoir for pathogenic *E. coli*, especially O157:H7 (Kudva *et al.*, 1996). The presence of these organisms in feces can lead to contamination of beef products, watersheds, and various other food sources, including apple cider made from contaminated apples (Besser *et al.*, 1993; Miller and Kaspar, 1994). Within the bovine intestinal tract, these organisms are in stationary phase and exposed to short-chain fatty acids, two conditions that can induce acid resistance. Once induced, these acid resistance systems will remain active until cells reenter log phase. If the organisms are refrigerated in a contaminated food, the AR systems can remain active for at least a month (Lin *et al.*, 1996). Pathogens taken from the cold and ingested will be poised for passage through the human stomach. The food ingested along with the organisms will provide levels of glutamate and/or arginine sufficient to fuel acid resistance.

Another important question is whether any of these systems really contribute to gastrointestinal survival. When cattle are orally inoculated with an *rpoS* mutant of O157, fecal shedding occurs at a considerably lower level than a simultaneously administered wild-type strain, suggesting that RpoS-regulated genes are essential for survival in the bovine intestinal tract (Price *et al.*, 2000). Considering the vital role RpoS plays in AR1 function, this finding could suggest a link between AR1 function and gastrointestinal survival (Lin *et al.*, 1995). However, it should be noted that an *rpoS* mutation affects all three stationary phase acid resistance systems as well as resistance to other environmental stresses (Hengge-Aronis, 1993; Cheville *et al.*, 1996; Castanie-Cornet *et al.*, 1999). Thus, the reason for decreased shedding of an *rpoS* mutant cannot be definitively ascribed to effects on acid resistance.

The contribution of AR2 toward survival in the bovine intestinal tract has been more definitively defined. In competitive mixing experiments, a mutation in the gene encoding the putative glutamate:GABA antiporter (GadC) causes a significant decrease in the level of fecal shedding compared to the wild-type strain (S. Price *et al.*, unpublished

observations). Given that decreases in the rate of fecal shedding occur with mutations in *gadC*, which functions only in glutamate-dependent acid resistance, it is likely that AR2 is essential for the survival of *E. coli* in the bovine intestinal tract. Before reaching the acidic abomasum stomach, where AR2 is thought to function, the organisms must pass through the less acidic rumen. It is in this environment that the organisms undergo challenge by short-chain fatty acids proposed to be important in the induction of acid resistance in enterohemorrhagic *E. coli* (O157:H7) (Lin *et al.*, 1996). AR2, providing the necessary resistance to survive the acidic abomasum, may facilitate shedding of pathogenic *E. coli* into the environment where entry into the host may occur (Lin *et al.*, 1996).

In contrast to AR2, arginine-dependent acid resistance has not been found to aid in the survival of *E. coli* in the bovine intestinal tract (S. Price *et al.*, unpublished observations). Where this system has value to the organism in nature has not yet been defined.

Most human outbreaks of enterohemorrhagic *E. coli* (EHEC), including O157:H7, can be traced back to contaminated beef (Duffy *et al.*, 2000). The beef industry commonly uses lactic and acetic acid sprays to reduce contamination of freshly slaughtered beef carcasses (Dorsa, 1997). However, the efficacy of organic acid washes and their bactericidal effect on *E. coli* O157:H7 are in question (Berry and Cutter, 2000; Dormedy *et al.*, 2000). Acid adaptation of these organisms, possibly in the bovine intestinal tract, decreases the effectiveness of organic acid sprays in the decontamination of beef carcasses (Deng *et al.*, 1999; Berry and Cutter, 2000; Cheng *et al.*, 2002). A similar phenomenon can be seen in studies of *E. coli* O157:H7 survival in foods preserved in low pH or organic acids in which acid adaptation increases the organism's ability to survive in these environments (Leyer *et al.*, 1995; Tsai and Ingham, 1997). These studies underscore the importance of *E. coli* acid resistance induction in the bovine intestinal tract and its relationship to survival during food processing, thus supporting the role of *E. coli* acid resistance in human pathogenesis.

Another product linked to outbreaks of O157:H7 is apple cider (Besser *et al.*, 1993; Miller and Kaspar, 1994). It is thought that water runoff from cow pastures, which can affect adjacent fruit and vegetable farms, is the source of this contamination. Recent experiments with O157:H7 wild-type and mutant strains have shown that AR1 is essential for survival in apple cider (pH 3.5). An *rpoS* mutant strain was found to have a significantly lower survival than wild-type, *gadC*, and *adiA* mutants, indicating that the effects of AR2 and AR3 are

negligible under these circumstances (S. Price *et al.*, unpublished observations). Although it is unclear whether AR1 contributes significantly to survival of *E. coli* in cattle, this finding, nonetheless, establishes AR1 as an important player in *E. coli* survival in acidic foods as the organism awaits ingestion.

In addition to its effects on *gadA/BC* expression, the regulatory protein GadX is also a transcriptional regulator of *perA* in enteropathogenic *E. coli* (EPEC). Per positively regulates genes located in the LEE pathogenicity island, which are essential for pathogenesis. One model suggests that GadX positively regulates *gadA/BC* expression and negatively regulates *perA* expression under acid stress conditions (Shin *et al.*, 2001). Upon passage of cells from the extremely acidic environment of the stomach to the relatively less acidic environment of the intestines, GadX would become a positive regulator of *perA* expression thereby conferring pathogenicity (Shin *et al.*, 2001). The mechanisms by which the *gadA/BC* and *per* genes are differentially regulated by GadX and GadW and how acid pH affects that regulation are unknown.

V. Summary

To colonize and cause disease, enteric pathogens must overcome environmental challenges that include acid stress in the host's stomach as well as short-chain fatty acid stress in the intestine of the host and reservoir. Three known inducible systems have evolved for stationary phase acid resistance in *E. coli*. These systems each provide a different level of protection with different requirements and induction conditions. Acid resistance system 1 (AR1) is acid induced in stationary phase, requires the presence of RpoS, and provides the least level of protection at pH 2.5. Acid resistance system 2 (AR2) is glutamate dependent and stationary phase induced, requires the presence of glutamate decarboxylase and a putative glutamate:GABA antiporter, and provides the highest level of protection. Acid resistance system 3 (AR3) is arginine dependent and acid induced under anaerobic conditions, requires the presence of arginine decarboxylase (AdiA), and provides only a modest level of protection. These three systems along with log phase acid tolerance protect cells from the acid stresses in both the reservoir and host, which can range from pH 2 to 4.5. They also protect against acid stress involved in food processing and facilitate the low infectious dose characteristic of *E. coli*, significantly contributing to the pathogenesis of this organism.

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Iron Chelation in Chemotherapy

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I. Association of Iron Mismanagement with Disease

Mismanagement is defined as the accumulation of excessive amounts of iron (for genetic, environmental, nutritional, or behavioral reasons) and the consequent dispersion of the metal into a great variety of cells and tissues to result in loss of tissue and organ functions. Excessive iron in specific tissue sites promotes infection, neoplasia, cardiomyopathy, arthropathy, and an array of endocrine and neurodegenerative diseases (Table I).

Iron loading can contribute to development of illness in several ways. Excessive amounts of the metal impede the iron-scavenging function of transferrin and lactoferrin. Nonprotein-bound ferric ions are reduced by superoxide, and the ferrous product is reoxidized by peroxide to regenerate ferric ions and yield hydroxyl radicals. The latter attack all classes of macromolecules. Hydroxyl radicals can initiate lipid peroxidation, depolymerize polysaccharides, inactivate enzymes, and, not least, cause DNA strand breaks (McCord, 1996).

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TABLE I

INCREASED RISK OF DISEASES IN IRON-LOADED CELLS OR TISSUES

Cells or tissues	Diseases
Alveolar macrophages	Respiratory tract neoplasia
Anterior pituitary	Gonadal and growth dysfunctions
Aorta, carotid, and coronaries	Atherosclerosis
Brain	Alzheimer's disease
Colorectal mucosa	Adenoma, carcinoma
Heart	Arrhythmia, cardiomyopathy
Infant intestine	Botulism, salmonellosis, SIDS
Joints	Arthropathy
Liver	Hepatitis, cirrhosis, carcinoma
Macrophages	Intracellular infections
Pancreas	Diabetes, carcinoma
Plasma and lymph	Extracellular infections
Skeletal system	Osteoporosis
Skin	Leprosy, melanoma
Soft tissue	Sarcoma
Substantia nigra	Parkinson's disease

Moreover, overabundant iron can serve as a readily available nutrient for invading bacterial, fungal, and protozoan organisms as well as for neoplastic cells. Heightened virulence is associated with the ability to acquire host iron. Indeed in iron-loaded tissue sites, even microbial strains that usually are not dangerous can cause illness. Furthermore, cells of highly invasive neoplastic strains can procure host iron more adroitly than can cells of either less malignant strains or normal host cells (Weinberg, 1999a).

II. Proteins That Function to Counteract Iron Mismanagement

A. NATURAL FUNCTIONS OF TRANSFERRIN AND LACTOFERRIN

Healthy hosts maintain an iron-withholding defense system designed to prevent accumulation of nonprotein-bound iron in extracellular fluids. Notable components of the scavenging arm of the system are transferrin (Tf) and lactoferrin (Lf). These 80-kDa glycoproteins each can bind strongly two atoms of iron. They function in a

complementary manner to continuously purge body fluids of ''free'' iron. Thus Tf is responsible for keeping the environment devoid of ''free'' iron in serum, lymph, and cerebrospinal fluid. Lactoferrin is assigned to exocrine secretions that are commonly exposed to normal flora: milk, tears, nasal exudates, saliva, bronchial mucus, gastrointestinal fluids, cervicovaginal mucus, and seminal fluid (Weinberg, 2001).

In addition to its iron-scavenging function, Tf has an important second activity, the conveyance of nutritional amounts of the metal to and from cells throughout the body. Quantities of iron delivered to cells by Tf that are not immediately needed for metabolic use are deposited in the intracellular iron-sequestering arm, ferritin. This 450-kDa protein can accommodate up to 4500 atoms of iron per molecule.

Lactoferrin likewise has a second indispenable function, that of deironing tissue sites that are being damaged by diseases that involve infection, neoplasia, inflammation, ischemia, and reperfusion. As a major constituent of the secondary specific granules of circulating polymorphonuclear neutrophils (PMNs), Lf is released on degranulation of the leukocytes in damaged areas. In such sites, the pH value is lowered by catabolic acids released from metabolically active invading cells as well as from PMNs. Unlike Tf, which cannot retain iron at pH values below neutrality, Lf withholds more than 80% of the metal at pH levels above 4. For disposal of iron-saturated Lf, hepatocytes might serve as a major depository (Brock *et al.*, 1994).

Although Tf and Lf were identified more than half a century ago, only within the past few years have they come to be considered as potential broad-spectrum therapeutic agents. Until recently, it was difficult to obtain the proteins at a sufficient purity and quantity for studies in which their therapeutic properties could be properly evaluated.

B. Development of Transferrin as a Pharmaceutical

Within the past several years, a research team of the Finnish Red Cross led by Dr. Jaakko Parkkinen has developed procedures for extracting Tf from human plasma (von Bonsdorff *et al.*, 2001). The starting material is a pool of 7,000–10,000 plasma units, each tested and found negative for HBsAg, anti-HIV-1 and -2, anti-HCV, HCVRNA, and parvovirus B19. The protein is extracted from Cohn fraction IV with the final steps involving cation-exchange chromatography and ultrafiltration. During the process, any remaining contaminating enveloped virus particles are inactivated by solvent detergents, whereas nonenveloped virus particles are removed by a 15-nm pore size hollow pipe filtration step.

Plasma Tf normally is 25–30% saturated with iron. In the extraction process, iron is mostly dissociated from Tf due to the acid pH and citrate buffer used for precipitation of Cohn fraction IV. Addition of ethylene diaminetetra acetic acid (EDTA) with application of the dissolved fraction IV paste to ion exchange removes nearly all of the remaining iron. The final product has an iron content of 0.3% and an iron-binding capacity of 94%. Upon iron saturation, the apoprotein is fully converted to the diferric (holo) form.

The extraction process has yielded a concentration of 49.7 g/liter. Impurity proteins [hemopexin, immunoglobulins A and G (IgA, IgG)] comprise 0.3–0.8% of the product. It has less than 1.0% dimers and contains no detectable polymers, aggregates, or zinc. The purified product is expected to be useful not only in hypo- and atransferrinemic patients but also in medical conditions in which iron saturation of Tf becomes severely elevated.

For example, bone marrow recipients are conditioned with a week of cytotoxic chemotherapy prior to the transplantation. This procedure temporarily halts red blood cell production and also damages hepatocytes, releasing iron deposits. The Tf iron saturation value in such patients consistently approaches 100% (Durkin *et al.*, 2000; Sahlstedt *et al.*, 2001). Likewise, in hemodialysis patients who receive intravenous iron dextran to improve their response to erythropoietin, Tf iron saturation becomes markedly raised (Parkkinen *et al.*, 2002). Patients in each of these categories have greatly increased risk for infection.

In many chronic infections, bacteria live in biofilms that are distinct matrix-encased communities specialized for surface persistence. Biofilms resist killing by host defense mechanisms and antibiotics. Apotransferrin has been reported to markedly lower gram-positive and gram-negative bacterial adhesion to synthetic and/or protein-coated surfaces (Ardehali *et al.*, 2002). Not surprisingly, lactoferrin, likewise by chelating iron, prevents bacterial biofilm development (Singh *et al.*, 2002).

C. Development of Lactoferrin as a Pharmaceutical

Except for colostrum, milk, and tears, concentrations of natural Lf in normal human body fluids are quite low (Table II). Accordingly, recombinant human Lf is being produced as a potential therapeutic by molds such as *Aspergillus awamorii* (Ward *et al.*, 1995) and in transgenic cows (Van Berkel *et al.*, 2002). The fungal cells initially express Lf as a glucoamylase fusion polypeptide that is immediately processed by an endogenous KEX-2 peptidase and secreted into the medium as

TABLE II

CONTENT OF APOLACTOFERRIN IN HUMAN BODY FLUIDS[a]

Fluid	Concentration (μM)	Underlying condition
Colostrum	100	Normal
Milk	20–60	Normal
Tears	25	Normal
Seminal fluid	1.4	Normal
Vaginal fluid	0.1	Before menses
	2.0	After menses
Saliva	0.05	Normal children
	0.11	Normal adults
	0.25	Children: cystic fibrosis
Cerebrospinal fluid	0.00	Normal children
	0.01	Children: aseptic meningitis
	0.13	Children: bacterial meningitis
Synovial fluid	0.014	Noninflammatory arthritis
	0.338	Inflammatory arthritis
Serum	0.005	Normal
	2.5	Acute sepsis

[a]Modified from Weinberg (2001, Table 1).

mature human Lf. With strain improvement, the yield has reached 2 g/liter. The glycopeptide retains its full range of biological activities including iron chelation, binding to Lf receptors, and *in vitro* and *in vivo* antibacterial potency.

Transgenic dairy calves carrying the hLf fusion gene have been generated by combining gene transfer technology with *in vitro* bovine embryology. The gene is under regulatory control of the bovine αS_1-casein promoter. Yields of rhLf of up to 3 g/liter of milk are obtained. With an annual output of 8000 liters of milk/cow, one animal can produce 24 kg rhLf/year. The product is 8% iron saturated (similar to that in human milk) and has less than 0.1% bovine Lf. The recombinant product has less glycosylation than natural hLf. However, it is identical to natural hLf in binding and release characteristics as well as in antibacterial action in mice infected with *Staphylococcus aureus* (Van Berkel *et al.*, 2002).

Even more so than with Tf, manifold potential therapeutic uses have been identified for rhLf. When used to treat animals infected with a

variety of pathogenic agents, Lf displays strong antibacterial and antimycotic activities (Weinberg, 2001). Moreover, the protein also has antiviral, antiinflammatory, antineoplastic, and antiangiogenic potencies. However, the extent to which iron binding might be involved in each of these latter activities is not clear.

An example of a therapeutic use of Lf that is associated with iron binding is that of the introduction of the protein into iron-loaded, inflamed joints (Guillen *et al.*, 2000). Sites in mice with either *S. aureus* septic arthritis or collagen-induced arthritis, when treated with hLf, showed significant suppression of local inflammation. In human volunteers, rhLf employed topically suppressed cutaneous inflammatory reactions (Griffiths *et al.*, 2001).

In our body, Lf and lysozyme often are present together, for example, in milk, tears, neutrophils, and tubotympanum mucus (Weinberg, 2001). Each of the two proteins alone tend to be bacteriostatic, whereas together they are bactericidal. Although the two proteins are not formed by plants in nature, strains of rice have been genetically modified to express production of the compounds. Chicks fed the modified strains have improved feed efficiency comparable to what would be achieved by feeding antibiotics (Humphrey *et al.*, 2002).

D. Potential Hazards in Pharmaceutical Use of Transferrin and Lactoferrin

A major advantage of the two iron-binding proteins over low-molecular-mass iron chelators is that they are human natural products and thus should be suitable for therapeutic use in patients. However, if recipients form polymorphic variants of the proteins, administration of Tf or Lf might trigger immune responses. Indeed, in patients with autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, or primary sclerosing cholangitis, antibodies to Lf have been detected (Weinberg, 2001). Moreover, in disorders such as asthma, Lf adherent to the respiratory tract epithelial lining may contribute to the activation of eosinophils that have infiltrated the airway lumen (Thomas *et al.*, 2002).

Another possible hazard of exogenous Tf and Lf is stimulation of growth of those pathogens that can obtain iron via the proteins. In systemic infections, of special concern are bacterial strains in the families Neisseriaceae and Pasteurellaceae that can form Tf or Lf receptors (Yu *et al.*, 1999). In the stomach, an important potential pathogen is *Helicobacter pylori*. Cells of this bacterium form a 70-kDa protein that binds human Lf. This pathogen also can acquire iron from heme but,

fortunately, not from human Tf (Dhaenens *et al.*, 1997). It is unable, also, to obtain iron from bovine or equine Lf or Tf.

In the female genital tract, *Trichomonas vaginalis* acquires iron from human Lf. Symptoms begin or increase during menses at which time the vaginal concentrations of Lf and iron are considerably higher than at midcycle. In the male urethra, the illness is largely asymptomatic. Although seminal fluid contains Lf, it has very little iron, as does urine. Providentially, *T. vaginalis* cannot obtain iron from human Tf and thus fails to cause systemic infections in either women or men.

Finally, for parenteral use, the protein products extracted from human plasma or from bovine milk must be free of possible prion contamination. As well, media constituents employed in product synthesis by fermentation should be derived from plant rather than animal sources.

III. Low-Molecular-Mass Compounds That Might Counteract Iron Mismanagement

A. General Principles

Discovery of natural products that chelate iron and designing of synthetic iron chelators are relatively uncomplicated tasks as compared with the development of selected compounds into clinically useful drugs. Despite recognition during the past four decades of the great medical need for chelators that specifically could treat iron loading, only two drugs presently are available for long-term clinical use: a natural product, deferoxamine (DFO), and a synthetic compound, deferiprone (DP) (Table III; Fig. 1).

It may be noted that several classes of commonly used drugs have members with iron-chelating properties. Examples include antiinfectives such as tetracyclines, rifamycin, and isoniazid; antineoplastics such as anthracyclines and bleomycin; and antiinflammatories such as salicylate and ibuprofen. However, these drugs were developed without emphasis on their iron-chelating feature or their possible ability to alter iron mismanagement.

The chemical principles underlying the design of clinically useful drugs whose function primarily is that of iron chelation are extensively reviewed in a recent publication (Liu and Hider, 2002).

To achieve *in vivo* efficacy and safety, an iron chelator must possess an array of properties including the following:

1. Chelate iron with high specificity and have low affinity for other metals of physiologic importance, especially zinc.

2. Remove the metal from iron-loaded tissues, cells, and subcellular sites but not from tissues that have normal iron content.
3. After combining with iron, the chelator must refrain from distributing the metal to potentially more dangerous sites such as cardiac tissue or the brain.
4. The chelator should not be able to donate iron to neoplastic and/or microbial cells that might have been overlooked or dormant in the patient.
5. When combined with iron, the chelator must readily be excreted in urine and/or bile.

Additional desirable properties are those sought for any drug such as ease of administration, metabolic stability, low toxicity, compatability with other drugs, and low cost.

An iron chelator that might be effective in cancer, infection, or other inflammatory illnesses need not also be effective or safe in therapy of whole-body iron loading. For the former conditions, the drug would be used for relatively brief periods, whereas employment of an iron

TABLE III

SELECTED FEATURES OF DEFEROXAMINE (DFO) AND DEFERIPRONE (DP)

Feature	DFO	DP
Iron-binding constant[a]		
For iron	30.6	37.2
For copper	14.1	21.7
For zinc	11.1	13.5
Ligand formation	Hexadentate	Bidentate
Ratio of chelator:iron	1:1	3:1
Removal of iron		
From Tf, Lf	No	Yes
From ferritin, hemosiderin	Yes	Yes
From small molecules	Yes	Yes
Redistribution of iron in body	Unlikely	Possible
Effective dose in humans	40–60 mg/kg subcutaneously	50–100 mg/kg orally
Elimination in humans	Urine and feces	Urine
Percentage of drug that binds iron in body	5%	5%
Cost per gram	$20.00 U.S.	30 cents

[a]Data from Liu and Hider (2002).

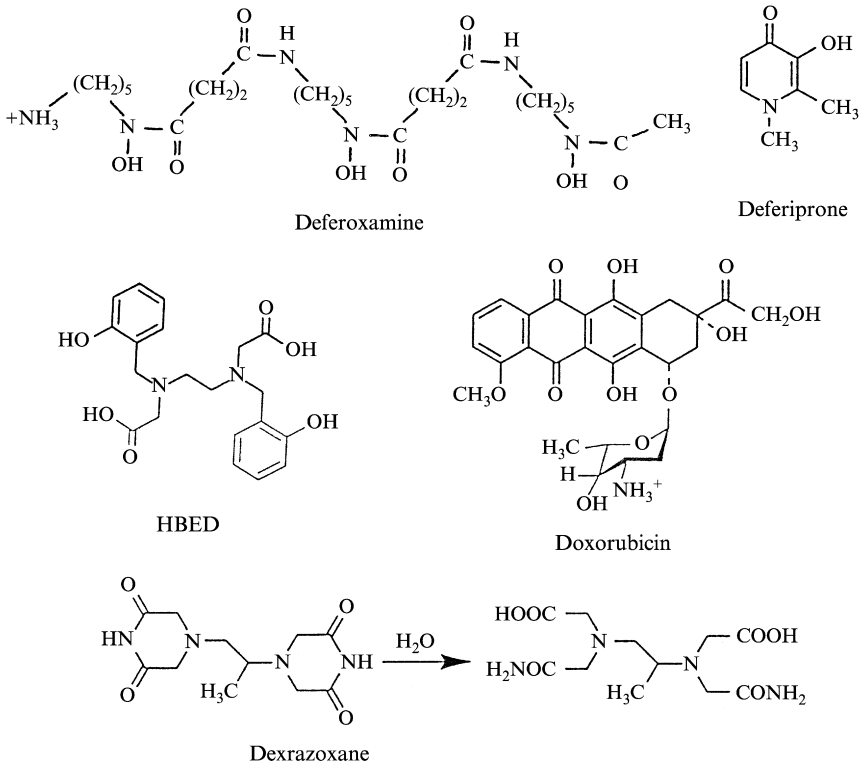

FIG. 1. Formulas of selected iron chelators.

chelator to protect persons who have chronic transfusional and hemoglobinopathic iron loading is required throughout life. Conversly, a chelator instrumental in therapy of whole-body iron loading might be dangerous in patients whose distribution of the metal is greatest at the site of the inflammatory lesion but who have a normal level of iron in the remainder of their body.

B. DEFEROXAMINE

The majority of laboratory and clinical studies on the effect of iron chelators on neoplastic, infectious, and other inflammatory disease conditions have employed DFO as a model. Since 1962, this trihydroxamic acid siderophore of *Streptomyces pilosus* has been the foremost drug available for therapy of transfusional and hemoglobinopathic iron overload. In the present stages of development of newer

iron chelators for possible drug use, clinical experience with DFO in thalassemic patients has been quite instructive.

Each molecule of DFO binds one atom of iron to form a highly stable hexadentate iron complex, feroxamine. The affinity of DFO for other metal ions of physiologic importance is much lower (Table III). The chelator can bind both intra- and extracellular iron. Hepatocyte iron is withdrawn into bile and excreted in feces. Iron derived from erythrocyte breakdown is excreted in urine (Porter, 2001).

In culture studies, DFO inhibits numerous lines of neoplastic cells as well as both intra- and extracellular microbial pathogens. Effective *in vitro* concentrations are compatible with levels of the drug that can be attained safely in humans. The site of inhibitory action generally is considered to be iron deprivation of ribonucleotide reductase, essential for DNA synthesis.

During the decade of the 1990s, several clinical studies of the use of DFO in malaria were conducted (Cabantchik *et al.*, 1999). In a group of 83 young patients, inclusion of DFO with standard antimalarial therapy enhanced clearance of parasitemia and hastened waking from coma. In a second study of 37 asymptomatic adults, infusion of DFO in half the patients lowered their levels of parasitemia. In a subsequent study of 352 children with cerebral malaria, a nonsignificant trend to faster recovery from coma occurred among the survivors of the 175 patients given DFO as compared with those who received placebo. However, no beneficial effect of the drug on clearance of parasitemia, on fever, or on mortality was obtained. The reduced efficacy of DFO in the latter study may have resulted from use of a loading dose of quinine at the time the patients were hospitalized.

In humans with neuroblastoma, cytoreduction of tumor mass was observed in 53 of 57 patients treated with 80 mg DFO/kg/day for 5 days followed by a combination of cyclophosphamide, etoposide, thiotriethylenephosphoramide, and carboplatin (Donfrancesco *et al.*, 1996). Unfortunately, in contrast, in an 85-year-old man with Kaposi's sarcoma (KS), five weekly interlesional injections of DFO resulted in development of numerous KS papules within the drug diffusion area (Simonart *et al.*, 2002b). Treatment with DFO of immunodeficient mice infected with human KS xenografts resulted in marked enhancement of tumor growth compared with controls (230 ± 134 mm^2 vs. 143 ± 70 mm^2) ($p < 0.01$) (Simonart *et al.*, 2002a).

Possibly, feroxamine can serve as an additional source of iron *in vivo* for sarcoma strains but not for neuroblastoma strains. It may be recalled that in dialysis patients, DFO stimulates growth of some *Yersinia* strains and some zygomycetes such as *Rhizopus* (Boelaert *et al.*,

1993). The ability of these strains to utilize feroxamine iron permits them to multiply in the infected host.

Another possible hazard of DFO therapy is ocular toxicity, which may be irreversible upon withdrawal of the drug (Szwarcberg *et al.*, 2002).

C. Deferiprone

Three molecules of 1,2-dimethy-3-hydroxypyridone-4-one (deferiprone; DP) (Fig. 1) are required to bind one atom of iron for bidentate chelation (Table III). This synthetic compound, about one-third the molecular mass of DFO, is lipid soluble and can chelate intracellular iron. Unlike DFO, which must be injected, DP is active on oral administration.

Moreover, in contrast to DFO, DP is much less expensive to produce and does not serve as a siderophore for microbial pathogens (Boelaert *et al.*, 1993; Lesic *et al.*, 1994). However, the quantity of DP required for deironing is double that of DFO. At high doses of DP over several months, cases of arthralgia and agranulocytosis have occurred (Porter, 2001). The side effects usually resolve spontaneously upon discontinuation or lowering the dosage of DP (Balfour and Foster, 1999). The iron complex of bidentate chelators is less stable than that of hexadentate chelators; possibly with DP, some iron might be redistributed to joints or bone marrow rather than excreted in urine.

In an *in vitro* system, low concentrations of DP or congeners enhanced growth of *Mycobacterium avium* within macrophages (Douvas *et al.*, 2002) apparently by facilitating iron uptake by the host cells. High concentrations of DP suppressed intracellular mycobacterial growth. Patients with untreated AIDS often become iron loaded (Weinberg *et al.*, 2002) and are susceptible to microbial opportunists such as *M. avium*. Thus, provided that the dosage is sufficiently high, DP might be a useful antiinfective for such patients.

Deferiprone also has potential use in chelating and removing aluminum, gallium, indium, uranium, and plutonium from metal-contaminated patients (Hoffbrand, 1996). Because it can rapidly penetrate the blood–brain barrier, DP might be a useful chelator of iron in the treatment of cerebral vasospasm (Arthur *et al.*, 1997). Moreover, excessive iron now is recognized to be a risk factor for ischemic heart disease (Sullivan and Weinberg, 1999). Deferiprone has been observed to prevent *in vitro* oxidation of low-density lipoprotein, and, in rabbits, the drug inhibits development of atherosclerosis (Matthews *et al.*, 1997).

In thalassemia major, half of the patients die before age 35. Of the deaths, 60% are due to iron loading in cardiac tissue (Anderson *et al.*, 2002). In a study of 15 patients on DP and 30 on DFO, the latter group

was 5.5 times more likely to have excess myocardial iron. However, patients on DFO tend to have less hepatic iron loading than do those on DP. Combined use of DFO and DP has been proposed for thalassemia patients (Giardina and Grady, 2001).

D. Other Compounds

The hexadentate phenolic aminocarboxylate, *N,N*^{1}-bis(2-hydroxybenzyl)ethylenediamine-*N,N*^{1}-diacetic acid (HBED) (Fig. 1) has been evaluated in monkeys for the treatment of acute iron poisoning (Bergeron *et al.*, 2002). Like DFO, HBED forms a high-affinity 1:1 complex with iron and the chelator is poorly absorbed on oral administration. Upon subcutaneous injection of the sodium salt, HBED was found to be two or three times more efficient than DFO in promoting iron excretion. Moreover, unlike DFO, rapid intravenous administration of HBED did not lower blood pressure or increase heart rate. Possible replacement of DFO by HBED for treatment of chronic transfusional iron overload remains to be determined. In such studies, zinc levels will need to be monitored because the affinity of HBED for this metal is over 1000 times greater than is that of DFO (Hider *et al.*, 1999).

The pyridoxal isonicotinoyl hydrazone (PIH) class of compounds is composed of tridentate ligands comparable to DFO in strength and specificity of iron binding. Substitution of salicylaldehyde or 2-OH-1-naphthylaldehyde moieties for pyridoxal improves *in vitro* antiproliferative activity against cancer cell lines (Richardson and Ponka, 1998). Analogues such as 2-pyridoxal-carboxaldehyde isonicotinoyl hydrazone effectively mobilize mitochondrial iron and thus might be potential therapeutics for patients with Friedrich's ataxia (Richardson *et al.*, 2001). Very strong antiproliferative activity has been demonstrated with the 2-OH-1-naphthylaldehyde analogue (Richardson, 2002). The compounds are easily synthesized, highly lipophilic, permeable in cell membranes, and absorbed from the gut. To prevent hydrolysis of the compounds in the low pH of the stomach, enteric coating is recommended.

Another tridentate iron chelator is 4-(3,5-bis-(2-hydroxyphenyl)-1,2,4-triazole)-1-yl)-benzoic acid (ICL670A). Two of the iron ligands are phenolates and the third is a triazole nitrogen atom. The nitrogen ligand is indicative of a high affinity for zinc (Liu and Hider, 2002). In hypertransfused rats, ICL670A given orally was four to five times more effective than parenteral DFO in promotion of iron excretion (Hershko *et al.*, 2001). In iron-loaded marmosets, the efficacy of the compound was 29% and the effective oral dose was 22 mg/kg (Galanello, 2001). In initial

studies in thalassemic patients, the compound has been well tolerated with no serious adverse effects.

A variety of additional compounds have been reported to possess specific activities by virtue of their ability to withhold or withdraw iron from biological systems. Examples are contained in Table IV.

IV. Low-Molecular-Mass Compounds That Require Iron for an Activity

A. Antimicrobial and Antineoplastic Activity

Some compounds require iron for specific, but not necessarily all, of their biologic activities. For example, the quinone antibiotic streptonigricin needs iron to effect hydroxyl radical–mediated damage in bacteria such as *Escherichia coli* (Yeowell and White, 1982) and *Neisseria gonorrhoeae* (Cohen *et al.*, 1987). Likewise, iron plays a central role in the antineoplastic cell activity of bleomycin (Dabrowiak, 1980). Combination of the metal with the drug releases hydroxyl- and possibly carbon-based radicals that produce single- and double-stranded DNA breaks.

The sesquiterpene lactone, artemisinin (ART), and its derivatives combine with iron or heme to form carbon-centered cytotoxic free radicals (Meshnick *et al.*, 1996). In their degradation of host hemoglobin, malarial protozoa accumulate hemozoin, a heme polymer. With access to this source of heme, ART compounds are useful in therapy of malaria. Moreover, loading iron into neoplastic cells via holotransferrin provides heightened ART activity against breast cancer cells (Singh and Lai, 2001).

B. Host Cell Toxicity

1. *Aminoglycosides*

The antibacterial action of the aminoglycoside antibiotics (amikacin, gentamicin, kanamycin, neomycin, netilomicin, streptomycin, and tobramycin) is not altered by iron. However, the metal forms both 1:1 and 2:1 drug:iron complexes and iron strongly increases toxicity of these drugs for patients (Forge and Schacht, 2000). The 1:1 complex is recognized to be dangerous because of its ability to catalyze formation of hydroxyl radicals (Priuska and Schacht, 1995). The tissues most likely to be harmed by iron-activated aminoglycosides are the proximal tubules of the kidney and the hair cells of the inner ear.

Damage to hosts could be predicted to increase if the drugs are given with iron and to decrease if given with iron chelators. Evidence from

TABLE IV

EXAMPLES OF MISCELLANEOUS IRON CHELATORS WITH VARIOUS BIOLOGICAL ACTIVITIES

Compound	Chemical designation	Activity	System	Reference
Ciclopirox	6-Cyclohexyl-1-OH-4-methyl-2(1*H*)-pyridinone	Antiangiogenic	*In vitro*	Clement *et al.* (2002)
Phytate	Inositol hexophosphate	Antineoplastic	Rodent	Ullah and Shamsuddin (1990)
Tachypyridine	*N*,*N*′,*N*″-Tris(2-pyridylmethyl-*cis*,*cis*-1,3,5-triaminocyclohexane)	Antineoplastic	*In vitro*	Samuni *et al.* (2002)
Triapine	3-Aminopyridine-2-carboxaldehyde thiosemicarbazone	Antineoplastic	Rodent	Richardson (2002)
Aminothiol	Ethane-1,2-bis(*N*-1-amino-3-ethylbutyl-3-thiol)	Antimalarial	*In vitro*	Loyevsky *et al.* (1997)
Daphnetin	7,8-Dihydroxycoumarin	Antimalarial	Rodent	Yang *et al.* (1992)
Dicatecholate	N^4-Nonyl-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine	Antimalarial	*In vitro*	Pradines *et al.* (2002)
Polyphenol	(−)-Epigallocatechin-3-gallate	Neuroprotective	Rodent	Levitas *et al* (2001)
Mimosine	3-OH-4-Oxo-1(4*H*)-pyridinealanine	Radiation sensitizer	*In vitro*	Samuni *et al.* (2001)
Feralex	2-Deoxy-2-[*N*-carbamoylmethyl-(*N*′-2′-methyl-3′-OH-pyrid-4′-one)]-D-glucopyranose	Acute iron detoxification	*In vitro*	Kruck and Burrow (2002)

several research groups indicates that this is indeed the case. When fed iron-enriched diets and injected with gentamicin, rats had increased tubular damage as compared with drug-injected controls on iron-normal diets (Kays *et al.*, 1991). Similarly, gentamicin-treated guinea pigs had elevated hearing injury on high-iron diet (Conlon and Smith, 1998). The amount of dietary iron enrichment in each study was comparable to that consumed by some humans who take iron supplements.

When DFO was injected along with gentamicin, rats had lower renal toxicity (Walker and Shah, 1988) and guinea pigs had lowered ototoxicity (Song *et al.*, 1997). Likewise, ototoxicity in guinea pigs due to injection of neomycin was decreased in animals injected with DFO (Conlon *et al.*, 1998). Note, however, that high prolonged exposure to DFO can cause auditory pathologic changes similar to those seen after much lower, briefer doses of gentamicin (Ryals *et al.*, 1997). The mechanism of toxicity of high concentrations of the DFO–Fe complex is not known.

An iron chelator with fewer side effects than DFO, 2,3-dihydroxybenzoate, was even more effective than DFO in protecting guinea pigs from auditory damage due to gentamicin, kanamycin, and streptomycin (Song *et al.*, 1998). Another iron chelator, salicylate, also successfully protected guinea pigs from gentamicin-induced hearing loss (Sha and Schacht, 1999). Drugs commonly ingested by humans such as aspirin and ibuprofen (Kennedy *et al.*, 1990) are iron chelators but it is not known if these could prevent the aminoglycosides from combining with nonprotein-bound iron in body tissues.

Due to the *large* quantity of iron in tobacco leaves, a one pack per day cigarette smoker inhales over one million picograms of iron per day (Weinberg, 1999b). In a study of nearly 4000 persons (Cruickshanks *et al.*, 1998), active as well as passive inhalation of cigarette smoke itself was observed to significantly contribute to hearing loss. Thus it would seem prudent for cigarette smokers to be especially cautious in using aminoglycoside antibiotics.

2. *Anthracyclines*

For over three decades, doxorubicin (DOX) (Fig. 1), isolated from *Streptomyces peucetius*, has been useful in the management of carcinomas, sarcomas, and lymphomas (Minotti *et al.*, 1999). Unfortunately, when the cumulative dosage of DOX or other anthracyclines exceeds 550 mg/m^2, chronic cardiomyopathy and congestive heart failure frequently occur. Moreover, the toxic effects of DOX are more common in the very young; the drug may severely limit myocardial growth in childhood (Doroshow, 1991).

Doxorubicin forms a 3:1 drug:iron complex that catalyzes generation of free radicals in such sensitive sites as mitochondrial membranes of cardiac myocytes (Hershko *et al.*, 1996). Elevated iron in cultured cardiac myocyes (Hershko *et al.*, 1996) and in humans (Halliwell, 1989) increases DOX toxicity. Removal of iron by chelators such as DP, DFO, or dexrazoxane (DEX) (Fig. 1) lowers DOX cardiotoxicity. Deferiprone efficiently protected rat cardiac myocytes (Barnabe *et al.*, 2002); DFO was active in iron-loaded but not in iron-normal mice (Hershko *et al.*, 1996); and DEX has been developed and approved for humans who are on DOX therapy (Elihu *et al.*, 1998).

Iron chelators are apparently far more potent in lowering cardiotoxicity of anthracyclines than in interfering with the antineoplastic activity of the drugs. This fortunate situation is analogous to the ability of iron chelators to lower nephrotoxicity and ototoxicity of aminoglycosides without significant interference in the antibacterial action of the antibiotics.

Patients with neoplasms often have elevated iron levels caused by multiple blood transfusions as well as by bone marrow failure due to cytotoxic therapy and tumor invasion (Beare and Steward, 1996). Dexrazoxane [(+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane] upon parenteral injection is enzymatically hydrolyzed to an open ring structure (Fig. 1) resembling edetic acid that binds iron (Beare and Steward, 1996). The chelator is effective not only in lowering the metal in cardiac myocytes but also in causing a marked increase in urinary iron excretion (Rajagopalan *et al.*, 1998).

Another possibly useful attribute of DEX is its ability to protect against bleomycin-iron induction of pulmonary fibrosis (Fukuda *et al.*, 1992). It will be of considerable interest to determine if DEX might protect against nephrotoxicity and ototoxicity in patients who are being treated with aminoglycosides.

V. Perspectives

The diversity of medical conditions in which iron loading is discovered to be an underlying risk factor continues to expand. For example, ultraviolet irradiation of skin activates iron catalysis of free radical formation (Kitazawa and Iwasaki, 1999). Excess irradiation plus cutaneous iron accelerates development of photosensitization, photoaging, and skin cancer. Novel amino acid–based iron chelators such as hydroxylbenzyl-serine and -glycine have shown protective activity against ultraviolet cytotoxicity in murine dermal fibroblasts (Kitazawa and Iwasaki, 1999).

Innovative kinds of potentially useful iron chelators would be expected to continue to emerge from screening of plant natural products. Not only do plants employ low-molecular-mass compounds (as opposed to such iron-scavenging proteins as transferrin and lactoferrin) for efficient iron management, but also some of these chelators are indispensable for animals. For instance, in the wild, black rhinos browse on a variety of species of plants, bushes, and trees and thus are able to maintain excellent iron balance (Paglia and Dennis, 1999). In captivity, although the animals are fed the same basic diet as in the wild, they become severely iron loaded and die prematurely of infection, cardiovascular decay, and/or liver failure. Similar iron-loading problems afflict many other kinds of zoo-housed birds and mammals. As the plant chelators become identified, they can immediately be used to benefit the captive animals and, quite possibly, be developed into useful drugs for humans.

Alternative mechanisms for denying iron to microbial and neoplastic cell invaders continue to unfold. For example, gallium is similar to iron in regard to transferrin binding, cellular uptake via transferrin receptors, and incorporation into intracellular proteins. The metal blocks cellular acquisition of iron and inhibits iron-dependent enzymes such as ribonucleotide reductase. Gallium is bactericidal for mycobacterial strains grown extracellularly as well as within human macrophages (Olakanmi *et al.*, 2002). In cancer therapy, gallium is synergistic with other antineoplastic agents. Conjugates such as doxorubicin–gallium–transferrin have become available and are showing useful activity (Collery *et al.*, 2002).

For release of growth-essential iron from transferrin, endosomes and lysosomes must maintain an acidic pH value. Chloroquine, a weak base, can elevate the pH of these organelles. The elevation blocks the intracellular release of iron from holotransferrin, an action that suppresses multiplication within human host cells of pathogens such as mycobacteria, *Legionella* bacteria, and *Leishmania* protozoa (Weinberg, 1999c; Boelaert *et al.*, 2001).

A third method of interfering with transferrin delivery of iron to unwanted invaders employs antitransferrin receptor antibodies (ATRAs) (Weinberg, 1999c). Multivalent ATRAs tend to cross-link the receptors at the cell surface; bivalent ATRAs are endocytosed with the receptors and decrease recycling of the latter. Combination of ATRAs with DFO or with gallium increases the efficiency of ATRAs. As with iron chelators, care must be taken with gallium, chloroquine, and ATRAs to avoid denial of iron to multiplying, normal host cells.

Potential bacterial vaccines that incorporate iron acquisition antigens of the pathogens can stimulate antibodies that starve the invaders of iron. Because the antigenic proteins function at the cell surface of the bacteria, the receptors are ideal vaccine candidates. For synthesis of the receptors, the microorganisms must be cultured in iron-restricted media (Weinberg, 1999a).

In conclusion, a considerable number and variety of iron chelators are being developed or have become available for use in the array of diseases that are exacerbated by iron mismanagement. When combined with methods for prevention of iron loading, the chelators can be expected to become important tools for reduction of disease morbidity and mortality.

Acknowledgments

This chapter is dedicated to Arthur Schade and Leona Caroline in recognition of their far-reaching discovery of transferrin.

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Angular Leaf Spot: A Disease Caused by the Fungus *Phaeoisariopsis griseola* (Sacc.) Ferraris on *Phaseolus vulgaris* L.

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I. Introduction

Angular leaf spot (ALS) is a disease of bean (*Phaseolus vulgaris* L.) caused by the fungus *Phaeoisariopsis griseola* (*Pg*). It occurs in tropical and subtropical countries and has significant economic consequences. In recent years, the incidence of the disease has increased in many

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areas where common bean is cultivated, becoming a serious problem causing losses of up to 30%. In this chapter we summarize the knowledge of many and diverse contributions, and we highlighted what is known and unknown about the disease.

ALS causes important economic losses in *Phaseolus vulgaris* L., mainly in Latin America and Africa, which are responsible for 20% and 12% of the world bean production, respectively. *Pg* has been found in more than 60 countries worldwide (Guzmán *et al.*, 1995). COSAVE (2002) reported the presence of the pathogen in Bulgaria (1936), France, Hungary, and Greece (1965), Iran and Rumania (1929), Russia (Mosque, 1912), Sardinia (1965), Yugoslavia (1929), Austria (1905), Germany (1932), Ireland (1966), Israel, Italy, and Holland (1920), Poland (1936), Portugal (1936), Spain (1934), Suez and Torque (1948), South, East, and West Africa, Asia, the United States, Mexico, and Central and South America. In 1948 and 1949 ALS was considered the most important disease of beans in South New Wales, Australia (COSAVE, 2002). In 1954, the disease caused yield losses of 50% or more in several commercial snap bean plantings in central Wisconsin (Cardona-Avarez and Walker, 1956), but it was not reported again until 1973. Numerous red kidney bean fields in the northwestern part of the state were found to have plants seriously infected late in the season, which resulted in premature defoliation (Hagedorn and Wade, 1974). Melzer and Boland (2001) reported the presence of the disease during the summer of 2000 in commercial fields of green beans in Ontario (Canada).

In recent years, the importance of the disease has increased in Central America, Mexico, Bolivia (Pastor-Corrales *et al.*, 1998), and Argentina (Vizgarra *et al.*, 1999, 2000, 2001; Ploper *et al.*, 2002). ALS is also a major constraint in Africa, where common bean is an important source of food, particularly in Malawi, Ethiopia, Kenya, Uganda, Tanzania, and the Great Lakes Region (which includes Rwanda, Burundi, and the Kivu Province of the Congo) (Pastor-Corrales *et al.*, 1998).

Barros *et al.* (1957) reported yield losses of 30–80% in Colombia; Golato and Meossi (1972) 50–60% in Ethiopia; Crispin *et al.* (1976) 80% in Mexico; Singh and Saini (1980) 79% in India; Ploper (1981) 35–40% in Argentina; Schwartz *et al.* (1981) 80% in BAT 394; and Brenes *et al.* (1983) reported 70% loss in yield in Brazil. Vizgarra *et al.* (1999, 2000, 2001) reported that ALS was one of the most destructive and widespread problems of common bean production in Argentina.

II. The Pathogen

A. Taxonomy

The causative agent of ALS of bean is the fungus presently known as *Phaeoisariopsis griseola* (Sacc.) Ferraris. It is an imperfect (*Deuteromycotina*) fungus in the class Hyphomycete, order Moniliales, family Stilbaceae, with different synonyms (Table I).

The fungus was originally described as *Isariopsis griseola* by Saccardo in 1878: ''*maculis ochraceis, hypophyllis, fasciculis stipitiformibus dense gregariis, 200∼30–40, fuscidulis, ex hyphis filiformidus, contiunuis, dense stipatis conflatis apiceque griseo-capitatis; coniciis ex hypharum apicibus patulis v. reflexis sublevibus oriundis, dense conglobatis, cylindraceo-fusoides, curvulis, 50–70 ∼ 7–8, 1–3 septatis, non v. vix constrictis griseis. In pagina inferiore foliorum Phaseoli vulgaris, Treviso Ital. Bor., Plezzo Austriae, Boca del Riachuelo Argentinae.*'' Ellis (1881) described a fungus in the United States under the name *Graphium laxum* with characteristics similar to *Isariopsis griseola* Sacc. In 1886, Saccardo considered *Graphium laxa* to be synonymous to *Isariopsis griseola* and renamed the species *Isariopsis laxa*. Later, Ferraris (1909) described the same fungus and used the name *Phaeoisariopsis griseola* (Sacc.) Ferraris to accommodate the species *Isariopsis griseola* Sacc. In 1971, Ellis confirmed this name.

TABLE I

Synonyms of *Phaeoisariopsis griseola* (Sacc.) Ferraris

Synonyms	Reference
Isariopsis griseola Sacc.	(1878) *Michelia* **1,** 273.
Graphium laxum Ell.	(1881) *Bull. Torrey Bot. Club* **8,** 65.
Cercospora solimani Speg.	(1886) *An. Soc. cient. Argent.* **22,** 214.
Isariopsis laxa (Ell.) Sacc.	(1886) *Syll. Fung.* **4,** 631.
Cercospora columnaris Ell. & Ev.	(1894) *Proc. Acad. Natl. Sci. Philad.* **46,** 380.
Arthrobotryum puttemansii Henn.	(1902) *Hedwigia* **41,** 309.
Cercospora stuhlmanni Henn.	(1904) *Bot. Jb.* **33,** 40.
Cercospora griseola (Sacc.) Raganath. & Ramakr.	(1968) *J. Madras. Univ. B.* **35–36,** 11.
Phaeoisariopsis laxa (Ell.) Jong & Morris	(1968) *Mycopathol. Mycol. Appl.* **34,** 269.
Pseudocercospora columnnaris (Ell. & Ev.) Yen	(1980) *Gardens Bull. Singapore* **33,** 172.

The accepted name is *Phaeoisariopsis griseola* (Sacc.) Ferraris. The genus *Phaeoisariopsis* Ferraris has one characteristic in common with *Isariopsis* Sacc., which is the development of synnemata (Hocking, 1967).

B. Morphological Characteristics

Pg develops conidiophores that are macronematous, mononematous, and cespitose or synnematous (Fig. 1). The synnema are brown-dark, 100 μm long or longer (250–500 μm), and 20–70 μm wide. The individual conidiophores terminate in slightly swollen integrated conidiogenous cells, which form successive conidia at the tip by sympodial growth, sometimes geniculate, cicatrized, pale brown to brown, smooth, septate, 2–4 μm wide and 4–6 μm wide at the apex. Conidia are acrogenous, becoming acropleurogenous, solitary, cylindrical to obclavate, rounded at the apex, truncate at the base, usually curved, pale olivaceus brown, 1–6 septate, 30–70 μm long, 5–8 μm wide, and 1.5–2 μm wide at the base (Miles, 1917; Llanos, 1957; Olave, 1958; Díaz *et al.*, 1965; Hocking, 1967; Ellis, 1971; Brown and Brotzman, 1979; Schwartz and Gálvez, 1980; CMI, 1986; Deighton, 1990; Barnett and Hunter, 1998; Melzer and Boland, 2001; COSAVE, 2002). Karanja *et al.* (1994) isolated the fungus from cultivar Rosecoco-GLP-2 in Nairobi, Kenya and showed that some conidia were Y-shaped. The number of septations varied from 1 to 11 and the size ranged from 17 to 95 μm in length to 7 to 24 μm in width. Additional physiological characterizations should be performed to confirm that this is a representative of *Pg*.

Walker and White (1991) studied the ontogeny of conidia and the sympodial proliferation of the conidiogenous cell. Conidia are formed holoblastically at the apex of the conidiogenous cell. When a conidium is shed, the conidiogenous cell grows on around the conidial scar, tearing the outer wall of the cell as it grows through it. Proliferation thus is enteroblastic and sympodial. The broken area of the wall, through which the conidiogenous cell proliferates, can be seen on the conidiophore as a thin jagged tear line, encircling it just above the spore scar. This process is repeated every time proliferation occurs, so that a series of conidial scars and wall tear lines develops on the elongating conidiogenous cell.

Miles (1917) and Monda *et al.* (2001) observed that young hyphae are nonseptate in the early stages of fungal growth and that there was no septum delimiting the appressorium-like structures from the germ tubes and/or young hyphae.

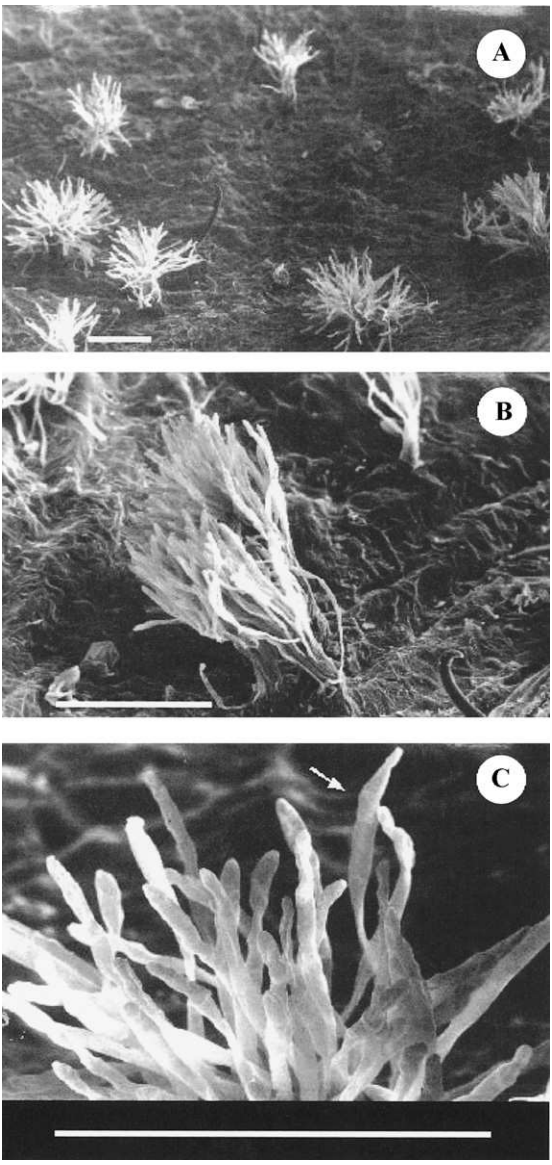

FIG. 1. Scanning electron microscopy showing synnemata on *Phaseolus vulgaris* L. cultivar TUC 500. (A) Synnemata on the abaxial leaf surface, ×150. (B) Typical synnemata, ×350. (C) Conidia at the tip of a conidiophore, ×1000. Scale bars = 100 μm.

Deighton (1990) remarked that the arrangement of the conidiophores in synnemata is not a good character for identification, because it can also be seen in other hyphomycetus genera. In addition, the degree of darkening of the conidial scars is variable in members of other groups such as the ''*Cercospora*-complex''. The most appropriate classification character, in combination with the synnematal conidiophores, seems to be the displacement of the old conidial scars so that they lie flat against the sides of the conidiogenous cells (Deighton, 1990).

C. Host Range

Dinoor (1974) defined host range as the list of species upon which a pathogen can provoke disease, though no standard procedures govern creation of such a list. Isolated fungi may be pathogenic on different plants but may have not been tested for pathogenicity on the host of economic interest. In fact, collateral hosts may be a source of pathogen populations with unknown pathogenic capacity on agricultural hosts. This is significant, because collateral hosts of *Pg* could play an important role as an inoculum source. Several species may function as alternative hosts of *Pg* since the disease has been detected on several species. Chupp [1929 results cited in Olave (1958)] mentioned the occurrence of ALS on *Pisum sativum*. Brock (1951) tested more than 150 *Phaseolus vulgaris* and two *P. coccineus* accessions, and observed infection on 140 cultivars of *P. vulgaris* and one cultivar of *P. coccineus*. Cardona-Alvarez and Walker (1956) described severe natural infections on beans (*P. vulgaris*) and also found symptoms of the disease on lima bean (*P. lunatus*). Although Abramoff [1931 results cited in Cardona-Alvarez and Walker (1956)] reported the presence of the disease on several varieties of soybean (*Glycine max*), Cardona-Alvarez and Walker (1956) were unable to find evidence of the disease on soybean. Díaz *et al.* (1965) detected low to moderate levels of infection on leaves of cowpea (*Vigna unguiculata*) plants. Campos and Fucikovsky (1980) evaluated susceptibility in a glasshouse on 30-day-old plants and found typical lesions in *P. acutifolius, P. angularis, P. calcaratus, P. coccineus, P. lumatus*, and *P. vulgaris*.

Pg is the causative agent of one of the most important diseases on cowpea in the humid mountains in Brazil (Pontel and Alameida, 1994). In addition, there also are records of the disease on *Mucuna capitata, Pachyrhizus* sp., *Phaseolus nanus, P. sinensis, Pisum sativum* (Jong and Morris, 1970), *Dolichos lablab* in Japan (Deighton, 1990), *Desmodium cephalotus, D. gangeticum, D. pulchellum, Phaseolus mungo*, and *Hibiscus esculentus* (*Malvaceae*) (CMI, 1986).

The fungus appears to have several alternative hosts. Should it become necessary, resistant species also could be useful as sources of resistance in wide-cross introgression breeding programs. The existence of alternative hosts of *Pg* is not only important, but it also might have epidemiological implications. Alternative host may reduce the value of rotations upon spore dispersal. Alternatively, identification of resistance in other species might well provide sources of resistance for breeding programs.

III. The Disease

The host–pathogen interaction is dependent upon the genome of each organism, their interaction, plus the effects of the environment. Year to year any given environment is likely to affect pathogen growth and reproduction, which is directly related to the different parasitic strategies that, in turn, may affect the genetics and epidemiology of the disease (Burdon, 1993). Whether the environmental conditions have been favorable for colonization, infection, and growth of the pathogen on a crop will be determined by the plant and pathogen genomes. Such interactions at plant, cellular, and molecular levels are of great interest for a better understanding of the processes involved in disease development. This knowledge may eventually lead to a more rational and effective control of the disease.

A. Symptomatology

Conidia of *Pg* inoculated on leaves of susceptible beans, germinated 4 h after inoculation at either one, or both ends of the spore (Wagara *et al.*, 1999; Monda *et al.*, 2001) or at its sides (Wagara *et al.*, 1999). Germ tubes grew following the contours of epidermal cells on the leaf surface (Monda *et al.*, 2001). Penetration occurred 24 h after inoculation (Wagara *et al.*, 1999) through stomata (Cardona-Alvarez and Walker, 1956; Wagara *et al.*, 1999; Monda *et al.*, 2001), either by formation of an apressorium-like swelling over the stoma or without it (Monda *et al.*, 2001). As the fungus grew in the substomatal cavity, chloroplasts of the cells adjacent to the stomatal cavity stained red, contrasting with normal green chloroplasts (Cardona-Alvarez and Walker, 1956). Three days after infection, necrosis of the guard cells and adjoining mesophyll cells occurred, and the chloroplasts showed signs of disintegration. The damage to the host plasma and chloroplast membranes has been attributed to the action of a toxin produced by the pathogen, though it has not been isolated yet (Monda *et al.*, 2001). After

infection, in 6–10 days, the fungus colonized most of the intercellular spaces of the palisade cells of the leaf, resulting in cell destruction. Hyphal growth was found to be intercellular by Cardona-Alvarez and Walker (1956) and Monda *et al.* (2001). Furthermore, Monda *et al.* (2001) found that *Pg* did not penetrate leaf veins, probably due to the lack of easily colonizable intercellular spaces. However, Wagara *et al.* (1999), as a result of observations made with a light microscope, reported intercellular but also intracellular colonization. Once hyphal growth occurred and the intercellular space was colonized by mycelia, the conidiophores emerged through the stomata (Monda *et al.*, 2001). Synnemata were formed in association with lesions and conidia were formed at the tip of the conidiophores, generally on the leaf undersurfaces (Cardona-Alvarez and Walker, 1956; Hagedorn and Wade, 1974; Schwartz and Gálvez, 1980; Monda *et al.*, 2001).

Wagara *et al.* (1999) found that resistance to the disease involves either inhibition of conidial germination, colonization, sporulation, or a combination of the three. These observations are in agreement with those obtained by Guzmán *et al.* (1995), who reported that host resistance to *Pg* appears to be partial rather than absolute. The resistance of *Phaseolus vulgaris* line M 26 to *Pg* was probably due to the inhibition of spore germination, colonization, or sporulation, whereas the delayed and limited sporulation of the fungus on common bean line M 29 was an intermediate response. Cultivars showed different levels of tolerance to *Pg*, which was related to the time it take symptoms to appear and the extent of the severity attained (Buruchara *et al.*, 1988). On susceptible cultivars infection started earlier and was spread rapidly along a larger leaf area. On resistant cultivars it took more time for symptoms to develop and the area affected by lesions grew more slowly (Buruchara *et al.*, 1988).

The role of spore adhesion and attachment to the plant surface in governing infection and ALS disease development is unknown. As in other plant–pathogen interactions like rust (Heath, 1997) and downy mildew (Kiefer *et al.*, 2002), spore germination and germ tube growth might be targeted by host factors. Microscopic observations in combination with biochemical analysis should provide evidence regarding the role of spore adhesion upon fungus–plant recognition, and also about the nature of the process that governs the direction of *Pg* germ tube growth and penetration.

In the field, the first symptoms of ALS appear during the early stages of plant growth on primary leaves. However, the disease does not become conspicuous until late flowering or early pod set (Barros *et al.*, 1958). The lesions on diseased plants are small (0.5 cm), irregular,

brown to gray spots lying in the angles of dichotomized branched leaf veins (Barros *et al.*, 1958; Hocking, 1967; Pastor-Corrales, 1985; Cardona *et al.*, 1997; Ribeiro do Vale and Zambolim, 1997). Symptoms are more evident on leaves 9 days after infection, when lesions are grayish turning to light brown and are assuming the typical angular shape (Hagedorn and Wade, 1974). Hocking (1967) observed and described a highly virulent form of *Pg* isolated from beans. Infected beans bore regular circular brown lesions up to 2 cm in diameter. High levels of lesions resulted in a substantial reduction of the leaf area, which reduces the plant photosynthetic capacity during seed filling when the demand for photosynthates is highest (Cole, 1966; Hagedorn and Wade, 1974; Schwartz and Gálvez, 1980; Cardona *et al.*, 1997).

Lesions may appear on different plant organs, including stems and pods. Lesions on the stems are elongate in shape and brown in color (Hagedorn and Wade, 1974; Schwartz and Gálvez, 1980; Cardona *et al.*, 1997). Pod lesions are oval to circular with reddish-brown centers surrounded by darker colored borders (Barros *et al.*, 1958; Hagedorn and Wade, 1974; Schwartz and Gálvez, 1980; Cardona *et al.*, 1997). Polanco (1970) obtained isolates from the hilum and superficial integument of certified and registered bean seeds. Infected pods may contain poorly developed or entirely shriveled seeds that may carry the fungus internally or on the surface. Seed became infected only when they were attached to the pod suture directly beneath a lesion (Pastor-Corrales *et al.*, 1998). Dhingra and Kushalappa (1980) showed that fungal growth always occurred at the hilum and that the seeds were infected by the pathogen only when the lesions were present at the suture. Sengooba and Mukiibi (1986) studied the behavior of the fungus in pods of several cultivars and found that it grows and sporulates on the hilum.

B. Physiological Specialization

The terms ''pathogenicity'' and ''virulence'' are likely to be used to describe the ability of an organism to cause disease. Pathogenicity is regarded as a general attribute of a species, whereas virulence is an attribute reserved for a particular strain of a pathogen in relation to a particular host genotype (Day, 1960). There exist virulent races of *Pg* that interact with common bean hosts in a highly specific manner. This suggests that host specificity attributes are superimposed on the general pathogenic ability of *Pg*.

Variation in virulence in the population of *Pg* is essential in understanding the interaction of the genomes involved in ALS. Studies of

the diversity of virulence within a pathogen population should help in the development of a successful disease management program, particularly resistant cultivars.

Several investigators have described diversity among *Pg* isolated from different areas around the world. Brock (1951) found that 13 Australian isolates differed in their virulence. Hocking (1967), based on the assumption that symptoms of infected plants are indicative of isolates virulence, described the identification of a highly virulent strain that provoked unusually large lesions on leaves. Alvarez-Ayala and Schwartz (1979) found that three of four isolates of *Pg* were virulent and caused disease on cultivar Caraota 260, which was known to be resistant to ALS in Brazil. The authors also found that *Pg* isolates showed different levels of virulence on specific cultivars. Lacerda *et al.* (1994) tested the ability of 14 isolates from Pernambuco (Brazil) to provoke disease on four bean cultivars, and found that isolates interaction resulted in a differential response. Pastor-Corrales and Jara (1995) were the first to study the genetic variability of *Pg* by means of a differential series of *P. vulgaris* cultivars and reported the existence of great variability among the pathogen.

Common beans have two pools of origin, and because both hold populations of the pathogen, several researchers have asked whether the organisms coevolved in both gene pools of origin. Guzmán *et al.* (1994), found that Mesoamerican materials bred for ALS resistance showed higher levels of resistance to Andean than to Mesoamerican isolates of the fungus. They included in their studies *P. vulgaris* cultivar A 240, a Mesoamerican material that is a poor source of disease resistance genes, as Brazilian isolates of *Pg* coevolved with Mesoamerican materials. By means of molecular markers Pastor-Corrales and Jara (1995), Maya *et al.* (1995), and Guzmán *et al.* (1995) showed a clear association between virulence and phylogeny of *Pg* by using 5, 6, and 20 random amplified polymorphic DNA (RAPD) primers, respectively. Both studies revealed polymorphisms among *Pg* isolates and clustered them into two major groups. The response of *P. vulgaris* cultivars challenged with *Pg* from both gene pools suggested that one group evolved with Mesoamerican beans and the other one with the Andean gene pool. Chacón *et al.* (1997) provided more evidence by means of three different tools: virulence, isozymes, and RAPD markers. They compared the genetic diversity of 33 isolates from Africa and 52 from South America. They found that Andean isolates, collected from large-seeded bean cultivars, were predominant in Ecuador, Colombia, and Africa. On the other hand, Mesoamerican isolates collected from small-seeded cultivars from the

Mesoamerican gene pool were predominant in Central America and Mexico.

Boshoff *et al.* (1996), by means of isoenzymatic analysis, studied the variability among 14 isolates of *Pg* from South Africa, 12 isolates from Malawi, and one isolate from Portugal, one from the Netherlands, and one from Zimbabwe. They found different electrophoretic patterns. Type I was the most common and included all South African isolates, 10 Malawian isolates, and the isolate from Portugal. Electrophoretic type II included two *Pg* from Malawi and the isolate from the Netherlands. The two different electrophoretic types among Malawian isolates are consistent with previous findings by Guzmán *et al.* (1995). They suggested the coexistence of two groups of *Pg* in Malawi, each with their specific hosts. Boshoff and colleagues suggested that only isolates from one group were associated with the large-seeded bean cultivars that are most commonly cultivated in South Africa. However, clustering of the isolates in only one group might have been the result of the number of isolates included in the study, which was not large enough to allow the detection of the second virulence group among South African isolates.

Coevolution of *Pg* and the host *P. vulgaris* in two different and distant geographic areas might lead to a more specific interaction between the host and the pathogen. Though the isolates can be clustered into Mesoamerican or Andean groups, finer levels of variability also exist. Liebenberg *et al.* (1996) inoculated 15 *Pg* isolates onto nine bean cultivars. Among the isolates of the pathogen, 13 were Andean specific and two were Mesoamerican specific. They found that all Andean isolates caused severe infection on two of the Andean cultivars, and low to moderate infection on the remaining cultivars. The reaction of the large-seeded cultivar G 5686 to the Andean specific isolates varied from resistance to susceptibility. These findings support the hypothesis of coevolution of the host and the pathogen proposed by Guzmán *et al.* (1995) and also demonstrate that African isolates are genetically similar to South American isolates. This suggests that the American continent was the common place of origin for the disease and most probably the source inoculum that was spread through contaminated seed.

Pastor-Corrales *et al.* (1998) characterized 433 isolates of *Pg* from 11 Latin American and 10 African countries by using the six Andean differential genotypes (Don Timoteo, G 11796, Bolón Bayo, Montcalm, Amendoin, and G 5686) and six Mesoamerican genotypes (PAN 72, G 2858, Flor de Mayo, Mexico 54, BAT 332, and Cornell 49–242), isozymes, and/or RAPD markers. The analysis clustered the isolates into

two major groups: Andean and Mesoamerican. Among each groups they found differences not only at the biochemical and molecular level, but also in virulence. The Andean *Pg* isolates were more virulent on common beans of Andean origin, confirming the coevolution hypothesis suggested by Guzmán *et al.* (1995). They concluded that the Mesoamerican *Pg* isolates, although more virulent on common beans from Mesoamerica, can also infect and provoke disease on Andean beans, thus exhibiting a much broader virulence spectrum.

Busogoro *et al.* (1999a) tested a collection of 54 isolates. Forty-four isolates originated from countries of the Great Lakes Region of Africa and 10 isolates were collected in Brazil and Colombia. When the isolates of *Pg* were inoculated on 29 plant genotypes (17 species of *Phaseolus vulgaris*, 6 species of *P. coccineus*, and 6 species of *P. polyanthus*) the isolates were highly divergent in virulence. They described the existence of many pathotypes, which were defined by both inoculation of different plant genotypes and RAPD markers. They demonstrated that most of the isolates (53 of the 54 isolates analyzed) exhibited different virulence profiles, whether isolated from the same place or from different locations. However, they were unable to cluster the isolates into two major groups in accordance with their place of origin like Guzmán *et al.* (1995) and Chacón *et al.* (1997). Busogoro *et al.* (1999a), based on the fact that genotypes of Andean gene pool are predominant within the African Great Lakes region, assumed that most probably the isolates were collected from the same common bean gene pool. Therefore it is likely that they all belong to the same group. Recently, Mahuku *et al.* (2002), by means of random amplified microsatellites (RAMS), restriction fragment length polymorphism of the amplified ribosomal intergenic spacer region (IGS-RFLP), and RAPD molecular markers demonstrated that Afro-Andean isolates do not represent a new group within *Pg* but are Andean isolates that have evolved to colonize beans from the Mesoamerican gene pool. In addition, this study revealed significant levels of geographic differentiation within the Andean group. All the isolates from Africa were grouped in a cluster distinct from that occupied by isolates from Latin America.

Guzmán *et al.* (1999) analyzed *Pg* isolates from Argentina, Brazil, Costa Rica, Malawi, and the United States by means of RAPD markers generated with primer, OPA 11. They amplified a 390- and 690-bp fragment from Andean and Mesoamerican *Pg* isolates, respectively. These fragments are conserved among the isolates, being a useful tool for typing isolates of the fungus. Guzmán and colleagues usually found Andean isolates on Andean genotypes, whereas Mesoamerican isolates were found on Mesoamerican genotypes (Guzmán *et al.*, 1999). These

findings confirmed those of Pastor-Corrales and Jara (1995) that challenged Mesoamerican and Andean cultivars with isolates of the fungus from both places of origin and found that susceptible interactions occurred when the organisms belong to the same pool of origin. Guzmán *et al.* (1999) was able to isolate conidia from one lesion that were identified by polymerase chain reaction (PCR) as members of both Andean and Mesoamerican gene pools, suggesting that more then one fungal isolate can coexist and be responsible for causing disease. In addition, this finding suggests that a Mesoamerican isolate incompatible with an Andean host can use an Andean isolate infection to evade the plant mechanisms of resistance.

Sartorato (2000, 2002) tested 12 differential cultivars (Pastor-Corrales and Jara, 1995; Pastor-Corrales *et al.*, 1998) with 42 and 51 isolates from Brazil, respectively, and identified seven different pathotypes. He found that the major pathotypes in Brazil were races 63.31 and 63.63 (see Section IV). Nietsche *et al.* (2001), by means of molecular markers, found that among 30 isolates from the state of Minas Gerais (Brazil) all belong to the Mesoamerican gene pool. However, they still found wide genetic variability of the pathogen, as among 30 isolates 13 races were identified. Race 63.63 was the most virulent and race 63.23 was the most frequent. A bean cultivar from the Mesoamerican gene pool, Mexico 54, was found to be a valuable source of resistance to the pathogen. Nietsche *et al.* (2001) suggested that the 12 differential cultivars should be revised so that additional sources of resistance are included. This was based on the observation that two isolates collected in Lavras-MG were classified as race 63.63 yet virulent in all varieties of the differential series. Five RAPD primers (OPA 02, OPA 03, OPA 04, OPA 10, and OPA 18), recommended by Pastor-Corrales and Jara (1995), were used for the study of genetic diversity. They found that most of the isolates belong to the Mesoamerican gene pool. These molecular data did not allow the authors to group the isolates in races or by place of origin, due to the low number of primers used (Nietsche *et al.*, 2001). They concluded that the genome of the isolates amplified by the primers may not be correlated with virulence genes.

It is interesting to note that though coevolution of bean and *Pg* resulted in specific interactions, both gene pools of origin still support quite diverse organisms. Furthermore, the Andean gene pool appears to support races of the pathogen with a narrow and specific cultivar range, with the ability to infect only Andean materials. On the contrary, the Mesoamerican gene pool of origin supports populations of *Pg* with a much broader and nonspecific cultivar range, and these provoke disease not only on Mesoamerican cultivars but also on Andean ones. This

suggests that coevolution of the pathogen in both gene pools of origin was not only different but also under different selection pressures.

IV. Race Determination

The identification of pathogenic races is important for disease resistance breeding and for the efficient use of available resistant cultivars. A physiological race is defined by a group of cultivars that can host the pathogen, where this race can express virulence at different levels. In general, most plant pathologists favor the use of a population originated from a single spore or isolates from a single lesion. Therefore spores are multiplied separately and then inoculated onto a set of differential hosts.

The most traditional method to test susceptibility uses a monosporic culture of *Pg* grown on V8 juice agar medium (per liter: 200 ml of V8 juice, 3 g $CaCO_3$, and 18 g agar). Differential cultivars are inoculated on both sides of the first trifoliolate leaf with a conidial suspension adjusted to 2×10^4 conidia/ml (Brenes *et al.*, 1983). The plants are incubated for 48–96 h in a humid chamber (relative humidity of 95%) at 24°C. The inoculated plants are moved to the greenhouse bench at 22–30°C for about 10 days, until they develop symptoms (Santos-Filho *et al.*, 1976a; Beebe and Pastor-Corrales, 1991; Guzmán *et al.*, 1995; Pastor-Corrales and Jara, 1995; Bassanezi *et al.*, 1998; Pastor-Corrales *et al.*, 1998; Busogoro *et al.*, 1999a; Guzmán *et al.*, 1999; Nietsche *et al.*, 2001).

Susceptibility or resistance to the disease is evaluated 10–17 days after inoculation on a **1–9** symptom scale (van Scoonhoven and Pastor-Corrales, 1991) as follows: **1**, plants with no symptoms; **3**, plants with 5–10% of the leaf area with lesions; **5**, plants with 20% of the area with lesions and sporulation; **7**, plants with up to 60% of the leaf area with lesions, frequently associated with chlorosis and necrotic tissues; **9**, plants with 90% of the leaf area with lesions, frequently associated with early loss of the leaves and plant death. Plants with a score of 3 or higher were considered susceptible.

Races were determined by means of a binary value proposed by Pastor-Corrales and Jara (1995) and Pastor-Corrales *et al.* (1998) (i.e., race ***7.31*** for isolate "**II**"). The first number was obtained by adding the binary values of the susceptible Andean differential cultivars. Each was given a letter: ***a, b,*** *c*; **1 + 2 + 4 = 7**. The second number was obtained by adding the binary values of the susceptible Mesoamerican cultivars: ***g, h, i, j, k***; **1 + 2 + 4 + 8 + 16 = *31*** (Table II).

Liebenberg (1995) described an alternative method for the identification of pathotypes of *Pg*, with detached leaves from each of the

TABLE II

DETERMINATION OF RACE

Lines	Gene pool	Binary value
a. Don Timoteo	Andean	1
b. G 11796	Andean	2
c. Bolón Bayo	Andean	4
d. Montcalm	Andean	8
e. Amendoin	Andean	16
f. G 5686	Andean	32
g. PAN 72	Mesoamerican	1
h. G 2858	Mesoamerican	2
i. Flor de Mayo	Mesoamerican	4
j. Mexico 54	Mesoamerican	8
k. BAT 332	Mesoamerican	16
l. Cornell 49–242	Mesoamerican	32

Example[a]

	Differential Andean beans						Differential Mesoamerican beans						
Isolate	**a**	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**	**k**	**l**	Race
I	a						g	h	i		k	l	1.55
II	***a***	***b***	***c***				***g***	***h***	***i***	***j***	***k***		***7.31***
III	a	b	c	d	e		g		i		k		31.21
IV	a	b	c	d	e	f	g	h	i	j	k	l	63.63

[a]Letters **a** to **l** indicate susceptibility of the respective differential common bean genotype to the specific isolate of *Pg*.

differential cultivars. Nietsche *et al*. (1999) compared the traditional method used to characterize the pathogen with a method that used rooted trifoliolate leaves. Four isolates were tested by both methods and they successfully characterized the pathotypes 63.23, 31.30, 31.23, and 31.21. Rooted leaves proved to be an important alternative in the characterization of *Pg* in pathotypes, as it allows examination of a large number of isolates in a reduced space and also because a genetically identical host can be used.

McDonald (1997) stated that knowledge of genetic diversity is needed for resistance deployment to be effective and also to identify shifts that may occur in race or population structure. Pathogenic tests are cumbersome and time consuming, require extensive facilities, and

are influenced by variability inherent to the experimental system. Furthermore, pathogenicity data alone provide no information about genetic diversity within, or relatedness among, races of the pathogen. Neutral markers are especially useful to identify races of pathogens with asexual reproduction, as no recombination occurs and the entire pathogen genome is effectively linked (Milgroom and Frey, 1997).

Methods involving analysis of mitochondrial, ribosomal, or total DNA may be desirable to have a more rapid, and less labor-intensive method of distinguishing pathotypes and/or races. Therefore molecular markers or characters to be used as a diagnostic tool for the fast and precise identification of ALS should be identified.

V. Disease Cycle

The disease cycle of ALS (Fig. 2, see color insert) depends upon host, pathogen, and temporal and environmental components of the pathosystem.

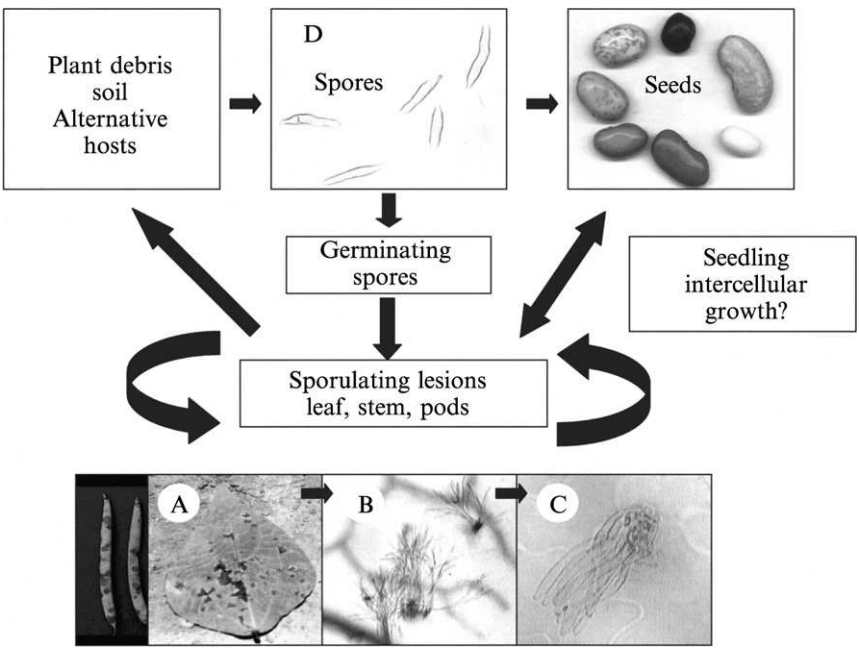

FIG. 2. Asexual cycle of *Phaeoisariopsis griseola* (Sacc.) Ferraris. (A) Pods and leaf showing typical lesions provoked by *Pg.* (B, C) Light microscope (LM) photographs of synnemata protruding from a lesion of a bean leaf infected with the fungus. (D) Conidia of *Pg* observed under the LM.

A. Infection

The site of contact between a pathogen and a host cell is known as the host–pathogen interface (Lucas, 1998). Factors influencing fungal spores, adhesion and germination are of special significance, and among them humidity, temperature, pH, and nutrient availability are the most significant.

Infection and colonization by *Pg*, as well as ALS disease development, occur within temperatures between 16 and 28°C with an optimum at 24°C (Cardona-Alvarez and Walker, 1956; Hagedom and Wade, 1974; Schwartz and Gálvez, 1980; Inglis and Hagedom, 1986; Saettler, 1991; Cardona *et al.*, 1997). No infection was detected with temperatures over 32°C (Cardona-Alvarez and Walker, 1956). Therefore, in temperate regions temperature is not a limiting factor for disease development (Cardona-Alvarez and Walker, 1956). Bassanezi *et al.* (1997) found that infection, on bean cultivars Rosinha G-2 and Carioca, occurred at temperatures between 15 and 30°C. In general, high temperatures with an optimum for disease severity between 24 and 28°C (Bassanezi *et al.*, 1998) favored the occurrence of ALS. Several authors have concluded that the most favorable environmental conditions for the development of an epidemic of the disease are moderate temperatures and high RH, >95% (or a water film on infected foliage, stems, and pods) for at least 48 h, with alternating periods of low humidity (Cardona-Alvarez and Walker, 1956; Schwartz and Gálvez, 1980; Beebe and Pastor-Corrales, 1991; Cardona *et al.*, 1997).

Once the fungus has infected the tissue, disease development proceeded even in a relativity dry atmosphere and even under these conditions stromata in substomatal cavities are formed. The stroma remaining among leaf tissue after destruction of the host cells remains dormant. When the environmental conditions become favorable, regrowth of the fungus occurs, conidiophores emerge and form synnemata, and finally sporulation occurs (Monda *et al.*, 2001). Although 24 h of high RH is enough to allow the development of synnemata, a longer period of high RH is necessary for the fungus to sporulate profusely (Cardona-Alvarez and Walker, 1956).

Whenever pathogens are not growing within a host, they face the problem of survival in a potentially hostile environment. The problem for any particular pathogen to survive depends not only on the period of time it takes to invade the host cells, but also on the relative hostility of the environment (Lucas, 1998). *Pg* successfully overwintered in stem and pod tissues of infected cultivar Montcalm plants that were left in the field during the winter. The pathogen was recovered from tissue

samples collected for five consecutive months starting in December 1983 (Correa and Saettler, 1987). The pathogen can survive up to 140 days (140–500) in soil or plant tissues (Cardona-Alvarez and Walker, 1956; Sohi and Sharma, 1967; Sindhan and Bose, 1979). The fungus that survived for 9 months on plant debris or those seeds left in the field after harvest are most probably more important sources of primary inoculum than stored seed (Orozco-Sarria and Cardona-Alvarez, 1959; Sindhan and Bose, 1979). Because the fungus is dependent upon viable conidia for primary infection, the stromata are the most important overwintering structures (Cardona-Alvarez and Walker, 1956). Under favorable environmental conditions, new synnema and conidia are produced, and they comprise the primary source of fungal inoculum (Cardona-Alvarez and Walker, 1956; Sohi and Sharma, 1967). Sengooba and Mukiibi (1986) studied the survival of *Pg* in crop debris and found that longer periods of storage (laboratory, shade, outside, and soil) increased the number of conidiophores in the synnemata, which also became fewer and less compact. Infected straw in the soil did not form synnemata, but after 31 days produced conidia on solitary conidiophores. No conidiophores were found after 63 days of storage of stroma (Sengooba and Mukiibi, 1986). The results described above suggest that crop management, including rotations, might alter the survival ability of the pathogen.

The host–pathogen interface is crucial to understanding the nature of the different host–pathogen interactions, where molecular communications between the two partners occur. This interesting process needs to be studied in detail, because it is not only the site but also the moment where the events involved in recognition may result in a susceptible or resistance response to infection.

B. Dissemination

The problem of dissemination is a fundamental feature of the life cycle of a living organism. Most pathogens occupy a narrow ecological niche, namely a living host. If this is the case, the pathogen is required to infect the host specifically or should have some sort of survival mechanism (Lucas, 1998).

Cardona-Alvarez and Walker (1956) concluded that wind-blown particles of recently infested soils, wind-blown spores, and water-borne spores are all effective agents of ALS disease dissemination. Although infected seeds have been reported to be a source of primary infection, Cardona-Alvarez and Walker (1956), using various lots of seed from diseased plants, concluded that the fungus was not carried by the seed

or on its surface. Orozco-Sarria and Cardona-Alvarez (1959) concluded that in areas where survival of the fungus in the soil is limited (extremes of heat and or cold), transmission of *Pg* through stored seeds was relatively important. Menezes *et al.* (1978) found that among 289 seeds examined 1.1% were infected with *Pg*, whereas Tanaka and Deslandes (1978) found infected seeds to be only 2.5% out of 400 seeds examined. However, seeds, crop debris, volunteer plants, as well as off-season crops are all possible sources of *Pg* inoculum. Sindhan and Bose (1979) and Sengooba and Mukiibi (1986) suggested that infected seeds play an important role in transmission of disease from one season to the next and that under circumstances where aerial inoculum is absent, seed-borne inoculum is most probably the most important way of spreading the disease.

Although spores are frequently disseminated by the wind, dispersal of conidia has not been quantified. It might be of special interest to establish the dispersal gradients of conidia from a source such as diseased plants and infested stubble. Seed infection, incidence of seed-to-seedling transmission, and the factors affecting the process of seed infection need to be studied in order to understand the epidemiology of the disease and to control the disease.

VI. Management

The development and implementation of integrated pest and disease management systems require a precise and accurate knowledge of the damage caused by pests and pathogens. Damage is defined as any reduction in the quality and quantity of yield that results from injury (Nutter *et al.*, 1993). Injury is any visible or measurable symptom and/or sign caused by pathogens and/or pests (Nutter *et al.*, 1993). Plant pathology evolved into a science not because plant pathogens are causative agents of disease, but because injury often results in damage that may result in loss of revenue or direct loss of a food source (Nutter *et al.*, 1993).

A. Yield Loss

Disease incidence represents the number of plant units infected and is expressed as a percentage of the total number of units assessed. Disease severity is the area of plant tissue affected by the disease, expressed as a percentage of the total area assessed (Parlevliet, 1979). In epidemiology characterization of the disease incidence and severity over time is of fundamental importance to understanding the

dynamics of the disease. Typically, a measure of the extent of the disease, such as the number of lesions, the amount of diseased tissue, or the number of diseased plants, is plotted against time, and this can provide an idea of the progress of the disease (Xu and Ridout, 1998).

Rava and Sartorato (1985) and Sartorato and Rava (1992) observed that every 10% increase in disease severity resulted in 7.88% bean yield loss. Furthermore, by increasing the number of inoculations, disease severity can be enhanced, and this may lower yield. Cultivar Rosinha G-2 was the more susceptible cultivar, losing up to 45% of yield due to ALS infection. Bergamin-Filho *et al.* (1997) found no relationship between yield and the area under the disease progress curve (AUDPC), calculated by trapezoidal integration in which the number of assessments, disease severity, and the interval between two consecutive assessments are included. They thought that the lack of relationship between yield and AUDPC in four trials, even when the data from each trial were analyzed individually, was probably due to three main reasons: intense defoliation caused by the pathogen, the lack of an estimate of defoliation in the disease assessment method, and the indeterminate growth habit of the host. Carneiro *et al.* (1997) studied disease severity in bean cultivars Rosinha G-2 and Carioca Común, aiming to determine the damage caused by ALS. They concluded that the severity of the disease at different time intervals of evaluation and AUDPC was not correlated with yield components. Silva *et al.* (1998) observed that the green leaf area (GLA), calculated as the percentage of nonaffected leaf, of healthy plants was always higher than the GLA of diseased plants, thus being a good example of the effect of the disease on growth. A reduction in GLA due to lesions induced by the disease cannot explain, by itself, the difference between the GLA of the healthy and diseased plants. In addition, there was a decrease in leaf number and this provoked a reduction of the GLA in all the pathosystems studied (Silva *et al.*, 1998). Therefore, the reduction of the total leaf area of diseased plants was not only the effect of leaves but also of defoliation that was reflected in yield (Jesús Júnior *et al.*, 2001).

Sengooba and Mukiibi (1986) found that ALS affected seed quality. Pericarps of infected seeds can lose up to 40% color. Seed decoloration means a reduction in quality standards and this results in economic losses for farmers. It still remains to be studied if the color change is related to softening of seed coats that may affect seed quality during storage.

VII. Control of the Disease

Several control strategies have commonly been advocated in attempts to reduce losses caused by ALS. These include planting pathogen-free seed, field sanitation, crop rotation, fungicides, and/or plant resistance.

A. Cultural Controls

A whole range of agronomic factors may influence the occurrence and development of disease, including sowing date, cultivars, nutrients and water, soil organic matter content, management of crop residues, and crop rotations.

Differences in yield as a result of *Pg* infection at different growth stages have been investigated. Bhardwaj *et al.* (1994) studied the effect of sowing date on yield of French beans and showed that early sowing is also a way to escape ALS. Moreno (1977) studied the impact of different rotation sequences on six different cropping systems. Beans were grown alone or in association with maize, sweet potato, cassava, maize plus sweet potato, and maize plus cassava. No differences between treatments were detected during the growing period up to pollination, but the highest severity of the disease was found during pollination and early fruiting stages on those systems that included maize in the rotation. The lowest disease severity was observed when beans were alternated either with sweet potato or cassava.

BAT 76 and A 285 are breeding lines that were chosen by Pyndji and Trutmann (1992) to evaluate the severity of ALS. In plots seeded with local cultivars it was found that supplementation of local mixtures with 25, 50, or 75% of either BAT 76 or A 258 resulted in reductions of disease severity. These results are significant, because they show for the first time that disease can be controlled in local bean mixtures by supplementing them with resistant cultivars. This suggests that modern plant breeding can have an impact on ALS by including ALS-resistant materials in cultivar mixtures (Pyndji and Trutmann, 1992).

So far the best cultural practices that can efficiently control ALS are the use of ALS, free certified seed, debris elimination, and strategies of crop rotation (Moreno, 1977; Schwartz and Gálvez, 1980; Pastor-Corrales, 1985; Sengooba and Mukiibi, 1986; Correa and Saettler, 1987; Cardona *et al.*, 1997).

Another alternative to reduce the attack of a crop by a pathogen is to use other living organisms for biological control. *Pg* survives in the soil

and in the seeds, and in these two environments the fungus should compete with other organisms that may affect fungal growth or viability. The identification of natural constraints to *Pg* and their use as biological control remain to be explored.

B. Chemical Controls

In an age of increasing environmental awareness, the use of chemicals to control pests, pathogens, and weeds is now questioned. However, this type of disease control is the prevailing system to reduce losses provoked by many diseases. In the case of ALS, fungicides can be applied to the seed before sowing or to the foliage. Seed treatments are effective to reduce seed-borne inoculum and also help to protect seeds and seedlings against soil-borne pathogens.

Spraying a fungicide on the foliage of bean plants may be beneficial only when the disease has been detected and identified. Schwartz and Gálvez (1980) reported that several fungicides such as Benomyl, Captafol, Ferbam-Sulfur, Mancozeb, Maneb, Metiram, Copper Oxichloride, Thiophanate, Zineb, Ziram, and Bordeaux Broth, control ALS. González *et al.* (1977), in Costa Rica, successfully controlled ALS on cultivar Mexico 80 and Turrialba 4 with Mancozeb, Captafol, and Metiram, 20, 30, and 40 days after sowing. Issa *et al.* (1982) reported that Maneb + Zn (2 kg/ha) resulted in reasonable control, which compared to the untreated control plants resulted in a yield of 75.4%. Pastor-Corrales *et al.* (1983) obtained the best control of the disease, a 41% yield increase over to the control plants, with Biloxazol, and a yield increment of 33% with Tribasic Copper Sulfate on cultivar G 2858 when they were applied 26, 40, and 54 days after sowing. Canteri *et al.* (1998a) studied the effect of fungicides in *in vitro* cultures of the fungus. They concluded that difenoconazole (0.12 kg/100 liters) and tebuconazole (0.3 kg/100 liters) controlled *Pg*, with an efficiency of 93% and 74% of control, respectively, when they were sprayed 4 days after inoculation (DAI). Canteri *et al.* (1998b) reported that spraying with fentin hydroxide 0.1% plus 0.04% Tebuconazole beginning 30 days after emergence (DAE) resulted in 65.6% control of ALS.

Sartorato and Rava (1998) controlled ALS on dry bean cultivar Carioca in Brazil by applying the fungicide uniformly through a central pivot, a procedure that is known as fungigation (Johnson *et al.*, 1986). They concluded that the method was efficient in controlling the disease, and that significant differences in yield were obtained only at the experimental place located at Unaí, where tebuconazole (0.25 kg/ha) was superior. Other studies, with bean cultivar Carioca, revealed

that benomyl (0.25 kg/ha), a fungicide extensively used in the control of *Pg*, proved to be more effective in controlling the disease in association with mancozeb (1.6 kg/ha), except for the experiment carried out in Acreúna (Sartorato and Rava, 1999). In the Northern region of Río Grande do Sul cultivated with bean cultivar Carioca, ALS control was higher than 70% for those treatments using 0.8–0.12 kg/ha azoxystrobin and chlorothalonil 0.15 kg/ha, thiophanate methyl + chlorothalonil (0.7 kg/ha + 0.75 kg/ha), propiconazol 0.125 kg/ha, tebucanozol 0.2 kg/ha, and triphenyltin hydroxide 0.2 and 0.3 kg/ha (Picinini and Fernández, 2000). Ploper *et al.* (2002) reported average yield increases between 16.8 and 28.1% for single fungicide applications (35 days after sowing), and 29.0 and 35.7% for double fungicide applications (35 and 50 days after sowing). Azoxystrobin, Benomil, Carbendazim, Thiophanate Methyl, and Difenocazole plus Propiconazole were the fungicides evaluated in two growing seasons using the bean cultivar TUC 500.

C. Genetic Resistance

A resistant plant has the ability to hinder the growth and/or development of the pathogen (Parlevliet, 1979). Resistance in a host can be vertical or horizontal. Vertical resistance is race-specific and is characterized by an interaction between the host and pathogen genotypes. Horizontal resistance is race-nonspecific, and it is characterized by the absence of a specific interaction between the host and each pathogen genotype (Agrios, 1999). In vertical resistance many major genes operate in a gene-for-gene manner. For each resistance gene in the host genome there is a corresponding avirulence gene in the pathogen genome (Flor, 1955).

Bean cultivar resistance to *Pg* was first detected in 1929 when Gardner and Mains observed that Kentucky Wonder was, among 40 common bean cultivars, a genetic source of resistance to *Pg* (Schwartz *et al.*, 1982). Olave (1958), Silvera (1962) [cited in Beebe and Pastor-Corrales (1991)], Buruchara (1985, 1988), Srivastava *et al.* (1995), and Wagara *et al.* (1999) evaluated resistance of several commercial cultivars and lines of beans to the causative agent of ALS. Based on cultivar response they proposed to group them as highly resistant, resistant, moderately resistant, susceptible, and highly susceptible materials.

Buruchara *et al.* (1988) found that on susceptible cultivars, infection as assessed through symptom development started at least a week earlier than on resistant cultivars. They concluded that reduction in disease incidence might be attributed either to long latent periods

of the fungus once the tissue was infected or to cultivar race-specific resistance. These results suggest that although some bean cultivars resistant to ALS prevent pathogen invasion, other mechanisms of plant resistance and tolerance might be able to reduce pathogen colonization, sporulation, germination, and growth on the plant's tissue. In addition, Srivastava *et al.* (1995) found that the environmental conditions also might have profound effects on plant response to the disease. It is feasible that resistance to *Pg* is under the control of multiple genes as it involves one or more genes with major effects or quantitative traits loci (QTL) with small effects, as occur with other common bean pathogens. Beans' resistance to anthracmose, rust, bean common mosaic virus, and bacterial blight is under the control of multiple genes, some of which are dominant and some of which are recessive (Singh, 2001).

Vertical resistance and not horizontal resistance seems to be the strategy used by beans to evade ALS. Sartorato *et al.* (1991), Paula Júnior *et al.* (1998), Nietsche *et al.* (1998, 2000a), and Faleiro *et al.* (2001) challenged bean cultivars with several pathotypes of the pathogen in order to evaluate the type of resistance of bean cultivars. They found that except for cultivar Rudá, which was fully susceptible to *Pg*, some showed a broader resistance response, that is, they were resistant to several pathotypes. Other cultivars were resistant to only one pathotype. In addition, they were unable to find cultivars of *P. vulgaris* fully resistant to *Pg*, suggesting that common beans lack horizontal resistance to *Pg*.

Although resistance sources to ALS have been found mainly in the Mesoamerican gene pool, Aggarwal *et al.* (1996) reported that CAL 143, a bean line from Andean origin, is consistently resistant to the disease. Furthermore, Fortes-Ferreira *et al.* (1999a) tagged by means of a molecular marker resistance genes in two Mesoamerican cultivars, Cornell 49-242 and Mexico 54, and on the Andean cultivar AND 277, suggesting the same resistance locus is present in materials from different places of origin.

Vertical resistance against *Pg* is not only present in *P. vulgaris* but also in *P. coccineus* and *P. polyanthus*. Busogoro *et al.* (1999b) inoculated 54 isolates of *Pg* on 29 genotypes of these three species. They confirmed results reported by Brock (1951), as all the *Pg*–bean interactions behaved in a typical vertical resistance manner. Furthermore, they crossed a *P. coccineus* cultivar that was resistant to *Pg* with cultivar Aroana, a *P. vulgaris* material susceptible to *Pg*, and the F1 hybrids were resistant. Therefore it appears that the interaction between the plant genotype and the isolates of *Pg* is the result of vertical resistance under

the control of genes within *P. vulgaris* as well as within *P. coccineus* and *P. polyanthus*.

Plant resistance to pathogens has been found to be under the control of single genes. They are most often dominant and less frequently recessive. Polygenic inheritance of resistance also has been reported, but at much lower frequency, most probably due to the fact that this type of resistance is more difficult to study (Ribeiro do Vale *et al.*, 2001). In 1957, Barros *et al.* found several highly resistant native and foreign cultivars with no commercial value. They made crosses and confirmed that resistance was dominant in a few cases. In addition, in most crosses, resistance appeared to be recessive and controlled by two or three independent ''factors.'' Similar results were obtained in Brazil (Santos-Filho *et al.*, 1976b). A single recessive gene controlled resistance in Caraota 260. Singh and Saini (1980) transferred from a *P. coccineus* (PLB 257) genotype to *P. vulgaris* cultivar Contender a gene for resistance to *Pg*. They found that resistance in PLB 257 was governed by one recessive gene.

Several bean cultivars carry single dominant genes of resistance to ALS. Cardona-Alvarez (1962), Carvalho *et al.* (1998), Fortes-Ferreira *et al.* (1999b), Sartorato *et al.* (2000), and Nietsche *et al.* (2000b) described the identification of single dominant genes that govern plant resistance to certain pathotypes of *Pg* in bean line 0258 and cultivars AND 277, MAR 2, Mexico 54, and Cornell 49-242, respectively. Correa *et al.* (2001) also studied the inheritance of resistance to *Pg* pathotypes 63.39 and 31.23 and found that a dominant gene also controlled resistance in cultivar Ouro Negro. In addition, they found that in US cultivar Pinto 111 resistance to pathotype 31.23 was under the control of a single recessive gene.

As in other plant–pathogen interactions, resistance to ALS may be conferred by single, duplicate, or complementary dominant genes as well as by recessive genes depending upon the parent and cross. It is predictable that the gene pool of origin of beans must hold a whole set of resistant genes, each effective against different pathotypes. Therefore the combination of these genes in a new cultivar might provide a cultivar with resistance against all tested pathotypes.

Guzmán *et al.* (1995) mentioned that breeding for disease resistance has been difficult because there is substantial pathogenic variation among fungal isolates. If breeders want to find durable resistance to disease, pathogenic variation should be understood and more sources of resistance should be found (Gridley and Buruchara, 1995; Pastor-Corrales *et al.*, 1998). In conclusion, any breeding program is dependent upon the study of pathogen variability and on the identification

of new resistant plant genes that may be transferred to well-adapted commercial cultivars (Sartorato *et al.*, 2000). Biological and molecular studies are needed to know more about the mechanisms of resistance that operate in this pathosystem.

VIII. Role of Markers Linked to Disease Resistance

With the advent of recombinant DNA technology, different types of molecular markers became available for breeders and geneticists (Staub *et al.*, 1996; Mohan *et al.*, 1997).

Monogenetic race-specific resistance genes are attractive to breeders, because they can be easily incorporated into susceptible material through simple backcrossing. However, resistance obtained in this way is a nondurable source of genetic resistance to highly variable plant pathogens (Kelly and Miklas, 1998).

Michelmore *et al.* (1991) developed the bulked segregant analysis (BSA), a procedure that allowed researchers to associate a phenotype characteristic with DNA fragments of the genome sparsely populated with markers. BSA allows the identification of molecular markers in any genomic region of an organism. Genetic mapping of disease resistance genes will help to improve the efficiency of plant breeding programs and should also lead to a better understanding of the molecular basis of resistance.

Several molecular markers have been found to be associated with genes that control resistance to *Pg*, and these findings should help breeders in the development of bean-resistant cultivars. Carvalho *et al.* (1998) identified a RAPD marker (OPH 13_{490}) linked to a resistance gene at 5.5 cM in AND 277. They proposed the use of *Ph-1* for designating this *Pg* resistance gene. Fortes-Ferreira *et al.* (1999b) evaluated a susceptible and resistant parent to *Pg* race 63.39, Rudá, and MAR 2, respectively. Resistant and susceptible DNA bulks, extracted from the F2 population, were used as a template to generate, with the primer OPE 04, a 500-bp fragment that cosegregated with the DNA fragment associated with resistance. Cosegregation analysis revealed that this marker was linked to the pathogen (race 63.39) resistance gene, at a distance of 5.8 cM. Sartorato *et al.* (1999) identified by sequence characterized amplified region (SCAR) primers a DNA region, originally revealed with RAPD markers, that is associated with resistance gene *Phg-2* in cultivar Mexico 54. Sartorato *et al.* (2000) identified three polymorphic DNA bands between the contrasting bulks constructed with the DNA from F2 plants derived from the cross Mexico 54 × Rudá. These bands were present in all resistant and absent in all susceptible individuals of

the bulk. These markers (OPN 02_{890}, OPAC 14_{2400}, and OPE 04_{650}) were mapped at 5.9, 6.6, and 11.8 cM from the resistant gene, respectively. The OPN 02_{890} fragment was transformed into a SCAR marker and the polymorphism observed was identical to the one revealed with the corresponding RAPD marker. Correa *et al.* (2001) analyzed resistance to races 63.39 and 31.23 in cultivars Ouro Negro and US Pinto 111 through the BSA, and identified two markers in Ouro Negro, OPM 02_{460c} and OPA 19_{600c}, which were linked to resistance. They were located at 5.3 and 10 cM of the resistance locus, respectively, but no markers were found to be linked to the resistance locus present in US Pinto 111. Alzate-Marin *et al.* (2001) showed a practical application of molecular markers as a tool in the selection process directed toward the commercial release of new cultivars. This suggests that pedigree information has a fundamental importance in the process of validating previously identified disease resistance gene sources. Elite lines A 774, FEB 163, TB 94-01, AN 9021334, AN 9021336, LM 93204453, LM 9220225, and LM 93203304 were used as templates for the successful amplification of a band, SCAR $N02_{890c}$, which was found to be linked to the *Pg* resistance gene in cultivar Cornell 49-242. These results confirmed that these lines carry at least one resistance gene for *Pg*. Elite lines LR 9115398 and LM 93204303 did not have the SCAR $N02_{890c}$ band, suggesting that the *Pg* resistance gene, present in one of the progenitors that was Cornell 49-242, was probably lost along the breeding process (Alzate-Marin *et al.*, 2001).

Pg is a pathogen with great genetic variability, therefore the identification of pathotypes and variability among *Pg* populations and of resistance genes in bean populations is extremely important for bean breeding programs.

IX. Concluding Remarks and Future Prospects

Phaeoisariopsis griseola (Sacc.) Ferraris, the causative agent of angular leaf spot, has been known for 125 years. However, major questions remain unanswered over the extent to which different approaches will provide solutions to this relevant disease. Epidemiology, in turn, is the foundation of information-based ALS management. Knowledge of spatial and temporal disease relations and fungal population biology should have an impact on disease control. Studies on *Pg* diversity and epidemiology are integral to characterize and understand the evolution of resistance genes to *Pg*. Parallel investigations of the molecular determinants of *Pg* pathogenicity and virulence are critical to understand not only the function but also the pathways involved in disease

resistance. Genetic studies should provide the bases for the development of bean cultivar resistance to *Pg*. Although the *Pg*–bean pathosystem is complex, continued basic and applied research should provide answers to solve many of the problems encountered by bean growers.

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The Fungal Genetics Stock Center: From Molds to Molecules

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I. Introduction

The Fungal Genetics Stock Center (FGSC) was established in 1960 following the recommendation of the Committee on the Maintenance of Genetic Stocks of the Genetics Society of America (GSA). At this time a number of the scientists who had established fungi in general and *Neurospora* in particular as premier genetic research systems were retiring or planning retirement and it was recognized that important strains needed to be maintained and made available to newer researchers. Moreover, the establishment of a stock center offered the surety that the same strains could be used by researchers in different places and

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times. A survey carried out by the GSA found that approximately 9000 *Neurospora* strains were being used around the world and, although the number of duplicated strains or trivial strains was not known, it was suggested that perhaps 1000 to 2000 stocks would be worthy of preservation. It was estimated that there were 21 laboratories using *Neurospora* at the time. A portion of the first grant proposal requesting the funds from the National Science Foundation (NSF) read as follows:

> This proposal seeks support to collect, maintain, publicize, develop and distribute important stocks of *Neurospora* and *Aspergillus* for research and teaching (educational) purposes . . . Stocks of mutant strains whose genetic analysis has been completed to the location of the locus on one of the seven linkage groups or otherwise published will be included. . . . Multiple mutants useful for linkage detection and mapping analysis will be developed and maintained. Recurrences at specific loci will be maintained when they have been published on or when other pressing reasons exist. Stocks containing cytoplasmic markers and wild types collected from various locations will be included . . . Mutant strains on which no genetic analysis has been undertaken will be accepted only when they appear of unusual interest.

This has been the guiding principle of the FGSC throughout its life and, although we have expanded to include other fungi and unanticipated molecular resources, it has been closely adhered to. This, as much as anything else, has contributed to making the FGSC the leading repository for fungal genetic materials in existence.

II. Chronology

The FGSC received support from the NSF and began operations at Dartmouth College under the direction of Dr. Raymond Barratt in July 1960. By the end of the year 469 stocks had been deposited in the collection. This number grew to 866 by the end of 1961 and has continued a similar rate of growth to the present day. Although the bulk of the original strains were mutants, a significant portion of the stocks at the FGSC today are wild-type stocks from around the world. Indeed, recent discoveries have opened new and unexpected areas to collection of wild *Neurospora* strains (Jacobson *et al.*, 2001). The FGSC moved to Humboldt State College in Arcata, California, in August 1970 when Dr. Barratt took a position as dean there. In 1985, Dr. Barratt retired as director and the FGSC moved to the University of Kansas Medical Center where Dr. John Kinsey took over as director. In Kansas City, the FGSC occupies approximately 700 square feet of laboratory space and the director of the collection has a separate laboratory. The FGSC

has proposed, in its pending NSF proposal, to move again in 2004 when Dr. Kinsey retires. The FGSC, with the help of the FGSC advisory board, has selected Dr. Michael Plamann of the University of Missouri, Kansas City (UMKC) to be the next director. This will allow the FGSC to move with a minimum of disruption to its operations. UMKC also offers a convenient and well-located environment for the operations of the FGSC.

III. Organization

The FGSC is comprised of a director, traditionally a researcher working with *Neurospora*, a curator, and two technicians. Drs. Barratt and Kinsey have been the only directors. Mr. Bill Ogata was the original curator and worked with the FGSC until his retirement in 1982. Mr. Craig Wilson took over as curator and moved with the FGSC from Humboldt to Kansas City. He stayed with the FGSC until 1995 when the current curator joined the FGSC. There have been a series of capable technicians at the FGSC over the years.

The FGSC is overseen by an advisory board selected from researchers around the country. They represent a variety of research organisms and areas and meet annually to review the progress of the FGSC and its goals. This group was established in 1999 following the advice of the NSF. Prior to that, the FGSC had used the Fungal Genetics Policy Committee as its guiding body, although the director maintained executive control. The Fungal Genetics Policy Committee (originally the *Neurospora* Policy Committee) is elected at the biannual Fungal Genetics Conference at Asilomar by the conference attendees. The committee balances domestic and international interests as well as the interests of different research emphases.

IV. Context

Most significant research organisms have a collection devoted to maintaining and distributing stocks. The National Science Foundation supports general collections, the National Institutes of Health supports clinically important collections, and the U.S. Department of Agriculture (USDA) supports agriculturally important collections, including plant germ-plasm. The FGSC, one of many culture collections in the United States, distinguishes itself by its involvement in its community and its responsiveness to community needs. Culture collections in general are part of a community connected by the U.S. Federation for Culture Collections in the United States and the World Federation

for Culture Collections (http://www.wfcc.info) internationally. A number of other organizations provide information on the breadth of collections worldwide, including the Microbial Strain Data Network (http://panizzi.shef.ac.uk/msdn/), sponsored by the United Nations, and the World Federation for Culture Collections—MIRCEN World Data Centre for Microorganisms (http://wdcm.nig.ac.jp/).

The FGSC is a genetic collection, and this distinguishes it from many other culture collections. The American Type Culture Collection (Manassas, VA, http://www.atcc.org/) is larger than many other public collections in the United States, but is known for its breadth but not its depth. Another large repository of fungi is the USDA collection at the National Center for Agricultural Utilization Research (originally the Northern Regional Research Laboratory, http://nrrl.ncaur.usda.gov/) in Peoria, Illinois. As this was originally the USDA internal collection, it emphasizes strains with agricultural uses. Many other microbial culture collections exist around the world. The Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl/), formerly in Baarn, now in Utrecht, The Netherlands, is notable for its fungal holdings. There are other specialized collections, like the *Fusarium* Research Center at Pennsylvania State University and the *Aspergillus* collection in the laboratory of Dr. A. J. Clutterbuck at Anderson College, Glasgow, UK, but few emphasize the depth of genetic materials that the FGSC does. The Yeast Genetic Stock Center, for many years at University of California at Berkeley, is now part of the ATCC. The Yeast collection is approximately 1200 stocks. Some collections are able to offer custom services, but the small size of the FGSC precludes this.

V. Holdings

A. Neurospora and Aspergillus

The FGSC holds over 16,000 strains, including 9642 strains in the main collection, 3910 strains in the Perkins collection of wild collected *Neurospora* strains (Turner *et al.*, 2001), and various smaller groups of strains that have not received FGSC accession numbers. *Neurospora* forms the bulk of the FGSC collection with 7871 strains in the main collection. Among these are 1417 wild-type strains and 5291 mutant strains. The majority of the *Neurospora* wild-type strains are *N. intermedia* (600 total) with fewer *N. sitophila* (243), *N. crassa* (258), *N. tetrasperma* (164), and *N. discreta* (71) strains. These strains were collected from wild and agricultural sites around the world (Fig. 1)

FIG. 1. Origins of *Neurospora* strains in the FGSC collection.

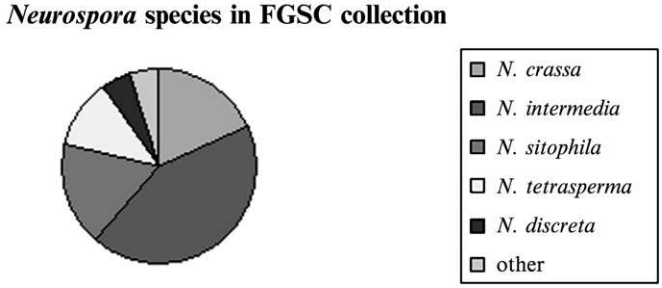

FIG. 2. Different *Neurospora* species in the FGSC collection.

by Dr. D. D. Perkins and others. They were predominantly collected as vegetative colonies from freshly burned substrate. Most have been through serial plating and have been tested by mating with known testers to determine both species and mating type. Most strains have been identified as a specific species and very few are either unknown or considered to be hybrids (Fig. 2).

The *Neurospora* mutant collection, by way of contrast, is primarily made up of *N. crassa* stocks. There are 4729 *N. crassa* mutants

TABLE I

STRAIN CATEGORY

Category of strain	Number of strains
Single mutant strains	3441
Multiple mutant strains	1978
Mitochondrial mutant stocks	85
Wild-type strains	1247
Chromosomal rearrangement stocks	884
Testers and stocks for special purposes	1252

currently active in the collection. These mutants represent over 1700 discrete lesions, including representatives of most of the approximately 1000 genes mapped in *N. crassa* (Perkins *et al.*, 2001). Sixty *N. intermedia* mutants, primarily biosynthetic mutants, 89 *N. tetrasperma* mutant strains, and 17 *N. sitophila* mutant strains comprise the remainder of the *Neurospora* mutant collection (Table I). The *N. crassa* wild-type strain that is most widely used is FGSC #2489 (74-OR23-IVA). This is the strain used by the Whitehead Institute Center for Genome Research (WICGR) to sequence the *N. crassa* genome.

The FGSC holds a number of collections that are part of the FGSC, but whose members do not receive individual FGSC numbers. Among these are the Perkins wild-type strain collection (3910 strains, Turner *et al.*, 2001), Dr. A. Lacy's *trp-3* strains, Dr. J. Fincham's *am* strains, Dr. M. Case's *pan-2* strains, and Dr. R. Davis' polyamine strains. Additional strains are maintained for historical purposes. Of such strains 388 are kept despite being officially retired. A strain may be retired because it is demonstrated to be unstable or to carry secondary mutations. Retired strains are often replaced by backcrossing them with a wild-type strain and the original strain is maintained as a source of the main mutation. The FGSC also has a collection of several hundred lyophils from the E. L. Tatum collection (Barratt, 1986). Certain strains are maintained as groups designed to be used together. Among these are the small and large restriction fragment length polymorphism (RFLP) mapping populations. Other strains are designed for the construction of heterokaryons or for the testing of mutagens, spore killer strains, mating type, or heterokaryon compatibility grouping. The FGSC holds a nearly complete set of known chromosomally abnormal strains. These come largely from the work of Dr. D. D. Perkins (1997). In total, the FGSC holds 842 *Neurospora* stocks with simple translocations, inversions, and duplications.

Some of these are simple, but others are complicated and involve four or more linkage groups. For a detailed description of *Neurospora* research, see Davis (2000). Perkins and Davis (2000) also collaborated on a recent retrospective on *Neurospora* research.

The *Aspergillus* collection is smaller numerically than the *Neurospora* collection. In total, the FGSC holds nearly 2200 *Aspergillus* strains. Of these 1100 are the *Aspergillus nidulans* temperature-sensitive mutant bank developed by Harris and Hamer (Harris *et al.*, 1994). The *A. nidulans* collection is made up of 844 stocks and 124 stocks are *A. niger*. A few *A. awamori*, *A. heterothallicus*, *A. oryzae*, and *A. flavus* stocks complete the *Aspergillus* collection. The FGSC, in cooperation with the *A. fumigatus* sequencing project at Manchester University in Manchester, UK, has recently accepted a strain of *A. fumigatus* pathogenic on humans. Also among the *Aspergillus* strains are 38 wild-type strains from six species and the main *Aspergillus nidulans* wild-type (FGSC A4) in use today. This strain is also the basis of the *A. nidulans* physical map (Prade, 2000). The *A. nidulans* strains include over 800 different genetic lesions at somewhat fewer gene loci. The mutant strains include single mutants as well as strains with all linkage groups marked and special mapping strains to be used as a kit. Other strains are used for targeting heterologous genes among other specialized uses. *Aspergillus* strains are held in a number of collections, largely due to the tremendous industrial importance of fungi in this genus (Jong and Birmingham, 1992).

B. Plant Pathogens

The FGSC *Fusarium* collection consists of mating type and vegetative compatibility group testers as well as strains for RFLP mapping (Xu and Leslie, 1996). Mostly these are *Fusarium moniliforme* but include a number of *F. oxysporum*, *F. graminearum*, and *F. solani* stocks. These are mostly from the collections of Drs. John Leslie, H. C. Kistler, and R. Ploetz. The FGSC *Magnaporthe* collection is comprised entirely of RFLP mapping strains, although it is expected that this will grow to include a large number of targeted gene disruption stocks in the near future.

C. *Sordaria*

The FGSC maintains, at the request of the GSA, a collection of *Sordaria* mutants. These are primarily from the collection of Dr. Y. Kitani and are similar to *Neurospora* in their handling characteristics. In total

there are 230 *Sordaria fimicola* strains and eight *S. brevicollis* stocks. The *S. fimicola* stocks include 46 different loci in five different linkage groups. The FGSC holds two *Sordaria macrospora* stocks.

D. Additional Fungi

Although not part of the main focus of the collection, the FGSC has a small number of other fungi, including *Gelasinospora*, *Podospora*, *Coniochaeta*, *Ascobolus*, and *Apiosordaria*. For the most part, these are type strains and are not genetic collections. They are useful, however, in providing outgroups for various studies. The FGSC also holds a collection of *Allomyces* strains from the collection of Drs. R. Emmerson and L. W. Olson (1984).

E. Molecular Genetic Materials

Since the late 1980s the FGSC has held an increasing number of molecular genetic materials. Originally, this was limited to cloned genes and cloning vectors, but soon included gene libraries for both *Aspergillus* and *Neurospora*. This has had a profound impact on both the relevance of the FGSC and the nature of research with *Aspergillus* and *Neurospora*. For the FGSC, it has maintained the position of the collection as the central resource for materials in fungal genetics. It has also allowed the progress made with these two main organisms to be extended and applied to other fungi. The collection of cloned genes and cloning vectors now numbers 206 with an additional 121 *Fusarium* RFLP markers and 182 RFLP markers for *Magnaporthe grisea*.

Among the 206 vectors and genes are cloning vectors encoding different antibiotic resistance, including benomyl, hygromycin, bialaphos, and sulfonylurea (Sweigard *et al.*, 1997). Other vectors are designed for library construction, DNA expression (Ebbole, 1990), or mutagenesis (Hamer and Gilger, 1997). The growth in the clone collection recently has slowed as more and more people are using clones from the several libraries associated with the genome projects rather than subcloned genes. Most of the information on clone identity is therefore resident on the genome server and not at the FGSC.

The collection of genomic DNA libraries has fostered research in a number of ways. The ordered libraries for *Aspergillus* and *Neurospora* have a great deal of information associated with them. The original *Neurospora* library is the pSV50 library and although it is known to be incomplete, the locations of over 50 genes are published in the FGSC

catalog. Similar information has long been available for the pMOcosX library, which has largely replaced the pSV50 library. The pMOcosX library was also used in the WICGR *Neurospora* genome project along with the pLORIST6xh library. In addition to these, the FGSC also holds an *N. crassa* YAC library, a BAC library, and numerous unordered *N. crassa* genomic libraries in both cosmid and phage vectors. The three *A. nidulans* ordered genome libraries are all related. The main set is the pWE15/pLORIST2 set, which is comprised of 60 96-well plates. Using data from the University of Georgia physical mapping program (Prade *et al.*, 1997), the FGSC picked both chromosome-specific sets and a minimal set from the original set. With the minimal set, the entire genome is represented on 16 96-well plates, with adjacent clones representing adjacent DNA on the chromosomes of *A. nidulans*. Since taking on these libraries, the FGSC has distributed nearly 140 copies in one form or another. The identities of numerous clones in this library are published in the FGSC catalog. The FGSC also holds one genomic library for *A. nidulans* in phage lamdba.

The collection of cDNA libraries for both *Neurospora* and *Aspergillus* is impressive. Although the *Neurospora* libraries outnumber the *Aspergillus* collection, both have been put to good use. The *A. nidulans* 24-h germinated conidia cDNA library was used at the University of Oklahoma as the basis of the *A. nidulans* EST bank (Kupfer, 1999). This library was distributed, in total, 67 times. A recent addition to the FGSC collection, the *A. nidulans* libraries in autonomously replicating vectors have become popular (Osherov *et al.*, 2000).

The *Neurospora* Genome Project (NGP) at the University of New Mexico, under the guidance of Drs. D. Natvig and M. A. Nelson generated a series of cDNA libraries that were made from RNA extracted from tissue in different developmental stages (Nelson *et al.*, 1997). Among these are the mycelial, conidial, and perithecial libraries. These, as well as their two-hybrid versions, have also been well used by the community. Other *N. crassa* cDNA libraries include the expression system for use in yeast or *E. coli* (Brunelli and Pall, 1993) and the nutritional-condition-specific cDNA libraries from Dr. R. Garrett (Exley *et al.*, 1993) and Dr. M. Sachs (Orbach *et al.*, 1990).

For a number of years, the FGSC distributed expressed sequence tag (EST) clones from the NGP and from the University of Oklahoma Advanced Center for Genome Technology. The NGP provided *N. crassa* ESTs from a variety of different tissue and the Oklahoma group provided *A. nidulans* ESTs from the 24-h germinated conidia cDNA library (Kupfer, 1999) and circadian rhythm-specific ESTs (Zhu *et al.*, 2001) from *N. crassa*. For a variety of reasons, including

the ease of generating full-length clones with PCR, the FGSC is no longer distributing ESTs.

VI. Source of Materials

Over 200 individuals have deposited materials into the FGSC collection. Dr. D. D. Perkins of Stanford University has deposited the most, with an impressive 3124 strains to his credit. This is in addition to the strains of the Perkins collection. Dr. F. deSerres deposited the second largest number of strains, with 933 to his credit. These are largely *ad-3B* strains with 439 containing that lesion. He deposited 700 *ad-3* strains in total. Dr. E. Kafer, now at Simon Fraser University, has deposited 829 strains of which 368 are *Neurospora* and the remainder *Aspergillus*. Dr. Kafer has deposited 32 *A. niger* strains out of a total 126 strains of that organism in the collection. She has also served as a consultant for the organization of the *A. niger* section of the FGSC catalog. Drs. J. Leslie, R. L. Metzenberg, M. Case, Y. Kitani, and D. Jacobson have all deposited over 200 strains each. Most of these strains are deposited at the initiative of the investigator, although the FGSC does request specific strains when they are described in the literature.

VII. Preservation

A. Fungal Strains

The FGSC has used proven long-term storage technology throughout its history but, because no one technology is foolproof, the FGSC has a strategy of reliability through redundancy. From the beginning of the collection, the primary means of preserving cultures has been to store them in anhydrous silica gel (Wilson, 1986). This has proven to be a very robust technique, and stocks that were preserved in the early days of the FGSC are still viable. This technique has been shown to preserve the genetic nature of strains (Jong and Davis, 1976). In addition to silica gel, stocks are also kept as lyophilized spores (Wilson, 1986). The record for longevity with *Neurospora* is 54 years (McCluskey, 2000b). Neither of these techniques is very useful for storing strains that do not sporulate profusely. For this reason, morphological mutants are now stored as both -80°C stocks (in 25% glycerol) and above liquid nitrogen (Wilson, 1986). Extending the strategy of reliability through redundancy, and to protect against catastrophic loss of materials, the FGSC maintains backups of certain aspects of the collection. This

applies primarily to primary mutants and wild-type strains. In this light, the *Neurospora* collection is backed up by a set of lyophils that is housed at the University of California, Santa Cruz in the laboratory of Dr. B. Bowman. This collection was stored at Stanford University in the laboratory of Dr. D. D. Perkins until recently. The *Aspergillus* collection is backed up similarly by a set of lyophils at the University of Texas M.D. Anderson Cancer Center in the laboratory of Dr. G. May. Many of the nonaccessioned stocks are stored as silica gel and freezer stocks for simplicity.

B. Molecular Genetic Materials

Cloned genes, cosmids, and BACs are stored at −80°C either in 2-ml screw-cap vials or in multiwell plates. Duplicate sets of each gene library are maintained in separate freezers. For cloned genes, DNA samples are maintained at −20°C. Because they are widely distributed, the gene libraries are not specifically duplicated, nor are the cloned genes.

VIII. Use of the Collection

The FGSC serves a steady demand and in 2001 fulfilled 387 orders. Of these, 185 were to foreign addresses and 202 were to U.S. addresses. Although the bulk of orders are to the United States, Asia, or Western Europe, materials were sent to 40 countries in 2001 (Fig. 3). The 387 orders in 2001 comprised 1081 fungal strains, 240 cloned genes, and 94 gene libraries. The FGSC presently distributes on average 110–115 cultures per month. This is similar to what it has distributed in the past and over the past 5 years (1997–2001) the FGSC distributed 6389 cultures and 452 gene libraries. In the entire history of the FGSC, over 55,000 cultures have been distributed.

IX. FGSC Clientele

The majority of the FGSC clients are researchers at academic institutions (Table II). This is reflected in the numbers of cultures that are distributed as well as the numbers of individuals on the FGSC mailing list. The number of researchers at U.S. institutions is roughly equal to the number at institutions outside the United States. Although the original focus of the FGSC was to serve the *Neurospora* and *Aspergillus* research communities, there are now researchers from a variety of fields among the FGSC constituency. The advent of molecular genetics has allowed many plant pathologists to use techniques and materials

FIG. 3. Destinations of strains from the FGSC in 2001.

developed with *Neurospora* and *Aspergillus* to conduct studies otherwise impossible. Other areas that have developed recently include medical mycology, industrial mycology, and applied chemistry.

X. FGSC Support

Although the FGSC has been supported by the National Science Foundation Division of Biological Infrastructure, Research Resources Cluster in the Support of Living Stock Collections program, it depends to a great extent on user fees to support its daily activities. Laboratory supplies, shipping, and printing are all supported by user fees. In the past year, the FGSC has switched from the U.S. Postal Service to a commercial courier (UPS) for most of our shipments. There are several reasons for this, but it has necessitated that a specific shipping fee be passed on to users. Notwithstanding the need to generate fees, the FGSC has a long tradition of providing materials to researchers without regard to their ability to pay. Moreover, the FGSC has a fee cap for fungal strains designed to allow new researchers to obtain the materials that they need without imposing onerous fees. The fee cap does not

TABLE II

NUMBERS OF STRAINS SENT TO DIFFERENT ORGANIZATIONS

	1997	1998	1999	2000	2001
U.S. academic	576	431	418	421	316
Foreign academic	519	593	339	306	273
U.S. company	55	136	52	41	14
Foreign company	20	28	13	11	7
U.S. governmental	9	5	0	0	0
Teaching	18	41	46	66	64
Internal use	3			55	0

apply to for-profit organizations or to molecular resources, although the FGSC does try to accommodate people's needs. Although recognizing the needs of academic laboratories, the FGSC has asked a substantially larger fee from commercial laboratories. This practice is typical among culture collections. The FGSC fee list is published in the FGSC catalog and online at the FGSC website.

XI. FGSC Website

In 1993 the FGSC established a site on the developing Internet. At first only strain lists and meeting abstracts were available on a gopher site, but this soon grew into an interactive site with various searches as well as listings of strains, clones, gene libraries, and meeting information. After going through a series of temporary site locations, the FGSC site is permanently located at http://www.fgsc.net where the FGSC hosts a bulletin board, online abstract submission for the Fungal Genetics Conferences, various interactive searches, back issues of the FGN, methods and protocols, and material for teaching with fungi. In the past few years, the FGSC site has garnered over 5,00,000 hits per year. The number of resources utilized is very high and follows academic year patterns. For example, the Fungal Genetics Conference material is most actively used in the weeks of online registration and in the weeks preceding the meeting.

As the FGSC database has developed, and as the *Neurospora* genome has become available, the FGSC has worked with the WICGR to provide links from our database to the *Neurospora* genome server and links to our database from WICGR. This is in addition to providing links to identified genes in Genbank, where appropriate. The FGSC is working with website developers and scientists to provide more

media content, micrographs, culture images, and video of cells, and micromanipulation of hyphae.

XII. Recordkeeping

Records of each strain were originally kept exclusively on paper FGSC deposit sheets in three-ring binders. This allowed for a variety of information to be maintained about each strain, including when it was received and preserved, what its requirements were, to whom it was sent, its genotype, and other relevant information. However, this did not allow the identification of strains by particular characteristics. To facilitate this, Mr. C. Wilson created an electronic database, using the database program dBase, for the FGSC. In keeping with developments in electronic and information technology, the FGSC updated to an interactive, relational database in Microsoft Access in 1998 (McCluskey, 2000a). This has enabled the strain database to be searched online and provides unprecedented access to the information to the FGSC clientele. The database also allows easy identification of strains with any combination of characteristics.

XIII. Publications

A. *Fungal Genetics Newsletter*

The FGSC publishes the *Fungal Genetics Newsletter* (FGN, originally *Neurospora Newsletter*) in cooperation with the editorial board of the FGN. The FGN is peer-reviewed and is published once per year. The FGSC distributes the FGN in print format and online. Being online since 1993 makes the FGN a pioneering journal. Originally published as part of the *Neurospora* Newsletter and later in the *Fungal Genetics Newsletter*, the FGSC catalog has grown to be too big for inclusion in the FGN. Although the FGSC catalog has information not found elsewhere, the number of people who request print catalogs is dropping precipitously. There are several reasons for this, the main being the ease of finding materials on the FGSC website. Moreover, the FGSC catalog is now available online at the FGSC website.

B. Program for the Fungal Genetics Conference at Asilomar

The FGSC has acted as a repository of information in a number of ways, including the organization, preparation, and publication of program books for the biannual Fungal Genetics Conference (FGC) and the

newly reestablished biannual *Neurospora* meeting. The FGC grew out of the *Neurospora* Information Conference and eventually became the primary meeting for fungal genetics. In years past, the FGSC has worked with the organizers of the European Congress on Fungal Genetics to host the meeting abstracts at the FGSC website. The FGSC has served as a means of disseminating information throughout its existence. We have maintained an extensive reprint collection, originally established by E. L. Tatum, and currently have over 5200 articles, dissertations, and chapters in our collection. This has helped the FGSC in providing information on the use of strains in the collection.

XIV. Future Directions

The FGSC will continue to hold and distribute the fungal strains, including the mutants and wild-type strains that make up the bulk of the collection, and will strive to add new materials as they develop. The rate of accession of new strains and related materials has been steady, with the exceptions of the large collections (Table III). It is expected that as established researchers retire, the FGSC will expand to hold a variety of new species. Already, the FGSC is expecting to receive a selection of *Schizophyllum commune* strains from the collection of Dr. C. Raper at the University of Vermont. The FGSC has also agreed to hold knockout strains of *M. grisea* and *N. crassa* as they are developed and expects to receive thousands of such mutants from the systematic knockout efforts planned, proposed, and in progress. Working with the fungal genome sequencing effort at the WICGR, the FGSC holds cosmid and BAC libraries used for *N. crassa* and has received commitments for the deposit of similar materials for *M. grisea*. A further extension of the

TABLE III

Recent Material Acquisition

Category	1997	1998	1999	2000	2001
Aspergillus	40	23	4	1154	33
Neurospora	42	11	3940	31	59
M. grisea	–	–	–	–	132
Other	5	4	18	3	0
Plasmids	33	18	11	5	4
Libraries	2	7	2	0	6
Totals	122	63	3975	1193	234

Whitehead effort will see the FGSC beginning to hold additional fungi and molecular resources relevant to the sequencing of these fungi. Early candidates likely include *Cryptococcus neoformans*, *Rhizopus arrhizus*, *Coprinus cinereus*, and *Ustilago maydis*. Although there is a risk of diluting the focus of the FGSC, the opportunity to expand our service to new and developing research communities is one that cannot be passed up. Moreover, much as the FGSC has expanded its mandate in the past, this will allow the FGSC to support research in a variety of fungal genetic systems.

XV. Summary

The FGSC, which began as a specialized repository for *Neurospora* and *Aspergillus* mutants, has grown to hold over 16,000 fungal strains as well as a variety of molecular genetics tools. The FGSC has expanded to include a variety of different fungi including *Magnaporthe* and *Fusarium* and is part of the *Magnaporthe* genome and knockout projects. The FGSC serves as a central clearing house for information in fungal genetics and has a role in facilitating the meetings of the *Neurospora* and fungal genetics communities. The FGSC is a model for the development of a culture collection much as *Neurospora* serves as a model organism.

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Adaptation by Phase Variation in Pathogenic Bacteria

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I. The Biological Significance of Phase Variation

The ability to adapt to changing environments is essential for the survival, development, and evolution of bacterial species. Often this takes the form of programmed responses to environmental stimuli. Such adaptability can also be generated through diversity within the population, which serves to increase the chances of survival of a portion of the population, if not the whole. Although diversity mediated by mutational processes is at times generated randomly, as is the case with point mutations, some species have developed a means of programming which genes will undergo mutations, within hypermutable loci, and through this mechanism generate frequent and reversible changes in phenotypes that result in a mixed population. The process is referred to as phase variation and involves predictable and predetermined changes in the structure of the bacterial DNA mediating the reversible expression of phenotypes that confer adaptive advantage in various environments.

The reversible switching between phenotypes in phase variation is due to genetic reorganization, mutation, or modification. Different species make use of different genetic switching mechanisms, such as alterations in the length of repeats, inversions, or insertions, but the phenotypic consequences are the same. To be considered to be phase

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variation the switching process must be reversible, and therefore must not result in the loss of coding potential. Compared to random mutations, these preprogrammed events occur at a high frequency, resulting in the maintenance of the alternative phenotypes in the population. The continuous generation of alternate phenotypes through the programmed, yet random, changes brought about by phase variation allows an otherwise clonal population to adapt to changing environmental conditions as it expands.

Modeling of the impact of phase variation on the population in which it occurs demonstrates some counterintuitive features (Saunders *et al.*, 2003). The generation of mutants at rates typical of this process results in the rapid presence of the variant subpopulations. However, the vast majority of the population remains phenotypically unchanged for periods of time that would include even prolonged periods of carriage. It is only when an alternate phenotype with a particular selective advantage arises, or when a negative selective pressure is applied to the predominant population, that significant changes in population composition occur. There is therefore a metastable situation in which the population exhibits flexibility in the context of stability, despite the relatively high switching rates that are observed.

It is useful to contrast the process of phase variation with that of programmed regulated systems. In a classic regulated system a change in the environment is detected and transduced, and leads to altered gene expression. The change in the environment therefore precedes the change in the organism, and the whole population can potentially respond to the new conditions. In phase variation new phenotypes are generated continuously. When one arises that has fitness advantages this will increase as a proportion of the population through clonal expansion and replacement. This is therefore different in two regards, the change in the organism precedes the alteration in the selective conditions, and it does not affect the whole population. This means that organisms using phase variation to affect transitions between environments potentially undergo many more clonal bottlenecks as they experience repeated waves of clonal replacement.

Different species have evolved different strategies with regard to the mechanisms used and the number of genes that are phase varied. These two issues are related. Some species, such as *Escherichia coli* and *Salmonella* spp., use predominantly recombination-mediated inversion mechanisms in gene switching, while maintaining relatively few potentially unstable simple sequence repeats in their genomes. This makes sense in a relatively clonal species with highly developed regulated responses to changes in environmental conditions. Other species,

such as *Neisseria* spp., have made use of predominantly slippage-based mechanisms within simple sequence repeats, and these species tend to have larger phase-variable gene repertoires and comparatively simpler transcriptional regulation networks. It has not yet been fully addressed, but there is probably a trade-off between permitting local functionally useful mutation and instability generally within the chromosome, which is reflected in the characteristics of the chromosome as a whole (N. J. Saunders, unpublished observations). There is therefore potentially a trade-off between phenotypic flexibility and mutability. This perhaps explains why this mechanism of gene switching is not used universally, and why the species that use it tend to be naturally transformable, and thus have a mechanism available to correct unwanted changes at secondary sites within the chromosome.

Although phase variation is often associated with the switching ON and OFF of the expression of virulence genes, a broader consideration suggests that these represent particular examples of environmental transitions to which the population is exposed. So, although the association between phase variation and virulence is consistent, it represents a general mechanism in the context of repeatedly encountered selective pressures, rather than a specific link. This chapter addresses the transitions that are facilitated by the switching of phase-variable genes, focusing on pathogenic bacteria.

II. The Types of Environmental Change to Which Phase Variation Is Adaptive

Phase variation has evolved as a means to allow a population to survive changing environmental conditions without the need for complex regulatory systems to respond to stimuli. Subpopulations are maintained expressing alternative phenotypes. For example, by stochastically changing the elements that comprise its cell surface, bacteria can evade host defenses. Antibodies raised in an immune response to one surface epitope will kill only those bacteria in the population expressing it. Other bacteria in the population will survive until they too become immunological targets. Through phase variation of surface structures, therefore, the bacteria can persist within the host even while there are ongoing immune responses. The adaptability afforded by phase variation in this situation is to survive the immunoselective transitions occurring in its environment and thus increase the colonization time. In some cases, where the variable repertoires are particularly large, it may be that there is sufficient capacity for sequential

diversification, that colonization for long periods, possibly even permanent, can be achieved even in the presence of effective immune responses. Some species have mechanisms that provide more direct resistance to immune defenses, such as capsules and surface modifications. However, these may be disadvantageous under other conditions and are also frequently phase varied.

Pathogenic bacteria make a variety of environmental transitions: movement to new hosts, new niches within the host, and often through either the environment or intermediate hosts. In some instances, sudden changes require some prepreparedness for survival. Through phase variation a population can include a variety of subpopulations expressing alternative phenotypes. Although these phenotypes may not be beneficial in the current environment, they may offer a significant advantage in the next and allow the bacterial population to survive an environmental transition. Varied functions involved in this process include those involved with adhesion to new host surfaces, nutrient acquisition, and environmental stress resistance. Likewise, through phase variation of structures involved in processes such as attachment, invasion, and motility, a subpopulation may be able to move from its current environment to exploit a different niche. In this sense the phase variation of specific genes is not only related to being suited to the next environment, but is an integral part of the process of making the transition, or ''moving on.''

It is frequently not possible to place any one switching gene into only one of these functional categories. However, the fitness of an organism depends primarily upon its ability to colonize, persist, and transmit from environment to environment and host to host. Phase variation is a mechanism integral to these processes in many species (Table I), and in the following sections examples will be used to illustrate its role in the broad categories outlined above.

III. Phase Variation Leading to Persistence

Whereas the physical location of the bacteria may not change, its environment may change dramatically, in particular during an immune response. Phase variation can provide a means to evade the host immune system through a variety of mechanisms that can broadly be divided into two categories: (1) evasion through the sequential production of diverse alternative structures, and (2) the switching ON and OFF of specific components that interfere with the immune system.

TABLE I

BACTERIA THAT UTILIZE PHASE VARIATION

Phase-variable bacteria	Repertoire of example phase-variable structures[a]	References
Bartonella henselae	Pili	Batterman *et al.* (1995)
Bordetella spp.		
B. avium	Surface protein	Gentry Weeks *et al.* (1991)
B. bronchiseptica	Toxin, fimbriae, hemagglutinin, regulator	Banemann and Gross (1997); van den Akker (1998)
B. pertussis	Toxin, fimbriae, hemagglutinin, regulator	Willems *et al.* (1990); Friedman *et al.* (1992); van den Akker (1998)
Borrelia spp.		
B. burgdorferi	Surface protein (VIsE)	Zhang *et al.* (1997); Zhang and Norris (1998)
B. hermsii	Surface proteins	Barbour *et al.* (1982); Stoenner *et al.* (1982)
B. recurrentis	Surface proteins	Meleney (1928)
B. turicatae	Surface proteins (Vmp)	Cadavid *et al.* (1994)
Campylobacter spp.		
C. coli	Flagella	Harris *et al.* (1987); Park *et al.* (2000)
C. fetus	Surface proteins	Dworkin and Blaser (1996)
C. jejuni	LPS, capsule, flagella	Linton *et al.* (2000); Parkhill *et al.* (2000); Bacon *et al.* (2001); Guerry *et al.* (2002)
Chlamydia pneumoniae	Uridine kinase	Read *et al.* (2000)
Citrobacter freundii	Vi antigen	Ou *et al.* (1988)
Corynebacterium spp.		
C. diphtheriae	Toxin	Kanel *et al.* (1978)
C. pilosum	Pili	Hiramune *et al.* (1991)
Dichelobacter nodosus	Surface proteins	Moses *et al.* (1995)
Enterococcus faecalis	Hemolysin/bacteriocin	Heath *et al.* (1995)
Escherichia coli	Fimbriae, pili, Ag43	Alkan *et al.* (1986); Henderson *et al.* (1997)
Francisella tularensis	LPS	Cowley *et al.* (1996)

(*continued*)

TABLE I (*Continued*)

Phase-variable bacteria	Repertoire of example phase-variable structures[a]	References
Haemophilus spp.		
H. influenzae	LPS, fimbriae (HifA, HifB), ChoP decoration, outer membrane proteins	Hood *et al.* (1996); Saunders (1999)
H. somnus	LPS	Inzana *et al.* (1992)
Helicobacter pylori	LPS, surface proteins, RMS, metabolic proteins	
Klebsiella pneumoniae	Fimbriae, capsule	Matatov *et al.* (1999); Ofek *et al.* (2001)
Legionella pneumophila	LPS	Luneberg *et al.* (1998); Luneberg *et al.* (2001)
Mannheimia haemolytica	Leukotoxin, RMS	Highlander and Hang (1997); Ryan *et al.* (2000)
Moraxella spp.		
M. bovis	Pili, surface proteins (Vsps)	Marrs *et al.* (1985); Marrs *et al.* (1988); Tobiason *et al.* (1999)
M. catarrhalis	LPS, surface proteins (UspA1), pili	Peak *et al.* (1996); Lafontaine *et al.* (2001)
M. lacunala	Pili	Marrs *et al.* (1990); Tobiason *et al.* (1999)
Mycoplasma spp.		
M. agalactiae	Surface proteins (Vpma)	Glew *et al.* (2000)
M. bovis	Surface lipoproteins (VspA)	Lysnyansky *et al.* (1996)
M. fermentans	Surface lipoproteins (P29, P78)	Theiss and Wise (1997); Leigh and Wise (2002)
M. gallisepticum	Lipoprotein hemagglutinins (pMGA)	Athamna *et al.* (1997)
M. genitalium	Adhesins (MgPa)	Peterson *et al.* (1995)
M. hominis	Surface proteins (Vaa)	Zhang *et al.* (1997); Ladefoged (2000)
M. hyorhinis	Surface proteins (VIpA, VIpB, VIpC)	Citti *et al.* (1997)
M. penetrans	Surface proteins (P35)	Neyrolles *et al.* (1999)
M. pneumoniae	Surface proteins (HMW2, HMW3)	Stevens and Krause (1990); Stevens and Krause (1992)

(*continued*)

TABLE I (*Continued*)

Phase-variable bacteria	Repertoire of example phase-variable structures[a]	References
M. pulmonis	Surface proteins (V-1), RMS	Bhugra *et al.* (1995); Dybvig *et al.* (1998); Gumulak-Smith *et al.* (2001)
M. synoviae	Lipoprotein hemagglutinins (vlhA)	Noormohammadi *et al.* (2000)
Neisseria spp.		
N. flavescens	Surface proteins (Opa), ChoP decoration	Wolff and Stern (1995); Serino and Virji (2000); Toleman *et al.* (2001)
N. gonorroheae	LPS, surface proteins, RMS	Snyder *et al.* (2001)
N. lactamica	Surface proteins (Opa), ChoP decoration	Stern and Meyer (1987); Wolff and Stern (1995); Serino and Virji (2000); Toleman *et al.* (2001)
N. meningitidis	LPS, surface proteins, RMS	Saunders *et al.* (2000); Snyder *et al.* (2001)
N. mucosa	Surface proteins (Opa), ChoP decoration	Wolff and Stern (1995); Serino and Virji (2000); Toleman *et al.* (2001)
N. sicca	Surface proteins (Opa)	Wolff and Stern (1995)
N. subflava	Surface proteins (Opa), ChoP decoration	Wolff and Stern (1995); Serino and Virji (2000); Toleman *et al.* (2001)
Photorhabdus spp.		
P. luminescens	Lipase activity	Wang and Dowds (1993)
P. temperata	Surface proteins	O'Neill *et al.* (2002)
Proteus mirabilis	Fimbriae	Zhao *et al.* (1997); Li *et al.* (2002)
Pseudoalteromonas atlantica	Surface protein	Bartlett *et al.* (1988); Perkins Balding *et al.* (1999)
Pseudomonas spp.		
P. aeruginosa	ChoP decoration, fimbriae, motility	Weiser *et al.* (1998a); Déziel *et al.* (2001)
P. brassicacearum	Extracellular proteases and lipases	Chabeaud *et al.* (2001)

(*continued*)

TABLE I (*Continued*)

Phase-variable bacteria	Repertoire of example phase-variable structures[a]	References
P. fluorescens	LPS	Dekkers *et al.* (1998); Sanchez-Contreras *et al.* (2002)
P. tolaasii	Adhesin, toxin	Han *et al.* (1997)
Salmonella spp.	Flagella/fimbriae, adhesin	Andrewes (1922); McWhorter *et al.* (1972); Isaacson and Kinsel (1992); Norris and Baumler (1999)
Serratia marcescens	Surface structure (H-antigen), flagella, color	Bunting (1940); Young *et al.* (1980); Paruchuri and Harshey (1987)
Staphylococcus spp.		
S. aureus	Adhesin	Baselga *et al.* (1993)
S. epidermidis	Adhesin	Ziebuhr *et al.* (1999)
Streptococcus spp.		
S. gordonii	Surface proteins	Jones *et al.* (1996)
S. pneumoniae	Surface proteins, capsule, teichoic acid	Weiser *et al.* (1994); Kim and Weiser (1998); Overweg *et al.* (2000)
S. pyogenes	Surface proteins (SclB)	Rasmussen and Bjorck (2001)
S. sanguis	Surface proteins	Tardif *et al.* (1989)
Treponema pallidum	Flagella, cell wall, transport	Saunders (1999)
Vibrio spp.		
V. cholerae	Adhesin (Tcp), toxin	Carroll *et al.* (1997)
V. parahaemolyticus	Surface proteins	Enos-Berlage and McCarter (2000)
V. vulnificus	Capsule	Wright *et al.* (1999); Wright *et al.* (2001)
Xenorhabdus spp.		
X. bovienii	Extracellular products	Pinyon *et al.* (2000)
X. nematophilus	Flagella, hemolysin	Givaudan *et al.* (1996); Givaudan and Lanois (2000)
Yersinia pestis	Urease	Sebbane *et al.* (2001)

[a]LPS, lipopolysaccharide; RMS, restriction-modification system.

A. Evasion through the Sequential Production of Diverse Alternative Structures

The size of the phase-variable repertoire in some species is formidable, and because each switch is independent of all others, this provides a very large combinatorial potential. Excluding restriction-modification genes, which cannot currently be clearly invoked to play a role in host interactions, in *Haemophilus influenzae* there were initially 11 phase-variable genes identified (Hood *et al.*, 1996) with the capacity to generate $(2)^{11}$ or 2048 genotypes. There are possibly at least 7 more giving $(2)^{18}$ or 262,144 genotypes (Saunders, 1999). The genome sequencing project of *Campylobacter jejuni* identified 19 phase-variable genes (Parkhill *et al.*, 2000) giving $(2)^{19}$ or 524,288 genotypes. Analysis of *Helicobacter pylori* strain 26695 identified 21 phase variable genes (Tomb *et al.*, 1997; Saunders *et al.*, 1998) with the capacity to generate $(2)^{21}$ or 2,097,152 genotypes. This number has now been refined through comparative analysis to 24 genes (L. Salaün and N. J. Saunders, unpublished observations) capable of generating 68, 719,476,736 genotypes. As an extreme example, *Neisseria gonorrhoeae* probably contains 74 or more switching genes (Snyder *et al.*, 2001) giving $(2)^{74}$ or 18,889,465,921,478,580,854,784 genotypes. The associated capacity to evade the immune system is self-evident.

The bacterial structures that interact closely with the host are precisely those most likely to be immunological targets because of their location and charge characteristics. Antigenic variation is the process by which the available epitopes and structures are changed. There are several mechanisms that have evolved to achieve this, involving gene replacement, formation of mosaic genes, alterations in the length of repeated structures by duplication and deletion events, as well as gene switching. In some instances these mechanisms are combined such that a gene that is phase varied is also subject to other variation processes, such as recombination or repeat component length variation. Combining the effects of these processes exponentially increases the potential for diversity of the structures presented on the surface of a population of bacterial cells. When these processes are combined, even in a relatively small number of genes, then organisms also achieve great flexibility.

1. *Persistence through Phase Variation of Surface Proteins*

One of the earliest studied examples of the coupling of antigenic and phase variation is in the *Borrelia* spp. During the course of an infection with *Borrelia recurrentis*, patients experience relapsing fevers, during

which the *Borrelia* proliferate. As the patient mounts an immune response, the levels of bacteria isolated from the blood decrease, as does the fever. With time, the fever returns as the cycle begins anew. The cyclic nature of this disease is associated with the antigenic variation of the *Borrelia* major surface proteins (VMPs) (Meleney, 1928). The population increases in the host, unchecked by the immune system, until such time as an immune response is generated against the VMP being expressed by the majority of the population. At that time the population expressing that VMP is killed by the immune system. However, subpopulations expressing different VMPs are not affected, and in time these fill the niche created by the immune response (Meleney, 1928; Coffey and Eveland, 1967). In this species these changes in the surface proteins are mediated by recombination, but since the repertoire is limited and the phenotypes are sequentially re-created in new hosts, this can be considered to be a form of phase variation, whereas some other systems involving recombination, such as pilus variation in *Neisseria* spp., cannot.

The *Mycoplasma* spp. have complex systems combining phase variation and antigenic variation mediated by other mechanisms. In *Mycoplasma hyorhinis*, a group of three lipid-modified surface proteins undergoes phase and size variation. The genes for VlpA, VlpB, and VlpC are highly repetitive at the C-terminal region, which is the location for duplications, deletions, and recombinations within the genes (Rosengarten and Wise, 1990, 1991; Yogev *et al.*, 1995). These proteins are involved in the interactions of the bacteria with the host cells (Rosengarten and Wise, 1991), yet as surface proteins they are targets for host antibodies (Citti *et al.*, 1997). So, the Vlp proteins need to be expressed for attachment and interaction of the bacteria with the host cell, but are antigenically and phase varied to avoid the antibody-mediated killing (Rosengarten and Wise, 1991; Citti *et al.*, 1997).

Related species have similar, but sometimes mechanistically different systems. *Mycoplasma bovis* also phase and antigenically varies its surface lipoproteins, called Vsps, which are different from the *M. hyorhinis* Vlps (Behrens *et al.*, 1994) yet share similar characteristics in their expression. Again, expression is phase varied and alterations in the repetitive structures of the C-terminus result in antigenic variation (Rosengarten *et al.*, 1994). *Mycoplasma hominis* has three major surface-exposed proteins that antigenically vary: P120, Lmp, and Vaa. The gene for Vaa undergoes phase variation in addition to antigenic variation (Ladefoged, 2000), much like the Vlp and Vsp proteins. As Vaa is involved in cell adherence, its presence is necessary for cellular persistence on the host cells, yet as an immunological target, the

antigenic and phase variation of Vaa allows persistence of the organism in the face of the immune response.

The Opa proteins of the *Neisseria* spp. are involved in adhesion to host cells (Dekker *et al.*, 1990; Makino *et al.*, 1991b) and also undergo both phase and antigenic variation (Stern *et al.*, 1986; Stern and Meyer, 1987). These were the first genes to be described with a repeat within the coding region of the gene mediating phase variation through the generation of frame-shift mutations controlling expression at the level of translation (Stern *et al.*, 1986). Strains of *N. gonorrhoeae* have up to 11 *opa* genes (Bhat *et al.*, 1991), therefore switching between the expression of these can provide a changing repertoire of these surface proteins in up to 2^{11} (2048) combinations. In addition, recombination between *opa* genes can provide a further level of diversification and antigenic variation of the protein (Bhat *et al.*, 1991; Hobbs *et al.*, 1994, 1998).

The phase-variable surface protein UspA1 mediates the attachment to epithelial cells of *Moraxella catarrhalis*, an unencapsulated bacterium that causes respiratory tract infections in humans. Due to the nature of the promoter-located switch, phase variation in UspA1 results in greatly reduced levels of this antigen on the surface of the bacterium. Variation in UspA1 expression may enable a population of *M. catarrhalis* to establish a balance between the requirement for adherence to human epithelial cells in order to colonize its human host, and the necessity to evade the host immune response in order to persist and subsequently cause infection (Lafontaine *et al.*, 2001). Alternatively, it may facilitate surface binding by other proteins at a particular level of expression.

2. *Persistence through Phase Variation of Lipopolysaccharide and Associated Structures*

The surface of Gram-negative bacteria is predominantly composed of lipopolysaccharide (LPS) (Nikaido, 1996). This is therefore one of the structures readily available for interaction with the host. Whereas Gram-negative bacteria such as *E. coli* and the *Salmonella* spp. have long O-antigen side chains as part of their LPS, the *Haemophilus* spp., *Neisseria* spp., and many others do not, which means that they lack the ability to mask the LPS core structures. Species that lack O-antigens frequently vary the sugars and substitutions of the LPS core through phase variation of the LPS biosynthetic genes (see Table I) (Kimura and Hansen, 1986; Weiser *et al.*, 1989; Gibson *et al.*, 1993; Shafer *et al.*, 2002). Due to the variety of structures that are produced and the complexity of the LPS interactions with the host environment,

the function of the phase variation of each of these different structures is not currently as clear as the ON and OFF switching of proteins such as Vlp and Vsp in the *Mycoplasma* spp. However, there are some examples that are particularly informative.

There are very few situations in which it is possible to study a human pathogenic organism in its natural host. Studies of *Haemophilus somnus* in its natural host, the cow, in which it causes a spectrum of infections similar to those associated with *H. influenzae* in humans, provides significant insights into the way in which these systems probably work generally. Variation in the LPS of *H. somnus* is known to involve repeat associated genes in a fashion similar to that present in *H. influenzae* (Inzana *et al.*, 1997). As seen in *H. influenzae*, the LPS phenotype is stable over weeks of daily subculture *in vitro* on solid media. However, during infection in the natural host, in a calf lung infection model over a 10-week period, rapid changes in the LPS of serial isolates were observed (detected by weekly sampling). The appearance of variant phenotypes is associated with the generation of specific immune responses to the phenotype that is lost, and the phenotypes occur sequentially as the animals are exposed and respond to each (Inzana *et al.*, 1992). This is the clearest demonstration of the fitness advantage to a colonizing population of phase variation of LPS resulting in prolonged carriage. It also illustrates apparent stability and then change in the absence and presence of specific selection pressures, and demonstrates that the LPS phenotypes that are present and expressed are immunogenic in the natural host. A parallel can therefore be drawn between the surface protein variation seen in *Borrelia* spp. and variation of LPS phenotypes. That similar processes occur during human infection is suggested by studies of repeat length variation indicative of gene switching in the related genes in *H. influenzae* in outbreaks involving a single bacterial clone. In these studies these genes are found to be among the most frequently switched between isolates obtained from different infected cases (van Belkum *et al.*, 1997a,b). Similar processes might be expected in other species with several phase-variable LPS biosynthetic genes such as *Neisseria, Helicobacter*, and *Haemophilus* spp.

3. *Phase Variation to Evade Preexisting Immune Responses*

The examples given so far have focused upon changes in expression leading to the evasion of developing responses. However, bacterial fitness is greatly enhanced if a previously colonized individual can be repeatedly colonized or infected. Further, the importance of this is even greater if protective antibodies can be induced by other species bearing

common antigens. This has been investigated in the context of *Salmonella*–host interactions (Norris and Baumler, 1999). The components of the flagellae of *Salmonella enterica* are frequently antigenically conserved between unrelated strains. This means that a prior colonization with a different flagellate strain of *S. enterica* that resulted in an immune response can provide cross-immunity to other flagellate strains. Phase variation of this structure provides a mechanism for evasion of cross-immunity in this species that can facilitate colonization, at the cost of loss of motility. The same may prove true for other common conserved antigenic epitopes.

B. The Switching ON and OFF of Specific Components That Interfere with the Immune System

1. *Capsules*

Capsules are polysaccharide outer-surface structures that are present in many forms on a wide variety of bacterial species. Although their importance in many aspects of host interaction and virulence is well documented, especially as it relates to resistance to complement-mediated killing and interactions with phagocytic cells, their primary functions in transmission and colonization cycles may be quite different. Especially in the context of bacteria that normally colonize a host without causing invasive disease, the roles of capsules in immune interference are probably incidental to other functions, such as avoiding desiccation during transmission. However, such structures would be expected to impede the interactions of those surface proteins that do not extend through the thickness of the capsule, and thus the way in which this structure is likely to be adaptive and maladaptive under different conditions is consistent with its frequent phase variation.

There are several representative examples. The phase-variable polysaccharide capsule of *Neisseria meningitidis* is perhaps the major determinant of serum resistance and survival in the bloodstream (DeVoe, 1982; Hammerschmidt *et al.*, 1994). Additionally, its presence reduces adherence and uptake into phagocytic cells and delays or prevents killing of phagocytozed bacteria (McNeil *et al.*, 1994; Read *et al.*, 1996). However, many meningococcal capsules are immunogenic so constitutive expression would lead to relatively rapid elimination. The bacteria avoid killing by serum components by expressing capsule, and avoid recognition of the capsule by the immune system by switching it OFF. The virulence of *Vibrio vulnificus*, the leading cause of fatal infections following seafood consumption, is associated with the

expression of capsular polysaccharide. Its expression correlates with lethality in mice and is a prerequisite for cytokine induction and for resistance to phagocytosis and complement-mediated lysis. A reversible deletion in the wza_{Vv} gene leads to the loss of the virulent phenotype by preventing translocation of the polysaccharide to the cell surface (Wright *et al.*, 1999, 2001). *Klebsiella pneumoniae* capsule phase variation leads to two types of colonies associated with different fimbrial expression. Mucoid opaque colonies are encapsulated and do not express type 1 fimbriae, whereas nonmucoid translucent colonies are unencapsulated and express type 1 fimbriae. Type 1 fimbriae and capsule are two of the most prominent virulence factors of *K. pneumoniae:* type 1 fimbriae allow the bacteria to bind to mannose residues on the surface of both epithelial and phagocytic cells, and capsule protects bacteria from the phagocytic cells of the host. As variants expressing both structures are not observed, it has been proposed that expression of capsule may interfere with the fimbrial assembly (Matatov *et al.*, 1999). *Streptococcus pneumoniae* presents a singular example of phase variation mediated by reversible sequence duplications, perhaps indicative of significant mechanistic differences in switching mechanisms between gram-positive and Gram-negative species. Capsule is switched ON and OFF through expansions and reductions within the coding region, in which the rate of reversion is proportionate to the size of the duplication that has occurred (Waite *et al.*, 2001).

2. *LPS and LPS-Related Structures*

There is evidence that particular LPS phenotypes protect *H. influenzae* against serum killing (Gilsdorf and Ferrieri, 1986). Phase variation of the *lic*-1 locus confers resistance or susceptibility to C-reactive protein–mediated killing through linkage of host-acquired choline to the LPS in the form of phosphorylcholine (ChoP) (Risberg *et al.*, 1997; Schweda *et al.*, 1997; Weiser *et al.*, 1997). Expression of the phase-variable *lic*-2 gene confers resistance to antibody-mediated serum killing through different structural changes to the LPS (Weiser and Pan, 1998). The commensal *Neisseria* have been found to possess a phase-variable *lic* gene, which is functionally similar to that found in *H. influenzae* (Serino and Virji, 2000, 2002). In both cases organisms expressing *lic*-1 decorate their LPS with ChoP, which is also present in the natural ligand for platelet-activating factor (Cundell *et al.*, 1995). Expression of a ChoP structure on the surface of the bacteria contributes to adherence to host cells and therefore to the persistence of these bacteria in the host (Weiser *et al.*, 1998b). The bacteria expressing the ChoP epitope are, however, more sensitive to serum killing mediated

by the C-reactive protein than bacteria that have turned OFF the expression of *lic*-1 through phase variation (Weiser *et al.*, 1997, 1998b; Serino and Virji, 2000, 2002). Cells that decorate their LPS with ChoP therefore have increased adherence to host cells but also have increased serum sensitivity, whereas those without are less adherent but are not targeted for serum killing through the recognition of ChoP by C-reactive protein, adapting them to distinct niches.

The LPS of *N. gonorrhoeae* can also be modified. The terminal sugar residue of the LPS is sialylated, which is dependent on the action of the phase-variable gene *lgtA*, responsible for adding the terminal LPS sugar substrate for sialylation (Apicella *et al.*, 1987; Parsons *et al.*, 1990; van Putten, 1993; Danaher *et al.*, 1995). Sialylation impedes adherence, uptake, and killing of the bacteria by neutrophils (Kim *et al.*, 1992; Rest and Frangipane, 1992). The altered structure of LPS also serves to mask epitopes on the LPS itself and exposed epitopes on other surface structures of the gonococcus from antibody recognition (Judd and Shafer, 1989; de la Paz *et al.*, 1995). In addition to its properties in the avoidance of neutrophil-mediated killing, it also provides resistance to serum killing (Parsons *et al.*, 1989; Gill *et al.*, 1996) due to altered complement activation (van Putten, 1993). These variants, although unaltered in their ability to adhere to host cells, are deficient in invasion of host cells, demonstrating the benefit of switching OFF the sialylated phenotype.

Several other species have less complex variable LPS biosynthetic pathways leading to smaller repertoires of LPS phenotypes. Serum resistance of *Legionella pneumophila* is mediated by the LPS carbohydrate moiety. Switching between two LPS phenotypes occurs mediated by the RecA-independent insertion/deletion of a 30 kb element located on a high copy plasmid (Luneberg *et al.*, 1998), affecting adhesion, serum resistance, and survival in biofilms (Luneberg *et al.*, 2001). *Francisella tularensis*, a facultative intracellular bacterium, is the etiologic agent of the zoonotic febrile illness tularemia. Phase variation occurs between two forms of LPS that differ both antigenically (at the O-antigen level) and functionally (at the lipid A level). One form of LPS induces nitric oxide production by macrophages, an effector for killing of intracellular pathogens, whereas the other does not. Thus *F. tularensis* could exploit the immune system for its own benefit, by prolonging its existence in the host or establishing a carrier state (Cowley *et al.*, 1996).

3. *Surface Proteins*

Serum resistance can also be mediated by surface proteins, which can act as protective layers in a fashion similar to capsules, and by providing substrates that either prevent complement activation or

induce activation remote from the cell surface. *Campylobacter fetus* subspecies *fetus*, an opportunistic pathogen in humans, and subspecies *venerealis*, which infects the genital tract of cattle, are covered by phase-variable monomolecular arrays of surface layer proteins. This ''S-layer'' prevents C3b binding by interfering with complement-mediated lysis and phagocytic killing. In addition, antigenic variation of this protein also protects *C. fetus* against antibody-mediated opsonization and killing by phagocytes (Dworkin and Blaser, 1996, 1997a,b; Dworkin *et al.*, 1997).

There are also other specific immune evasion strategies potentially available, although less well characterized. For example, the Omp1 protein of *Dichelobacter nodosus*, the etiologic agent of foot-rot disease in ruminants, is exposed on the cell surface. Omp1 undergoes phase variation that involves rearrangements by multiple site-specific DNA inversion events, leading to structural variations in the fimbrial subunit protein (Moses *et al.*, 1995). It has been proposed that Omp1 could have a role as a decoy antigen diverting host immune responses away from other nonvariant surface structures essential for virulence (Borst, 1991; Wise, 1993).

IV. Phase Variation Providing Adaptation to New Niches

Thus far, consideration has been almost exclusively focused upon adaptation to changes involved in evading immune responses and to different local immune defenses. These are undoubtedly among the most strongly selective and rapidly changing pressures to which bacteria that depend upon prolonged carriage are exposed, and the ability to evade these is central to features of virulence associated with invasive disease potential. However, bacterial systems are also exposed to other frequent and predictable environmental transitions, and some species also use phase variation to facilitate this aspect of adaptation. Because of the focus of research upon disease processes these are relatively less well studied, but interesting trends can nevertheless be discerned that are consistent with the general model of the nature of genes that are likely to be switched in this way. Functions associated with local environmental stresses, nutrition, and intracellular compartments serve as examples of this type of phase-variable adaptation.

Helicobacter pylori, the etiologic agent of gastritis, gastric ulcers, and gastric cancer in humans, can persist in its host for decades. *H. pylori* adherence on the gastric mucosa involves the expression of adhesins that bind to specific host-cell receptors. Among the 21

genes in strain 26695 (20 in strain J99) encoding for Hop proteins, a family of outer membrane proteins believed to function as key adhesins, six are demonstrated to be, or putatively, phase variable (HP0009, HP0638, HP0722, HP0725, HP0896, HP1243) (Tomb *et al.*, 1997; Saunders *et al.*, 1998; Alm *et al.*, 1999; Peck *et al.*, 1999). Two phase-variable adhesin–receptor interactions involving the bacterial adhesins BabA and BabB (encoded by HP0896 and HP1243) (Ilver *et al.*, 1998) and SabA (encoded by HP0725) (Mahdavi *et al.*, 2002) have been characterized. BabA adheres to the fucosylated Lewis B and related ABO blood group antigens expressed on gastric epithelial cells (Ilver *et al.*, 1998). SabA binds to sialyl-Lewis X antigens (sLeX) and also to the structurally related sialyl-Lewis A (sLeA) present on the gastric epithelial cells of different individuals (Mahdavi *et al.*, 2002). These sialylated antigens, weakly expressed on healthy gastric mucosa, are more prominent during gastritis. Binding of SabA to sLeA, a tumor antigen and marker of gastric dysplasia, may facilitate binding to areas of local tissue damage (Mahdavi *et al.*, 2002).

In addition, *H. pylori* phase variably express blood group antigens on their LPS that are similar to those of the host. In the gastric mucus layer the pH gradient ranges from pH 1.5 on the luminal side to almost pH 7 on the cell surface. Although the mucus layer partially isolates the bacterium from the gastric acidity, *H. pylori* may encounter significant changes in pH conditions (Lee *et al.*, 1993). Besides urease production by the bacterium, which is a key element in the colonization of the stomach (Eaton *et al.*, 1991), variations in LPS composition also influence survival at different pH conditions. *H. pylori* grown at neutral and low pH have different colony morphology, cellular lipid composition, and virulence properties (Bukholm *et al.*, 1997). Liquid culture studies have demonstrated that this transition is associated with phase-varied LPS changes in which sLeX is expressed at pH 7, and mixed sLeX and sLeY is expressed at pH 5 (Moran *et al.*, 2002). So, changes in surface structures may play a direct role in adaptation to local conditions through mechanisms other than altered cell–cell interactions or available targets.

Iron is an essential nutrient that, due to the presence of high-affinity binding within the host, is effectively present in low concentrations to colonizing bacteria. The pathogenic *Neisseria* spp. can utilize transferrin, lactoferrin, hemoglobin, hemoglobin–haptoglobin complexes, heme, and heterologous siderophores as sources of iron. The receptors for most of these are phase variable, although the repertoire of which ones are varied differs between strains (Chen *et al.*, 1998; Lewis *et al.*,

1999; Richardson and Stojiljkovic, 1999; Saunders *et al.*, 2000; Carson *et al.*, 2000; Guerry *et al.*, 2002; Hobbs *et al.*, 2002; Jerse *et al.*, 2002). It has been demonstrated in a mouse model of gonococcal vaginal infection that the presence of hemoglobin in the inflammatory exudates selects for hemoglobin receptor ON variants during infection (Jerse *et al.*, 2002). Strains that cannot use transferrin and lactoferrin are noninfectious in gonococcal human male urethral infection (Hobbs *et al.*, 2002). While iron is needed for growth, the receptors for these nutrients are also immunological targets (Ala Aldeen *et al.*, 1994; Carson *et al.*, 2000). Through phase variation of these receptors, the *Neisseria* spp. can adapt to available iron sources while not expressing potential immunological targets when they are not necessary.

H. influenzae requires a porphyrin source for growth, which it can acquire in the form of heme, hemoglobin, and related complexes. Different strains contain three or four copies of variant proteins with homology to hemoglobin receptors that are associated with repeats indicating phase variation (Hood *et al.*, 1996; Morton and Stull, 1999; Cope *et al.*, 2000); in addition a heme-utilization gene may also be phase variable (Hood *et al.*, 1996; Saunders, 1999). The phase variation of this gene family has been confirmed (Jin *et al.*, 1999; Ren *et al.*, 1999; Cope *et al.*, 2000), but differences in their substrate specificities have not been fully elucidated to date.

Mannheimia (Pasteurella) haemolytica specifically uses transferrin as an iron source via the surface expression of the transferrin-binding protein (Tbp) complex and also via a novel phase-variable TonB-dependent outer membrane iron receptor (*irp*). Irp is distinct from the Tbps, and may act as an alternative pathway for iron acquisition (Graham and Lo, 2002). Genome sequences of other pathogens suggest other examples of phase variation of iron acquisition genes. For example, the hemerythrin-like putatively phase-variable iron-binding protein (Cj0045c) of *C. jejuni* possesses a poly(C) tract (Parkhill *et al.*, 2000).

Iron is only one variable nutrient that organisms must obtain within the host. Although this aspect of host interaction has not been addressed in detail experimentally, candidate phase-variable genes indicate that this may be important in niche adaptation. For example, although the substrates and the direction of transport have yet to be determined, potentially phase-variable ABC transporter systems have been recognized in *Mycoplasma fermentans* (Theiss and Wise, 1997), *Treponema pallidum* (Saunders, 1999), *N. meningitidis* (Snyder *et al.*, 2001), and *H. pylori* (Saunders *et al.*, 1998).

Adaptation mediated by phase variation has been demonstrated between extracellular and intracellular compartments. In *Bordetella bronchiseptica* the BvgAS two-component regulator controls the expression of adhesins and adenylate cyclase toxin (Banemann and Gross, 1997). Avirulent phase variants that do not express the BvgAS system have higher rates of survival in macrophages, as do BvgAS mutants, suggesting that production of the controlled genes may be disadvantageous to the intracellular bacteria. In *Bordetella pertussis*, in which the BvgAS regulatory system is also phase variable (Carbonetti *et al.*, 1993), it is known that adenylate cyclase can induce apoptosis in phagocytic cells (Khelef *et al.*, 1993). Therefore the phase variation of a response regulator of virulence gene expression may increase the survival of these bacteria in the intracellular milieu.

Through phase variation–mediated changes in its LPS, *Francisella tularensis* has been shown to reduce nitric oxide production of macrophages (Cowley *et al.*, 1996). Decreased nitric oxide production allows the bacteria to survive inside the macrophages. In this way the bacteria can avoid the immune system both by antigenically varying its LPS and can reduce the nitric oxide production of macrophages as a means to escape the immune system by residing within these host cells (Cowley *et al.*, 1996).

Oxygen tension is likely to be significantly lower in intracellular as opposed to extracellular locations on the respiratory mucosal surface. In this context the possible phase-variable nature of a *fixP* homologue in *N. meningitidis* (Saunders *et al.*, 2000) is notable. This gene encodes a homologue of a component of an alternative electron acceptor that is adaptive to microaerophilic conditions. However, under other conditions it is leaky of electrons and inefficient. As another example of a potentially phase-variable gene affecting metabolic functions directly, comparison of two *Chlamydia pneumoniae* genomes suggests phase-variable expression of uridine kinase, a key enzyme in nucleoside metabolism in these bacteria (Read *et al.*, 2000). Since this species is an obligate intracellular parasite it is unclear to what different conditions these two states would be adaptive. This may represent adaptation to different types of cell or intracellular compartments rather than to intracellular versus extracellular life.

A more macabre pattern of phase-variable metabolic behavior in pathogens is illustrated by *Xenorhabdus* and *Photorhabdus* spp., two bacterial species that form entomopathogenic symbioses with soil nematodes. These bacteria undergo a complex life cycle that involves a symbiotic stage, in which the bacteria are carried in the gut of the nematodes, and a pathogenic stage, in which susceptible insect prey

are killed by the combined action of the nematode and the bacteria (Forst *et al.*, 1997). Several traits of these bacteria such as motility, the production of antibiotics, crystal protein, extracellular enzyme, pilus and glycocalyx, and numerous other products are phase variable (Givaudan and Lanois, 2000; Pinyon *et al.*, 2000; O'Neill *et al.*, 2002). Although the two phase-variable forms equally kill the insect larvae, only the P1 form produces antibiotics. These antibiotics prevent the putrefaction of the larval cadaver by other microorganisms, leading to a favorable environment for nematode growth and reproduction (Pinyon *et al.*, 2000). The mechanism responsible for phase variation in these species is not well understood. However, it appears that the expression of this gene repertoire is controlled by a combination of phase variation and programmed regulation (Forst *et al.*, 1997; O'Neill *et al.*, 2002).

V. Phase Variation as a Mechanism for Making Environmental Transitions

There have been many phase-variable genes studied that encode surface proteins, particularly those involved in adhesion and colonization. The switching and modulation of these structures dictate the colonization potential of the bacteria, and in some cases the invasion and movement of the bacteria through the host. Phase variation of such genes also allows for the bacteria to let go of, or to actively move on from, their current environment so that subpopulations can explore new niches.

The most obvious example of this type of switched phenotype is the varied expression of motility determinants. The pili of *E. coli* are instrumental in the maintenance of the bacteria within the urinary tract during infection through their adhesive properties and motility-conferring ability to migrate through the urinary tract (Schaeffer, 1991). In two mouse models of *E. coli* infection, the phase-variable expression of pili has been examined in relation to the site of colonization within the host. Following inoculation of piliated cells in chambers implanted intraperitoneally, the *E. coli* isolated after 5 days were almost all nonpiliated. In contrast, piliated bacteria were more effective at colonizing the bladder urothelium following inoculation through a urinary catheter (Hultgren *et al.*, 1985). Switching OFF pili expression would release the *E. coli* from the host cell surface allowing movement to a new host via the urine. Additionally, the OFF phenotype is advantageous should the pilus become a target for antibody and phagocyte-mediated killing (Schaeffer, 1991). Similar processes appear to occur

in *Proteus mirabilis* that express MR/P fimbriae almost uniformly in the bladder but expression varies greatly in deeper sites such as the kidney (Zhao *et al.*, 1997; Li *et al.*, 2002).

Pili and fimbriae can also be involved in adhesion and subsequent invasion of host cells. In *Salmonella enterica* serovar Typhimurium the Peyer's patches of the ileum are the main point of bacterial entry. The *lpf* operon encodes the genes for the phase-variable long polar fimbriae (Norris *et al.*, 1998). There is selection for ON expression of the fimbriae in the Peyer's patches, where they play a role in adhesion and penetration. In the mesenteric lymph node and spleen, however, there is selection for the OFF phenotype, presumably based upon immune counterselection that can be induced by immunization with LpfA (Norris *et al.*, 1998). This suggests that the ON phase variants are selected for in Peyer's patches interactions, but the OFF variants are selected for when the fimbriae may be immunological targets.

Vibrio cholerae causes epidemic severe secretory diarrhea in developing countries. The expression of its two major virulence factors, cholera toxin and the toxin coregulated pilus (Tcp), an adhesin, is regulated in response to environmental signals. The transcription of the *tcpH* gene is activated by ToxT, whose expression is dependant on the activation of the *toxT* gene by the transcriptional factor TcpH. Phase variation of the *tcpH* gene leads to *V. cholera* variants with reduced transcription of *toxT* in response to environmental signals. These variants would be expected to be excreted into the environment and have an enhanced growth potential when virulence factors needed for human infection are no longer required (Carroll *et al.*, 1997).

Biofilms are frequently encountered niches, and are clinically important both as bacterial reservoirs and as sites that are relatively resistant to antibiotic penetration. *Pseudomonas aeruginosa* phase varies between large and small colony variants. The small variants are hyperfimbriated, with reduced flagellar and twitching motilities, and the ability to rapidly form strongly adherent biofilms (Déziel *et al.*, 2001). In contrast, the large variants are highly motile and allow the bacteria to spread to new niches. There is an interesting parallel in the nonpathogen *Pseudoalteromonas atlantica*, a marine bacterium, in which phase variation of its capsule-like extracellular polysaccharide affects its adhesive properties to marine surfaces (Bartlett *et al.*, 1988; Perkins-Balding *et al.*, 1999).

The common theme in each of the above selected examples is that there is a resident, invariant, main population. In each instance a small proportion of variants is continuously generated that is motile, gains an ability to transition to a new niche, or is no longer bound to the surface

on which they are resident. This process therefore provides for the maintenance of a stable colonizing population, while continuously generating subpopulations suitable for extension into new sites or transmission.

VI. Complex Interactions and Phase Variation

Normal experimental methods address one gene at a time, and most studies of phase variation focus upon single genes, or sometimes groups of genes with related functions. However, although this is a fair representation of these processes in organisms such as *E. coli* with small repertoires of switching genes, the situation is very different in those species using this mechanism in multiple genes. One gene may have a selective advantage only in the context of another, and if a fitness difference of 1% or greater is created through the interaction of one phase-variable gene with another, their expression can effectively become coordinated even though the switches themselves are stochastic and discrete (Saunders *et al.*, 2003). In some instances phase-variable genes may be regulators of larger networks, components of which may themselves be phase variable. Unraveling these processes is experimentally challenging; however, studies of these processes are likely to reveal significant insights into the nature of the bacterial–host interaction.

Neisseria spp. represent a good example of a system in which multiple independent phase-variable genes interact. In the *Neisseria* spp., the phase variation of surface components presents a highly dynamic surface structure to the environment. In addition to the meningococcal capsule and various neisserial LPS structures, other known phase-variable surface structures in these species include pili (Jonsson *et al.*, 1991), outer membrane adhesions such as Opa (Stern and Meyer, 1987) and Opc (Sarkari *et al.*, 1994), outer membrane pore structures such as PorA (van der Ende *et al.*, 1995), and iron acquisition proteins (Chen *et al.*, 1998). The expression and interplay of each of these structures in some way affect the adhesion and niche adaptation of the bacteria. A parent and variant of *N. meningitidis* were studied to determine the optimum expression of these components for the niches of the host (de Vries *et al.*, 1996). The parent was encapsulated, piliated, and had sialylated long LPS, whereas the variant isolated from the parent was unencapsulated, nonpiliated, had nonsialylated short LPS, and expressed Opa proteins. The capsule is a disadvantage for penetration of the mucosal barrier (Hammerschmidt *et al.*, 1996) but is important for survival during dissemination (Kahler *et al.*, 1998). The sialylated

LPS hampers the ability for the Opa proteins to mediate bacterial adhesion and entry into host cells (Virji *et al.*, 1993; van Putten and Robertson 1995) but allows escape from host defenses (van Putten, 1993). The differential expression of Opa and Opc influences tissue tropism with respect to the ability of the bacteria to invade epithelial cells when expressing different Opas, and the inhibitory affect of coexpression of Opc on the function of Opas (de Vries *et al.*, 1996). It has therefore been suggested that *N. meningitidis* moves into a new host encapsulated and piliated. Through pili it makes the initial attachment, which must become a closer attachment to the cells through loss of capsule and possibly pili and high levels of expression of Opc. Entry to the mucosal cells is gained through the expression of the appropriate Opa protein, loss of LPS sialylation, and switching OFF of Opc (de Vries *et al.*, 1996). During attachment and entry there is a switch from the heterogeneous expression of Opa proteins to almost complete expression of one Opa protein, suggesting a selection for distinct Opas in certain bacteria–host cell interactions, adding an additional layer of complexity to the phase variation–mediated invasion of the *Neisseria* spp. into host cells (Makino *et al.*, 1991a; Weel *et al.*, 1991). So, clearly a complex interplay of independently switching genes is involved, even in the simple process of establishing a stable colonization.

The phase-variable virulence determinants and the interplay between phase-varied and regulated systems has been extensively studied in *B. pertussis*, the causative agent of whooping cough. *B. pertussis* has a number of identified virulence determinants, including pertactin, filamentous hemagglutinin (FHA), and pertussis toxin (PT). Pertactin is a 69-kDa protein that contains two Arg-Gly-Asp (RGD) adherence motifs where the amino-terminal motif promotes adherence to cell lines. Knockout of the gene reduces adherence of *B. pertussis* in *in vitro* models, and it elicits substantial immune responses acting as a protective antigen in animal models (De Magistris *et al.*, 1988; Shahin *et al.*, 1990; Leininger *et al.*, 1991). FHA is a large filamentous protein containing an RGD adhesion motif that is both secreted and associated with the bacterial cell surface and mediates adherence to both ciliated and nonciliated cells (Tuomanen and Weiss, 1985; Urisu *et al.*, 1986; Relman *et al.*, 1989; Cotter *et al.*, 1998). FHA stimulates an immune response in humans after clinical disease (De Magistris *et al.*, 1988) and protection can be synergistic with that produced by pertussis toxin (Robinson and Irons, 1983; Sato and Sato, 1984). PT is a 105-kDa A-B toxin composed of five subunits (Locht and Keith, 1986; Nicosia and Rappuoli, 1987), which includes a surface-associated adhesin that binds to cells through a lectin-like mechanism to carbohydrate receptors

on the host cell surface (Brennan *et al.*, 1988; Tuomanen *et al.*, 1988) as well as having cilia-specific, cell surface receptor, and heparin-binding properties (Locht *et al.*, 1993). The toxic subunit is an NAD-dependent ADP-ribosyltransferase that causes irreversible uncoupling of the regulatory GTP-binding proteins from their membrane receptors. The other subunits act as the targeting and delivery system. This affects several metabolic pathways and its effects include inhibition of adenylate cyclase (Katada and Ui, 1982; Hsia *et al.*, 1984) and transducin (Manning *et al.*, 1984; Van Dop *et al.*, 1984). In addition, PT is a mitogen, an adjuvant, releases fatty acids from fat cells, interferes with chemotactic migration, and alters vascular permeability (Munoz *et al.*, 1981a,b; Hewlett *et al.*, 1983). PT is immunogenic and antibodies against PT are protective. It also generates a hemolytic colonial phenotype on blood agar.

Phase variation in *B. pertussis* affects the coordinated expression of pertactin, FHA, PT, and also fimbriae (of which there are two serotypes). In *B. pertussis* strain Tohama, this is mediated by altered transcription of a gene with homology with two-component regulatory systems at the *vir* locus (Stibitz *et al.*, 1989). In this case programmed regulation and stochastic switching by phase variation are closely integrated, demonstrating that phase variation of a gene does not preclude the possibility of additional regulation. The expression of these factors is affected by growth conditions such as temperature and the concentration of $MgSO_4$. Under nonpermissive conditions such as a temperature of 25°C or the presence of 20 m*M* $MgSO_4$ these genes are repressed. When the cells are returned to permissive conditions then expression is resumed (Lacey, 1960; Idigbe *et al.*, 1981). These genes are under the control of the *bvg* (or *vir*) locus, which encode three proteins involved in sensory transduction (Weiss *et al.*, 1983; Knapp and Mekalanos, 1988; Stibitz *et al.*, 1988; Aricó *et al.*, 1989). Therefore several of the virulence determinants of *B. pertussis* are part of a single regulon that is positively regulated by *bvg*, and the sensor/*bvg* system includes a gene that undergoes phase variation. When *bvg* is in the OFF state none of these genes are expressed, but when in the ON state they are expressed according to the environmental conditions.

However, this is not the full extent of the regulation of these phase-variable virulence genes. There is an additional regulatory factor that affects toxin expression but that has no effect on the adherence determinants (FHA and pertactin) (Carbonetti *et al.*, 1993). In addition, the fimbrial genes are independently phase variable. The two serologically distinct fimbriae are composed of subunits of different molecular weight (Ashworth *et al.*, 1982; Irons *et al.*, 1985; Zhang *et al.*, 1985)

and an individual strain can express both types, either type singly, or have no fimbriae at all (when the *bvg* is in the ON state)—a process that can be observed to occur *in vivo* (Preston *et al.*, 1980). The *fim* genes in *B. pertussis* are phase varied through alteration in the length of a promoter located homopolymeric tract of Cs. The promoter of the *ptx* gene also contains a (shorter) homopolymeric tract at an equivalent location (Locht and Keith, 1986; Nicosia *et al.*, 1986; Nicosia and Rappuoli, 1987), which suggests that this might also be phase varied independently. Taken together these mechanisms would provide the capacity to express at least 32 different phenotypic combinations of the components of the virulence regulon.

In addition to the altered expression of toxins and adhesins, *Bordetella* spp. also express variable LPS phenotypes that are influenced by environmental signals that are similar to those that influence the genes controlled by the *bvg* locus (Peppler, 1984; Peppler and Schrumpf, 1984; Caroff *et al.*, 1990; van den Akker, 1998). Alteration between the LPS phenotypes has been associated with altered susceptibility to antibacterial peptides (Banemann *et al.*, 1998), which form part of the defenses present on mucosal surfaces and that act to control intracellular bacteria. This may contribute to the effects of phase variation on survival within phagocytes (Banemann and Gross, 1997). In some strains of *B. bronchiseptica* this phenotypic variation is under the control of the phase-variable gene in the *bvg* locus. The mechanism of regulation and variation in other bordetellae is different and currently unknown (van den Akker, 1998). The true complexity of phase-varied systems is likely to be increasingly recognized as they are systematically investigated.

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What Is an Antibiotic? Revisited

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I. Introduction

Antibiotic substances such as penicillin, streptomycin, and other ''miracle drugs'' represent the triumph of twentieth century medicine over numerous infectious diseases. These natural products with antimicrobial properties transformed therapeutic medicine and changed the character of the pharmaceutical industry. In the English language, these substances are collectively called ''antibiotics,'' and the term itself has come to imply therapeutic power. Nevertheless, an examination of the literature shows that there is wide variation in the way both the lay public and scientists circumscribe and define these drugs. For many people in the public at large, an antibiotic is simply a kind of pill, often expensive, providing almost miraculous relief from whatever ails them. It is commonplace for patients to demand antibiotic treatment against all sorts of diseases (e.g., the common cold) for which it is futile. The overuse and misuse of antibiotics have contributed to the development of antibiotic-resistant strains of human pathogens.

What is the origin of the term antibiotic? When did it enter the English language? How is it defined in the scientific literature? How is it used in general language? What accounts for the frequent

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academic arguments over whether or not certain new compounds with antimicrobial (or other) activity deserve to be labeled as antibiotics?

One of the most widely quoted definitions of antibiotic comes from a paper published by Selman A. Waksman in 1947 entitled ''What Is an Antibiotic or Antibiotic Substance?''

> An antibiotic is a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms. The action of an antibiotic against micro-organisms is selective in nature, some organisms being affected and others not at all or only to a limited degree; each antibiotic is thus characterized by a specific antimicrobial spectrum. The selective action of an antibiotic is also manifested against microbial vs. host cells. Antibiotics vary greatly in their physical and chemical properties and in their toxicity to animals. Because of these characteristics, some antibiotics have remarkable chemotherapeutic potentialities and can be used for the control of various microbial infections in man and animals. (Waksman, 1947)

This definition, requiring 117 words, was proposed 2 years after Alexander Fleming, Howard Florey, and Ernst Chain shared the 1945 Nobel Prize for Medicine or Physiology for the discovery of penicillin, and 5 years before Waksman himself was awarded a Nobel Prize for the part he played in the discovery of streptomycin.

Waksman was a major figure in microbiology with a strong personality and considerable influence. His attempts to stipulate a definition for the new class of drugs held considerable authority. Nevertheless, words and their definitions have a way of evolving on their own. In this chapter, we trace the etymology of antibiotic before Waksman, review his various attempts to impose a definition, and document something of the current inconsistency and multiplicity in meaning associated with the word.

II. Etymology

The noun ''antibiosis'' comes from the French word, *antibiose*, coined by Vuillemin in 1889–1890 to describe antagonistic effects between microorganisms (Vuillemin, 1890). Antibiosis was the antonym of symbiosis: ''C'est exactement l'inverse qui se passe dans la *symbiose*.'' Vuillemin also used the adjective, *antibiotique*, as in ''*action antibiotique*.''

Writing shortly thereafter in English, Kanthack and Hardy (1894) observed a different kind of antimicrobial action, almost certainly immunological in origin: i.e., the action of frog lymph on *Bacillus*

anthracis and *B. filamentosus* (probably *B. cereus*.) They noted that eosinophilic leukocytes had a ''distinctly harmful action on the vitality and growth of the bacilli,'' and that when a chain of bacterial cells was attached by a sufficient number of the leukocytes ''all growth is suspended.'' They concluded that these cells contained an antagonistic substance and stated, ''The eosinophile cells are highly specialized bodies endowed with . . . glandular powers directed to the production of a bactericidal, or at least antibiotic, substance.''

Another nineteenth century use of ''antibiosis'' in English appeared in a long paper by Ward (1899) dealing with symbiosis. Ward described different degrees of association between organisms, asserting that there were extreme cases ''where one of the two associated organisms is injuring the other, as exemplified by many parasites, but these cases I leave out of account here. This state of affairs has been termed *Antibiosis*.'' This is the *only* use of this word in the article. Moreover, Ward provided no material about its etymology, although in a synopsis at the beginning of the paper he cited Vuillemin's paper after the following cryptic statement: ''Galls not necessarily due to insects, but may be due to the irritating action of Fungi or Bacteria. Phytocecidia of the Aleppo pine, & c.'' (Ward, 1899).

By the 1930s, well before Waksman's successful campaign to turn ''antibiotic'' into a noun, the words ''antibiosis'' (noun) and ''antibiotic'' (used as an adjective) had become fairly common in the general biological literature. (It should be noted that grammarians label ''antibiotic'' as a substantive, i.e., a word carrying the functions of a noun.)

A French text, *Les Associations Microbiennes. Leurs Applications Thérapeutiques*, published in 1928, was organized in three parts: Généralités, Les Associations Microbiennes, and Applications Pratiques des Antagonismes Microbiens (Papacostas and Gaté, 1928). In Section A of the second part, Chapter 2 is titled ''L'Antibiose'' with 80 pages dealing with *in vitro* work with mixed cultures. Antibiosis (used without attribution) was further categorized as ''antibiosis réciproque'' where the antagonistic action was on two components and ''antibiosis unilatérale'' where only one was involved. Antagonism affecting growth (végétabilité) was ''antibiosis vitale'' or affecting a function such as toxicity was ''antibiosis functionnelle.'' The literature review contained approximately 600 citations concerning antibiotic actions. Although claiming completeness, important early work was omitted.

The noun ''antibiosis'' and the adjective ''antibiotic'' were also being used in English textbooks. For example, Buchanan and Fulmer (1930)

published Volume III of *Physiology and Biochemistry of Bacteria*. The first chapter of this volume (Chapter XIV of the complete work) was titled ''Symbiotic and Other Special Interrelationships Among Microorganisms.'' They stated that,

> In some cases the two organisms growing together are more or less reciprocally beneficial, they live in a condition of *symbiosis*. In other cases one of the organisms lives on the growth or waste products of another without being either definitely beneficial or injurious, the organisms are said to live in a condition of *commensalism*, or in some instances, *metabiosis*. If the host is injured by the growth of the organism, the condition is that of *parasitism*, or if one organism is injurious to another, the condition may be termed *antibiosis*.

It was noted that these conditions intergrade and are sometimes difficult to distinguish. Many examples of antibiosis between different species of bacteria were given as well as examples of protozoal–bacterial antibiosis.

Several research papers published in the period 1931–1942 also used antibiotic in the adjectival sense. For example, in a 1931 paper entitled ''Bacteria antibiotic to *Ustilago zeae*,'' the isolation of bacteria with a deleterious effect on *Ustilago zeae* was described. The organisms were called ''antibiotic bacteria'' and the action was called ''an antibiotic effect'' (Bamberg, 1931). In further work on this topic, ''The antibiosis of certain bacteria to smuts and some other fungi,'' Johnson (1930) provided many uses of both terms. She described ''four types of bacteria antibiotic to certain smuts and other fungi,'' and commented that although it was impossible to ''make any statement as to the economic importance of this antibiosis, the study suggests that antibiotic processes occur in nature.''

Working in France, Lasseur and his colleagues studied antagonisms between *Pseudomonas chloroaphis* and *B. caryocyaneus* (*B. chloroaphis*) (Lasseur and Marchal, 1934; Lasseur *et al.*, 1934). On several occasions the authors wrote of ''L'action antibiotique'' and ''le pouvoir antibiotique.'' The title of the first paper was ''Associations bacteriennes. Antagonisme–Antibiose'' (Lasseur and Marchal, 1934).

In the late 1930s, the prominent mycologist Constantine J. Alexopoulos and his colleagues published three papers dealing with the possible inhibition of fungi by common species of bacteria under the generic title, ''Studies in Antibiosis between Bacteria and Fungi'' (Alexopoulos *et al.*, 1938; Alexopoulos, 1941; Alexopoulos and Herrick, 1942). In the first paper published in 1938, it was stated that

''the antibiotic phenomena herein described are due to the presence of some substance which is toxic to the fungus,'' and also used the phrase ''antibiotic phenomena'' (Alexopoulos *et al.*, 1938). Over 80 *Actinomyces* species were screened, thus presaging Waksman's work.

In *Biological Abstracts*, ''antibiosis'' had first appeared as an index heading in Volume 2 (1928) for a German paper dealing with induced antagonisms (Schiller, 1927). The abstract itself did not contain the word ''antibiosis,'' rather, reference was made to ''lytic substances.'' From 1929 to 1932, there were no ''antibiosis'' entries. In 1933 there were two, and in one of them the words ''action antibiotique'' appeared in the title (Dujardin-Beaumetz, 1932). There were three ''antibiosis'' entries in 1935, one in 1938, and three in both 1939 and 1940. In those years, the terms ''antibiotic action'' and ''antibiotic substance'' also appeared in the Abstracts themselves. The number of articles indexed under ''antibiosis'' increased to seven each in 1941 and 1942; four of the abstracts in 1942 were about penicillin. The major change came in 1943 with three headings: ''antibiosis'' (5 abstracts), ''antibiotic action'' (24 abstracts), and ''antibiotic substances'' (listing the names of 18 specific substances).

Dictionaries often list definitions in order of their chronological appearance in the language. Thus, it is worth noting that ''antibiotic,'' employed as an adjective, had appeared in the English language prior to Vullemin, in an entirely different sense. *A New English Dictionary on Historical Principles* [that later became *The Oxford English Dictionary* (OED)] indicated rare usage with the meaning of ''opposed to a belief in the presence or possibility of life,'' giving citations from 1860 (''I incline to the antibiotic hypothesis'') and 1877 (''the antibiotic prejudice''). This was the only definition given for antibiotic. The 1933 reissue of the OED contained a Supplement in which a second and biological meaning was given for antibiotic as an adjective: ''injurious to or destructive of living matter'' (Murray, 1933). This was supported with reference to the previously noted Kanthack and Hardy (1894) publication. In the more extensive Supplement of 1987, ''esp. microorganisms'' was added to the above and the adverb form ''antibiotically'' was given. ''Antibiotic'' was now described as coming from French, ''antibiotique,'' with a citation of Vuillemin, with the other references all to Waksman. A third meaning was also given: ''Hence as sb., [substantive] an antibiotic substance: one of a class of substances produced by living organisms and capable of destroying or inhibiting the growth of micro-organisms; spec. any of these substances used for therapeutic purposes. Also used of synthetic compounds having similar properties.'' The 1987 Supplement now listed antibiosis for

the first time as ''A condition of antagonisms between organisms, esp. micro-organisms (opp. *Symbiosis*)'' with derivation via Vuillemin.

III. Waksman's Usage

Waksman was somewhat imprecise in explaining the reasons that led to his adoption and then promotion of the unembellished noun form ''antibiotic'' for a group of compounds that had been previously called ''antibiotic substances.'' In the 1947 ''What Is an Antibiotic or Antibiotic Substance?'' paper, Waksman wrote that he had been asked to provide a term for materials such as penicillin and other new materials with similar antimicrobial properties ''in July, 1941, by Dr. A. [*sic*] Flynn, editor of *Biological Abstracts*.'' Many years later, he indicated that he had received a letter from Dr. Flynn ''around 1941'' (Waksman, 1981). In another publication, where he gave the correct initials J. E., for John E. Flynn, then Editor-in-Chief of *Biological Abstracts*, he stated that the request came in July 1942 (Waksman, 1975). To the best of our knowledge, Waksman's reply to Dr. Flynn was not in the form of a letter. It is likely that it was made verbally because in a videotape of an interview between Dr. Waksman and Dr. Boyd Woodruff in 1973, Waksman referred to a phone call (Waksman, 1973).

While doing research for this chapter in 2002, we contacted Dr. Boyd Woodruff. Although he could not shed light on the date of the request from Dr. Flynn, he recollected that

> The term antibiotic was introduced, I would state sometime in 1940, during a post literature club luncheon. Waksman had asked us students to spend a week thinking seriously about what name should be used to describe new antibacterial substances . . . and to present our suggestions to him at the next week's meeting. (Woodruff, personal communication, February 16, 2002).

At the next meeting, the students made no striking suggestions. However, ''it was obvious that Waksman had already made his decision and he put the word antibiotic forward with enthusiasm, with the suggestion that it be applied solely to antibacterial products produced by microorganisms active against other microorganisms.'' Dr. Woodruff reiterated his belief that this happened in 1940 but noted that he was recollecting events of more than 60 years ago.

Although it is difficult to establish the date when Waksman became aware of the terms ''antibiosis'' and ''antibiotic substance,'' most sources confirm that his adoption of the word ''antibiotic'' was precipitated by the Flynn request. In 1981, the introduction of an historical

perspective published by Merck, Sharp and Dohme, printed a letter, or a draft thereof, apparently meant for Dr. J. E. Flynn, that was dated 1962 (Cowen and Segelman, 1981). Waksman wrote that after going over Flynn's initial request carefully:

> I decided on an old word, like taking an old skin and putting a new wine into it. I said, ''Let us take the word 'antibiotic' which was formerly used in 1891 as an adjective, meaning against life, comparable to the word 'symbiotic', a phenomenon designating collaboration in living systems. Let us make it into a noun which will include microbes that have an injurious effect on other microbes.''

By using the date 1891, he was indirectly referring to Vuillemin's use of ''action antibiotique.''

In retrospect, it is surprising that Waksman almost completely ignored both the noun, ''antibiosis,'' and the adjective, ''antibiotic,'' in most of his pre-1940 publications. In 1937 he had written a two-part review, ''Associative and Antagonistic Effects of Microorganisms.'' Part I, ''Historical Review of Antagonistic Relationships'' contained 107 citations. Beyond citing Ward's ''definition'' of antibiosis, and quoting Johnson about ''bacteria antibiotic to certain smuts'' he made no further use of these words and did not quote Vuellemin (Waksman, 1937). Part II, ''Antagonistic Effects of Microorganisms Grown on Artificial Substrates,'' was an experimental paper containing no references to antibiosis (Waksman and Foster, 1937). One is almost forced to the conclusion that Waksman was unaware of Vuillemin's term until the early 1940s.

Dr Boyd Woodruff confirms that Waksman ignored the term ''antibiosis'' before about 1941. ''According to my recollection of the early screening period, late 1939 to late 1940, possibly into early 1941, the word 'antibiosis' was not used in the Waksman laboratory. The words 'antibacterial agent' or 'bactericidal agent' were the general terms used, with considerable effort to see which applied to specific situations'' (Woodruff, personal communication, February 16, 2002). Moreover, in a 1940 preliminary note about actinomycin, the destruction of bacterial cells had been attributed to a chemical interaction ''similar to that of other antiseptics'' (Waksman and Woodruff, 1940).

In a 60-page paper in *Bacteriological Reviews* published in 1941, with the title, ''Antagonistic Relationships of Microorganisms,'' Waksman referred to ''antibiosis'' as follows: ''The terms 'association' and 'symbiosis' are used to designate mutually beneficial relations, as contrasted to 'antagonism' and 'antibiosis', which refer to a reduction in growth and in activities as a result of the living of organisms

in mixture'' (Waksman, 1941). In the same review he stated that ''antagonism *in vivo* was often designated as antibiosis'' supporting this statement with reference to the papers by Lasseur's group (Lasseur and Marchal, 1934; Lasseur *et al.*, 1934). This *Bacteriological Reviews* paper contained only a single, rather casual use of ''antibiotic''—as an adjective—as follows: ''Four types of bacteria antibiotic to smuts and to certain other fungi . . .'' This exact phrase occurred in the paper by Johnson (1931). As noted above, Waksman had already quoted this same phrase in an earlier review published in *Soil Science* (Waksman, 1937). Again, Vuillemin's paper was not among the 373 references listed in this major review (Waksman, 1941).

Jumping ahead to his 1947 ''What Is an Antibiotic or Antibiotic Substance?'' paper, Waksman stated therein that ''the terms 'antibiotic' and 'antibiotic agent' were first used in the present sense by Waksman and his collaborators in several papers published or written in 1942'' (Waksman, 1947), i.e., two papers published in the *Journal of Bacteriology*, a paper published in *Mycologia*, and another in *Soil Science* (Waksman and Woodruff, 1942; Waksman and Horning, 1943; Waksman *et al.*, 1942, 1943). Later, in 1951, Waksman moved back his claim to priority by a year, stating that ''The word antibiotic, in the presently accepted sense, was first proposed by the writer in 1941. He and his collaborators first used this word in a series of scientific publications published in 1942'' (Waksman, 1951).

A careful reading of the four papers ''published or written in 1942'' to which Waksman referred shows an extensive adjectival use of ''antibiotic,'' as in ''antibiotic substances'' etc. There is, however, only a single use of antibiotic as a noun. In describing fumigacin, Waksman writes it was ''considered as the true antibiotic produced by *A. fumigatus*'' (Waksman *et al.*, 1943). (Note: fumigacin was later found to be a mixture of helvolic acid and gliotoxin.)

In a footnote to the 1942 *Soil Science* paper (Waksman *et al.*, 1942), *bacteriostatic* as used by Gardner and Chain (1942) was equated with ''*antibiotic* previously suggested by us to designate the various bacteriostatic and bactericidal substances produced by microorganisms.'' This suggested use was attributed to the *Bacteriological Reviews* paper (Waksman, 1941). However, as already noted, this lengthy paper provides no substantive use of antibiotic and in the 1947 *Mycologia* paper it was not listed to claim priority.

Surprisingly, in none of these priority-establishing recollections did Waksman point out that he and Woodruff had published a paper in 1941 on a new soil organism producing actinomycin in which they named the organism *Actinomyces antibioticus* n. sp. (Waksman and

Woodruff, 1941). Woodruff has stated that Waksman wrote the actinomycin paper and coined the *antibioticus* species name. Further,

> This is one of the reasons I feel the meeting when the word ''antibiotic'' was proposed came prior to July 1941. The *Actinomyces antibioticus* paper was published in 1941, I believe prior to July. If the word ''antibioticus'' had been in use then, proposal of the word ''antibiotic'' for the substance would have been obvious, and according to my recollection the proposal by Waksman at the luncheon meeting was not obvious. (Woodruff, personal communication, February 16, 2002).

Be that as it may, the *Actinomyces antibioticus* paper does not refer to the noun ''antibiotic'' in the text. The material produced by the newly described organism was simply labeled a ''bacteriostatic substance'' with a description of its bacteriostatic, bactericidal, and fungistatic properties (Woodruff and Waksman, 1941).

In his 1947 *Mycologia* paper, ''What Is an Antibiotic or an Antibiotic Substance?,'' Waksman cites himself for having defined antibiotic in two previous publications. The first of these was a Harvey Lecture on ''Production and Nature of Antibiotic Substances'' (Waksman, 1944–1945) where he pointed out that these substances ''possess certain chemical and biological properties which distinguish them from the common antiseptics and disinfectants'' (see later). There followed a wordy listing of 10 properties of ''antibiotic substances''— not exactly a definition in the usual sense of that word. In no place in the published Harvey Lecture did Waksman use ''antibiotic'' in the noun form.

The attributes of ''antibiotic substances'' that Waksman noted in his Harvey Lecture may be paraphrased as follows:

1. They are produced by living organisms and able to inhibit the growth of or destroy bacteria and other microorganisms. Some synthetic materials have similar properties.
2. They exhibit mostly bacteriostatic action but may have pronounced bactericidal properties.
3. Their actions are selective.
4. The ''substrate'' may alter the antibiotic action, e.g., blood may adsorb the material, thus inactivating it.
5. Antibiotic substances show a wide range of chemical structures.
6. They show a wide variety of toxic responses in animals.
7. They differ in mechanisms of action.
8. Some organisms produce more than one antibiotic substance.
9. Some (or similar) materials may be produced by different fungi.

10. The nature of the antibiotic substance produced may be influenced by media composition and cultural conditions.

In summary, in giving this list of attributes, ''antibiotic'' was always used as an adjective to describe ''substances.''

The second pre-1947 definition was in the text *Microbial Antagonisms and Antibiotic Substances* (Waksman, 1945), in which the Glossary provided the following somewhat circular meanings:

> *Antibiosis*—The inhibition of growth of one organism by another. *Antibiotic*—inhibiting the growth or the metabolic activities of bacteria and other microorganisms by a chemical substance of microbial origin. *Antibiotic substance, antibiotic*—A chemical substance, of microbial origin, that possesses antibiotic properties.

In summary, it appears that this 1945 textbook is the first published citation with use of ''antibiotic'' in the substantive noun form, i.e., ''antibiotic'' is equated with ''antibiotic substance.'' This conclusion is supported by Chain's statement: ''These substances are now known as antibiotics, a name coined in 1945 by S. A. Waksman'' (Chain, 1972).

Vuillemin was cited neither in the brief reference list for the Harvey Lecture (Waksman, 1944–1945) nor in the 1016 references listed in the textbook (Waksman, 1945). However, both Vuillemin (1890) and Ward (1899) were cited in ''What Is an Antibiotic or Antibiotic Substance? where Waksman wrote ''The use of the words 'antibiosis' and 'antibiotic substance' to designate antiliving processes in a very broad sense is found in the older biological literature as well as in many dictionaries'' (Waksman, 1947).

After 1947, Waksman continued to promote and republish definitions of antibiotic. Table I lists several of these later versions. Most of them simply restate the first sentence of the definition put forth in the 1947 paper, with one important modification. Starting about 1951, Waksman sometimes added the caveat that antibiotics were effective in low concentrations, a change intended to exclude materials such as acids and alcohols.

Waksman felt possessive about his definition of antibiotic. In 1952, P. R. Burkholder wrote an article in *American Scientist* in which he stated ''The word 'antibiotic' was coined by Vuillemin in 1889, and ten years later extended by Ward to include the antagonistic relations among microbes'' (Burkholder, 1952). Waksman immediately rebutted with a letter: ''Actually, Vuillemin never used the word 'antibiotic' in its presently accepted sense,'' he wrote. Then he retold the story about the 1941 request from Dr. Flynn, reviewed the discovery of gramicidin,

TABLE I

EXAMPLES OF SELMAN WAKSMAN'S PUBLISHED DEFINITIONS OF ANTIBIOTIC

''An antibiotic is a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other microorganisms. The action of an antibiotic against micro-organisms is selective in nature, some organisms being affected and others not at all or only to a limited degree; each antibiotic is thus characterized by a specific antimicrobial spectrum. The selective action of an antibiotic is also manifested against microbial vs. host cells. Antibiotics vary greatly in their physical and chemical properties and in their toxicity to animals. Because of these characteristics, some antibiotics have remarkable chemotherapeutic potentialities and can be used for the control of various microbial infections in man and in animals'' (Waksman, 1947).
''An antibiotic or antibiotic substance is a substance produced by microorganisms, which has the capacity of inhibiting the growth and even of destroying other microorganisms'' (Waksman, 1949).
''An antibiotic is a chemical substance, produced by microorganisms, which has the capacity to inhibit the growth and even to destroy bacteria and other microorganisms, in dilute solutions'' (Waksman, 1951).
''Chemical substance produced by microorganisms, which has the capacity to inhibit the growth and even to destroy bacteria and other microorganisms'' (Waksman, 1953b).
''Antibiotics are chemical substances that are produced by living organisms and that have the capacity to inhibit the growth of microorganisms or other living cells'' (Waksman and Lechevalier, 1962).
''Chemical substances, produced by microorganisms, which have the capacity, in dilute solutions, to inhibit the growth of and even destroy bacteria and other microorganisms'' (Waksman and Bianchine, 1994). (Note: Waksman died in 1973; Bianchine was giving posthumous credit 21 years later.)

penicillin, and actinomycin, and concluded, ''A suitable term was needed to include all these substances. I suggested then the use of the word 'antibiotic' to designate a 'chemical substance, produced by microorganisms, which has the capacity to inhibit the growth and even to destroy bacteria and other microorganisms.' This term first appeared in published papers in 1942. It was first employed for indexing purposes in Biological Abstracts in 1943'' (Waksman, 1953a). Similarly, in the taped interview with Boyd Woodruff shortly before his death at age 85 in 1973, Waksman stated, ''I came up with the word antibiotic . . . a French botanist had used antibiotic as an adjective. . . . I suggested that we take that word and change in into a noun'' (Waksman, 1973).

According to David Pramer, one of his graduate students, and later director of the Waksman Institute, Waksman took ''special pride'' in

having defined antibiotic and what Waksman considered errant definitions ''evoked philological discussion and debate on numerous occasions'' (Pramer, 1988). Moreover, Waksman worked hard to ensure that ''antibiotic'' be used according to his own stipulated meaning. Pramer remembered that Waksman had a ''sensitivity to what he considered abuse of the word'' and has described the way in which Waksman once chided a cancer researcher for using the word ''antibiotic'' to describe an agent active against tumor cells. When Pramer asked Waksman what term should be used to describe a microbial product active against cancer cells, he replied in good humor that when such a material was discovered he would tell Pramer what it should be called (Pramer, 1988). Paradoxically, in describing the objectives of the new Institute of Microbiology at Rutgers University in 1949, Waksman noted that particular attention was to be paid to ''isolation of antibiotics active against *Mycobacterium tuberculosis*, against viruses, and possibly against tumor cells also'' (Waksman, 1949).

In summary, Waksman and his colleagues, beginning in the early 1940s, promulgated the use of ''antibiotic'' based on the words antibiose and antibiotique, introduced by Vuillemin. Although Waksman can be criticized for not being more generous in giving credit to Vuillemin, he clearly derived the use of antibiotic as a substantive noun and can be credited for his attempts to stipulate a standard definition for the class of new drugs that was revolutionizing the treatment of infectious diseases.

IV. A Myriad of Definitions

A. Acceptance Despite Alternate Terms and Definitions

Whatever Waksman's part in popularizing the word ''antibiotic,'' by 1945 it was in wide use by many scientists. For example, a major 1945 article in *Annual Review of Biochemistry* entitled ''The Chemistry of Antibiotic Substances Other Than Penicillin'' contained the following definition:

> For the present purpose an antibiotic is defined, subject to the qualifications set out below, as a soluble, organic substance, which is produced by a microorganism from a harmless constituent (or constituents) of a medium and which has been found to be markedly inhibitory to the growth or activity of a second microorganism, when it is dissolved in a medium otherwise suitable for the normal growth or activity of second microorganism. (Oxford, 1945)

Oxford went on to include a criterion that Waksman had ignored, namely concentration, and proposed that an antibiotic substance should show an inhibitory action *in vitro* of ''the order of 50 p.p.m or less.'' Finally, Oxford also suggested that the active substance should have been isolated and tested in a pure state. Waksman did not like this modification. It is interesting to note that Fleming had coined the name penicillin not for a purified chemical compound, but simply to avoid repetition of the phrase, ''mould broth filtrate'' (Fleming, 1929).

Fleming himself was quick to adopt ''antibiotic.'' By 1946 he wrote, ''Penicillin belongs to a class of antibacterial substances produced by living organisms which as far back as 1889 were termed antibiotics'' (Fleming, 1946). Although Fleming did not give a precise citation, the date of 1889 indicates that he was referring to Vuillemin's paper. Fleming did not cite Waksman.

On the other hand, not every one adopted Oxford's, Fleming's, and Waksman's choice of the word ''antibiotic.'' The bacteriologist J. H. Cohn, in Geneva, suggested the alternate term ''antibiotin'' (Cowen and Segelman, 1981)—luckily for work on biotin this was never seriously considered. Another suggestion made in 1943 by German authors was that fungal antibacterial materials should be termed ''mycoine'' (Vonkennel *et al.*, 1943). According to Milton Wainwright's book, *Miracle Cure. The Story of Penicillin and the Golden Age of Antibiotics* (Wainwright, 1990), Florey ''objected to the word because, taken literally, it meant 'against life' and clearly, he argued, a substance that could snatch a person from the 'jaws of death' is not best described in this way.'' Florey actually preferred ''bacteriostat'' (Wainwright, 1990). The previously noted use of bacteriostatic (Gardner and Chain, 1942) came from colleagues of Florey.

In a brief letter published in *The New England Journal of Medicine*, Wingo (1945) wrote that ''so useful a word as antibiotics'' should not be limited ''to one particular category of substances that are destructive to life'' and indicated that arsenicals and mercurials, as well as different types of radiant energy were ''true antibiotics.'' He proposed that bacterial and fungal products be termed ''mycoantibiotics,'' whereas synthetic compounds would be ''chemoantibiotics'' with ''actinoantibiotic'' referring to the action of radiant energy. Waksman opposed them suggesting that such variation would require the addition of ''phytoantibiotics'' (products of green algae and higher plants) and zoöantibiotics (animal products) (Cowen and Spegelman, 1981). These modifications never came into general use. Nevertheless, in the 1949 treatise, *Antibiotics*, Florey and his co-authors included plant products with antimicrobial activities, although ''strictly speaking it is doubtful

whether the term should be applied to them''; substances of animal origin such as lysozyme were excluded (Florey *et al.*, 1949).

In 1947, in the first volume of *Annual Review of Microbiology*, Benedict and Langlykke titled a review with the unadorned substantive noun: ''Antibiotics.'' Ignoring Waksman's attempts to standardize a stipulated definition they wrote, ''The term 'antibiotic' as used in the sciences is not yet of fixed definition; it connotes many different meanings, depending on individual interests . . . we choose to define an antibiotic as a chemical compound derived from or produced by living organisms, which is capable, in small concentration, of inhibiting the life processes of microorganisms.'' The article then classified known antibiotics according to the organisms that produced them with sections on antibiotics from bacteria, antibiotics from yeasts, antibiotics from molds, antibiotic activity of phycomycetes, antibiotics from basidiomycetes, and antibiotics from algae and lichens.

The *Handbook of Antibiotics* (Baron, 1950) also did not adopt Waksman's stipulated definition, stating that ''In the most parsimonious sense of the word, an antibiotic is merely a member of the group of substances generally referred to as antiseptics, disinfectants, bactericides, fungicides, etc.'' (Parsimonious seems an odd word to describe such a commodious definition.) Baron then ''cut the Gordian knot by proposing no new definition at all.'' However, he considered that an antibiotic should satisfy ''more or less'' all of the following conditions: (1) It was a product of metabolism (although it might be duplicated or even have been anticipated by chemical synthesis), (2) it antagonized the growth and/or survival of one or more species of microorganisms, and (3) it was effective in low concentration. A desirable feature, although not a prerequisite, was a low toxicity to higher plants and animals.

B. Deconstructing Waksman's Definition

An examination of Waksman's published definitions of ''antibiotic'' (Table I) shows that Waksman emphasized the fact that antibiotics were natural products. Moreover, his stipulated definition limited antibiotics to natural products synthesized by microorganisms. He maintained that ''antibiotic'' should be used only with this narrow meaning; an antibiotic was neither a material produced by a plant nor a substance produced by animal cells. At a 1956 Symposium, he made this point forcibly:

> As I understand antibiotics, we are dealing here entirely with microbial products. We are not dealing with plant products; we are not dealing with

> animal products. Blood has antibacterial properties. Quinine has antibacterial properties. Therefore, we would be lost if we consider the whole system of life, because we will find that all plants, all animals, insects and what not, produce in one way or another antimicrobial activity. Therefore, we must adopt the concept that has been said time and again by some of the greatest philosophers: ''In order to understand one another, let us define our terms first of all.'' (Waksman, 1956)

Waksman's insistence that the term ''antibiotic'' be limited to microbial products has generated decades of heated debate about whether a given antimicrobial compound can legitimately be called one. When an expert six-person ''Antibiotic Definition Committee'' was convened by the *Journal of Antibiotics* in 1992, for example, the majority of the committee felt that an antibiotic could be defined ''as a natural product produced by any type of cell, microbial, plant or animals'' but one member felt that only microbially produced products should be called antibiotics (A. Demain, 2002, personal communication). The Committee ended up delivering both a majority and a minority report.

Do antibiotics have to be microbial products? Contemporary usage answers this question with a resounding ''no.'' Current usage, by most workers in the field, is extremely inclusive with multicellular plants and animals both being accepted as legitimate sources of antibiotic materials.

The next question is: Do antibiotics have to be natural products? Waksman stressed that antibiotics came from living organisms. Perhaps this emphasis was an attempt to distinguish penicillin and streptomycin from the sulfonamides. During the 1930s, the sulfa drugs had come into extensive clinical use and had provided an early demonstration that a chemical substance could provide antibacterial activity with low toxicity in humans. Nevertheless, the early classifications of antibiotic drugs, and many contemporary ones, exclude the sulfonamides from the rubric.

On the other hand, even Waksman conceded that antibiotic materials could be made by chemists. In his Harvey Lecture, Waksman (1944–1945) had postulated derivation from a living organism but added, ''Some synthetic materials have similar properties.'' Indeed, research on the penicillin family soon blurred the difference between natural and synthetic compounds. The widely used semisynthetic material, amoxicillin (β-amino-*p*-hydroxybenzylpenicillin), was readily accepted as a genuine antibiotic; it contained much of the structure of the usual benzylpenicillin molecule and was derived from a fermentation product. Thus, it was an easy step to accept that aztreonam, the first wholly synthetic monocyclic β-lactam, was also

an antibiotic. Benzylpenicillin, amoxicillin, and aztreonam all contained a β-lactam structure, the so-called ''enchanted ring'' (Sheehan, 1982), whether or not the compounds were synthesized by microbial fermentation, laboratory bioconversion, or chemical synthesis. In summary, the general consensus has been that synthetic compounds should be accepted as antibiotics if they have otherwise appropriate biological activity.

What is this otherwise appropriate biological activity? Waksman's definition specified that antibiotics ''inhibit the growth or even destroy bacteria and other microorganisms.'' Thus, his version of the antibiotic umbrella covered activity against algae, bacteria, fungi, protozoa, and viruses, but not—except as a secondary effect—activity against multicellular animals and plants. Waksman's antibiotic was synonymous with ''all antimicrobial substances of microbial origin.''

A difficulty not foreseen by Waksman, or the other early pioneers, was that almost all the clinically significant antibiotics isolated for most of a half century were active only against bacteria. Antifungal, antiprotozoal, and antiviral agents were—with few exceptions—too toxic for therapeutic use. Therefore, the clinical application of antibiotics was largely restricted to treatment of diseases caused by bacterial pathogens. Because these drugs were so efficacious, many lay people came to expect that they should be effective against *all* infectious disease. To correct this misconception, it became commonplace for health care workers to warn patients that antibiotics did not work against influenza, malaria, and other microbial diseases of nonbacteriological etiology. This practical advice has affected the vernacular and clinical meaning of antibiotic (see below).

As noted earlier, Waksman added the phrase ''in dilute solution'' to his short definition to highlight the fact that antibiotics were active in low concentration. The long version of his definition also addressed another important aspect of antibiotics: their selective nature. This selectivity was manifested in two ways. First, each compound had a characteristic antimicrobial spectrum. Some antibiotics killed gram-positive bacteria and others killed gram-negative bacteria. Some antibiotics affected a large number of pathogens; others were effective against only a few types of bacteria. The former are now called broad-spectrum antibiotics; the latter are called narrow-spectrum antibiotics.

The second manifestation of selectivity, Waksman lumped together with several nonbiological attributes, almost as a throw-away line: ''Antibiotics vary greatly in their physical and chemical properties

and in their toxicity to animals.'' At no point did he specify that antibiotics should have little or no toxicity.

In summary, the definition of antibiotic, *sensu* Waksman, emphasized three criteria: the source of the compound (a microbe), the target of its pharmacological action (another microbe), and activity in low concentration. The attributes of differential selectivity against target organisms, and—most importantly, differential toxicity to the host species—were of lesser importance.

C. Toxicity and Antibiotics

From the medical point of view, the objective of antibiotic research is ''to find new substances that are active in the body against pathogenic micro-organisms and can be used in chemotherapy'' (Abraham and Florey, 1949). For clinical use, an antibiotic must have selective toxicity—sufficient to incapacitate a pathogen but with little or no ill effect on the host. Thus, some early antibiotic substances, e.g., tyrocidine, became limited to the topical treatment of localized infections because of their systemic toxicity.

As noted above, it is striking to note how little Waksman was concerned about the toxicity of the substances he called antibiotics. By stating that ''Antibiotics vary greatly . . . in their toxicity to animals'' he was creating a ''big tent'' definition, close to the meaning originally given by Vuillemin for ''antibiosis.'' Perhaps in the context of the times, this disregard for toxicity was not surprising. When Ehrlich opened the gates of modern therapeutics, at the beginning of the twentieth century, arsenous acid had been injected into mice suffering from trypanosomiasis. The trypanosomes were killed—but so were the mice. The experiment was rated a modest success, however, since the animals had ''died cured'' (Albert, 1968). When Ehrlich later discovered organic arsenicals, they were used in human therapy against syphilis despite high toxicity. There were no superior alternatives. (The drugs currently used in cancer chemotherapy provide a contemporary analogy. With few exceptions, they are quite toxic, but they are used anyway because they are better than nothing.)

Many of the active antimicrobial compounds found during the Golden Age of Antibiotic Discovery ultimately were declared too toxic for therapeutic use. For example, aspergillic acid was first described by White (1940), who mistakenly claimed that it was the second fungal antimicrobial agent discovered after penicillin. During the 1940s, hydroxyaspergillic acid, flavicol, neohydroxyaspergillic acid, and several other pyrazones related to aspergillic acid were studied intensively

because of their potent antimicrobial activities. All were found to be dangerously toxic to host animals. They are now called mycotoxins (Wilson, 1971). In another case, the antibacterial material, clavacin, isolated by Waksman *et al.* (1943), was independently obtained by other investigators and given the names claviformin, expansin, myocin c, and penicidin. This compound is now known as patulin (Ciegler *et al.*, 1971). At one time, patulin was thought to have promise in treating the common cold and was tested as both a nose and throat spray. It was also formulated as an ointment for treating fungal infections. During the 1950s and 1960s, however, it became apparent that patulin's antiviral and antifungal properties were overshadowed by its toxicity. Patulin also has been reclassified as a mycotoxin (Ciegler *et al.*, 1971; Ciegler, 1977).

Pharmaceutical companies have invested millions of dollars to come up with a relatively small number of useful materials. Most substances with antimicrobial activity have undesirable attributes (e.g., toxicity to humans and other animals) and are then abandoned as antibiotic candidates.

Finally, it should be pointed out that some of these antibiotic substances have several pharmacological activities, which means that sometimes they can be ''recycled'' for nonantibiotic drug use. Mycophenolic acid is a case in point. Originally isolated in 1893 as a crystalline fungal metabolite with a possible connection to pellagra, it was shown to inhibit growth of the anthrax bacillus (Gosio, 1893, 1896). In addition to its antibacterial activities, it has been investigated over the years as an antifungal, antiviral, and antitumor material, and as an agent with activity against psoriasis. Because of its toxicity, mycophenolic acid has also been called a mycotoxin. Nevertheless, it has now emerged in recent years that it can be used as an immunosuppressant in the prodrug form, mycophenolate mofetil (brand name, CellCept) (Bentley, 2000).

D. How Antibiotics Are Defined Now

Representative examples of definitions of antibiotics from general purpose dictionaries and encyclopedias are given in Table II, from textbooks in Table III, and from scientific and medical monographs and dictionaries in Table IV. The power of Waksman's posthumous influence is conspicuous. Many of these published definitions still limit the meaning of ''antibiotic'' to microbial natural products, despite the fact that most scientists and physicians find this distinction ''rather

TABLE II

DEFINITIONS OF ANTIBIOTIC IN GENERAL-PURPOSE DICTIONARIES AND ENCYCLOPEDIAS

''A substance, such as penicillin or streptomycin, produced by or derived from certain fungi, bacteria, and other organism, that can destroy or inhibit the growth of other microorganisms. Antibiotics are widely used in the prevention and treatment of infectious diseases'' (Soukhanov, 1992, *American Heritage Dictionary*).
''As adjective: **1**: tending to prevent, inhibit, or destroy life. **2**: of or relating to antibiosis. **3**: of, with, or relating to an antibiotic. As noun: a substance produced by a microorganism (as a bacterium or fungus) and in dilute solution having the capacity to inhibit the growth of or kill another microorganism (as a disease germ)'' (Anonymous, 1993, *Webster's Third New International Dictionary, Unabridged*).
''An antibiotic is a substance derived from living organisms, usually bacteria or molds that kills microorganisms or inhibits their growth. Some antibiotics also interfere with life processes in higher organisms, but the term usually applies only to substances that act against microorganisms. Synthetic drugs also used to treat bacterial, fungal, or other parasitic infections may be called antibiotics, but strictly speaking the term is reserved for substances derived from living agents. The more general term might be antibacterial or antimicrobial'' (Simon, 1995, *Academic American Encyclopedia*).
''Chemical substance produced by a living organism, generally a microorganism, that is detrimental to other microorganism'' (Anonymous, 1997, *New Encyclopedia Britannica*).
''As adjective: injurious to or destructive of living matter, esp. microorganisms. As substantive: an antibiotic substance: one of a class of substances produced by living organisms and capable of destroying or inhibiting the growth of micro-organisms, spec. any of those substances used for therapeutic purposes. Also used of synthetic organic compounds having similar properties'' (Simpson and Weiner, 1997, *The Oxford English Dictionary*, 2nd ed.).
''Chemical substances produced by microbes (very small cells that usually cause disease) that are capable of killing or inhibiting the growth of another cell. In most cases the cell affected by the antibiotic is also a microbe'' (White, 2001, *The Encyclopedia Americana International Edition*).

academic'' (Pratt and Fekety, 1986) and accept synthetic, plant, and animal products as valid antibiotics.

Nearly all of the definitions state, in one way or other, that antibiotics inhibit or kill microbes, but some of the contemporary definitions have narrowed Waksman's ''antimicrobial'' to the more specific meaning of ''antibacterial.'' [See for example, Clayman (1989), *A.M.A. Encyclopedia of Medicine*, and Creighton *et al.* (1999), *Encyclopedia of Molecular Biology* in Table IV.] The 1995 *Science Desk Reference* (Table IV) actually warns that ''they are used to treat bacterial infections'' and that ''they do not work on viral infections'' (Barnes-Svarney, 1995).

TABLE III

DEFINITIONS OF ANTIBIOTIC IN SELECTED GENERAL BIOLOGY AND MICROBIOLOGY TEXTBOOKS

''Substance produced by one organism having adverse effect on other species'' (Hardin, 1966).
''Substances produced by living organisms that are toxic to other organisms, for example penicillin, a bacterial product that is toxic to other kinds of bacteria'' (Case and Stiers, 1971).
''Substances secreted by fungi and some kinds of bacteria; these substances are capable of killing or inhibiting the growth of various kinds of bacteria'' (Luria *et al.*, 1981).
''A substance of microbial origin that has antimicrobial activity in very small amounts'' (Pelczar *et al.*, 1986).
''An organic molecule that is produced by a microorganism and kills or retards the growth of other microorganisms'' (Raven and Johnson, 1986).
''A chemical agent produced by one organism that is harmful to other organisms'' (Brock and Madigan, 1988).
''Any microbial product which, in low concentrations, is capable of inhibiting or killing susceptible microorganisms'' (Ketchum, 1988).
''A product of metabolism of a microorganism that is inhibitory to other microorganisms'' (Alcamo, 1991).
''A chemical substance from one microorganism that can inhibit or kill another microbe even in minute amounts'' (Talaro and Talaro, 1993).
''Literally, 'anti-life'; a chemical produced by a microorganism that is able to inhibit the growth of or kill other microorganisms'' (Tortora, 1994).
''A metabolic product of an organism that kills or inhibits the growth of microorganisms'' (Ingraham and Ingraham, 1995).
''Any of a large number of substances, produced by various microorganisms and fungi, capable of inhibiting or killing bacteria and usually not harmful to higher organisms; for example, penicillin, streptomycin'' (Wallace *et al.*, 1996).

Only a few of the definitions mention selective toxicity and therapeutic efficacy. To coin an awkward term: they are extremely ''microbiology-ocentric.'' Two nondictionary definitions in Table IV, both from outside of the tradition in microbiology, do allude to toxicity (Abraham, 1949; Albert, 1968). But even they fall under the Waksman–Vuillemin penumbra: they do not exclude compounds too toxic for medical use (e.g., patulin). An exception is a recently proposed definition (Table IV) by Forsdyke (2000a). In short, most of the compounds that fit the standard definitions for ''antibiotic'' would never be approved as antibiotics by the U.S. Food and Drug Administration! In a curious reversion to old meanings, the clinically excluded toxic compounds may be described as having antibiotic action (the classic

TABLE IV

SELECTED DEFINITIONS OF ANTIBIOTIC IN MEDICAL–SCIENTIFIC MONOGRAPHS AND DICTIONARIES

''Substances with diverse chemical structures and biological activities. They range in their action from those which inhibit the growth of certain strains of bacteria in a highly selective manner to those which are relatively toxic to all living cells'' (Abraham, 1949).

''Substance derivable from living organisms and capable of adversely modifying the vital functions of specific microorganisms'' (Karel and Roach, 1951).

''Antibiotics are toxic substances, of low molecular weight, secreted by few bacteria but by many other prokaryotes, and a few fungi, most of them the lower fungi known as moulds. Most antibiotics are insufficiently selective to be used in medicine'' (Albert, 1968).

''A bacteriocidal or bacteriostatic substance produced by certain microorganisms'' (King, 1968).

''Antibiotics are natural organic compounds produced by microorganisms'' (Korzybski *et al.*, 1978).

''Substances produced by some microorganisms that destroy or arrest the growth of others'' (Medawar and Medawar, 1983).

''Substances produced by microbes which kill or inhibit the growth of other microorganisms. In contrast to general cell poisons, the A. are selective. (Further extensive material follows.) Note: the A. are produced industrially by chemical synthesis, and more often, by microbial techniques'' (Scott and Eagleson, 1988).

''A group of drugs used to treat infection caused by *bacteria*. Originally derived from molds and fungi, antibiotic drugs are now made synthetically'' (Clayman, 1989).

''A natural substance of relatively low molecular weight, produced by a microorganism, which in dilute solutions inhibits growth or destroys other organisms. Toxicity is generally selective. Most natural antibiotics, whose structures vary widely, are derived from the Streptomyces, an exception being penicillin'' (Combs, 1992).

''A chemical substance that is important in the treatment of infectious disease, produced either by a microorganism or semisynthetically having the capacity in dilute solutions to either kill or inhibit the growth of certain other harmful microorganisms'' (Morris, 1992).

''A chemical substance (derived from a mold or from bacteria) that inhibits the growth of other microorganisms (McDonough, 1994).

''Chemicals used as drugs to kill or inhibit the growth of microorganisms. The drugs are derived from molds or bacteria and are used to treat bacterial infections (they do not work on viral infections)'' (Barnes-Svarney, 1995).

''Substances isolated from microorganisms, especially moulds, that destroy or inhibit the growth of other microorganisms, particularly disease-producing bacteria and fungi'' (Daintith, 1996).

''Antibiotics are a collection of natural products and synthetic compounds that kill bacteria'' (Creighton *et al.*, 1999).

(*continued*)

TABLE IV (*Continued*)

''Any of numerous substances of relatively low M_r produced by living microorganisms (and also certain plants) that are able selectively and at low concentrations to destroy or inhibit the growth of other organisms, especially microorganisms. Also included are the many semi- or wholly synthetic organic compounds with similar antimicrobial properties. Many are useful chemotherapeutic agents'' (Smith *et al.*, 2000).

''An antibiotic is a chemical (of natural or synthetic origin) which (usually at low concentrations) inhibits microorganisms of some type within a host organism, while not unacceptably interfering with life of that organism'' (Forsdyke, 2000a).

adjectival sense of Vuillemin) but not as being therapeutic antibiotics (the substantive noun sense championed by Waksman).

In summary, today, ''the central concept of antibiotic action is that of 'selective toxicity'—that is, growth of the infecting organisms is selectively inhibited, or the organism is killed, without damage to the cells of the host. The ideal antibiotic would have no deleterious effect on the patient but would be lethal to the organism'' (Pratt and Fekety, 1986). These authors point out that no such ideal antibiotic exists, with the caveat ''perhaps penicillin G in the nonallergic patient comes as close to this goal as any antimicrobial drug.''

V. Science and Semantics

In a recent essay titled ''Evolution of Knowledge Encapsulated in Scientific Definitions,'' Gest (2001) noted that confusion still abounds with respect to the definition of key scientific terms. As examples, he discussed ''gene'' and ''photosynthesis,'' but the same is true for many (all?) other important scientific words, including ''antibiotic.''

Scientists, more than most people, try to communicate using words that have unambiguous meanings. By restricting the range of meanings, scientific discourse is stabilized. Waksman recognized this need, and was effective in his promulgation of the word ''antibiotic'' as a noun with a circumscribed definition. Because of the force of his personality, and because of the need for a term collectively to describe β-lactams, aminoglycosides, and other antibacterials discovered during the 1940s, ''antibiotic'' was widely adopted. However, because Waksman insisted on the microbial origin of ''antibiotics,'' his definition spawned decades of rather academic debate about whether it should embrace antimicrobial compounds from plants and animals, or semisynthetic and synthetic agents. Most importantly, Waksman's definition did not highlight the concept of selective toxicity. He did not

consider therapeutic efficacy to be a significant feature of the class of compounds he was defining. Waksman was following Vuillemin more than he realized.

Because ''antibiotic'' has become so deeply entrenched in the medical and scientific literature, and because it is so widely used in vernacular language, it is usually used now without giving a definition. Everybody is supposed to know what an antibiotic is. For those who do not know, and choose to look the word up in a dictionary or glossary, a state of perplexity may ensue. To a large extent, Humpty Dumpty's dictum, ''it means just what I choose it to mean—neither more or less,'' has held sway when defining an antibiotic. Most contemporary glossaries and dictionaries follow in the tradition of Waksman and emphasize the source of antibiotics and their antimicrobial effects rather than their selective toxicity.

To his credit, Waksman realized that the word ''antibiotic'' would ''in time lose its specific meaning and join the 'antiseptics' and 'disinfectants' in their loose and overbroad characterization and application'' (Waksman, 1955). Indeed, Waksman's prediction has been amply fulfilled, but as we have seen, the evolution of specific meaning has been in ways he did not foresee. Usage has become narrower not broader. Much of contemporary usage makes ''antibiotic'' synonymous with ''antibacterial.'' Further, his largely successful efforts to stipulate a specific definition have led to a problematic gap between the way ''antibiotic'' is generally used in medicine and common language, and the way in which it is formally defined in dictionaries and books. Most of the latter still adhere to some form of Waksman's phraseology. Yet, in medicine and agriculture, where antibiotics are critical, selective toxicity is the most important attribute. In addition, to counter patient demands for antibiotic treatment when it is not warranted, health professionals regularly call attention to the fact that most antibiotics have only antibacterial activity. In recent years, with the discovery of efficacious agents for the treatment of human immunodeficiency virus, the trend has often been to come up with new names, often combining forms such as ''antiviral antibiotic,'' ''antiviral agent,'' or simply ''antivirals.'' A similar trend is in place for other antimicrobial agents, e.g., ''antifungal antibiotics'' (''antifungals'') and ''antiprotozoal agents.''

It is much debated whether antiviral agents should be termed antibiotics. One writer argues that agents such as AZT (3′-azido-3′-deoxythymidine, azidothymidine) are not antibiotics—because they are synthetic molecules (Carlberg, 2000). Others refer to antiviral materials as antibiotics but point out that it was predictable that since AIDS was caused by a retrovirus, antibiotics alone would be unlikely to eradicate

latent HIV (Forsdyke, 2000b). Significantly, AZT is not listed as an antibiotic by the *Merck Index* (12th ed.)

More than five decades after Waksman's 1947 paper, the question, ''What Is an Antibiotic or Antibiotic Substance?'' is still difficult to answer with precision. The word has multiple meanings. For clinicians, an antibiotic is an organic chemical of natural or synthetic origin that inhibits or kills pathogenic bacteria at low concentrations and possesses selective toxicity, i.e., maximal toxicity for a pathogen and minimal toxicity for the host. For many microbiologists and chemists, an antibiotic is still defined as any antimicrobial substance, irrespective of its toxicity. Some ''purists'' in the Waksman tradition may still insist on limiting antibiotics to antimicrobial substances of microbial origin. All of these definitions coexist.

Does it matter? Beyond the philological aspects of etymology and the practical semantic matter of scientific communication, the definition of antibiotic does have practical ramifications. The choice of screening strategies used by researchers looking for new antibiotics is guided by their sense of what it is they are looking for. Microbial toxicity, or some target associated with so-called ''cidal'' effects, is usually the first criterion for screening. As already noted, most ''hits'' fail to proceed to clinical stages because of the subsequent discovery of mammalian toxicity. Would the failure rate in drug screening have been so high if the definition of antibiotic had been different? It is difficult to answer this question because our experimental protocols are guided by the language that we use to design our goals, but it would seem that many scientists were slow to emphasize selective toxicity.

Recently, genomics research has promised to bring a new approach to screening for antiinfective drugs, taking into account the differential biology of pathogen and host. Presumptive antibiotic targets are selected based on several criteria: ideally they are unique to the pathogen. Moreover, the targets are essential genes, i.e., knocking them out results in death for the pathogen. This is the classic meaning of antibiotic (''against life'' and ''antimicrobial''). Using robotics, combinatorial chemical libraries, high throughput screening, and the other brute force techniques of modern drug discovery, hundreds of thousands of candidate antibiotic substances can be tested against the selected targets. To date, results have been disappointing. Despite the vast number of compounds tested, few hits have been found. Most of the selected targets cannot be attacked therapeutically. Although scientists still do not understand why this is so it would seem that the logic of our criteria for target selection may be part of the problem. The power of the word antibiotic, a ''substance that has the capacity to inhibit the growth of

and even to destroy bacteria and other microorganisms,'' continues to guide our thinking in ways most of us never consider.

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An Alternative View of the Early History of Microbiology

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Most histories of early microbiology and the germ theory are remarkably similar and provide what might be a called the ''standard account'' of the development of this science. Such accounts usually begin with the first sighting of microorganisms by Antonie van Leeuwenhoek, progress to Pasteur (the so-called ''father of microbiology''), make passing reference to the heroic work of Jenner (vaccination), Semmelwies (contagion and antiseptic hand washing), and finally Lister (antiseptic surgery). Authors of older texts, perhaps because they were closer to the events, often provide different, more complex narratives. Over the past century or so, however, the ''standard account'' has been pared down to a bare minimum, leaving us with a few heroes whose contributions are set in stone and are rarely questioned. Unfortunately, much of what is taught about the history of microbiology has been oversimplified to the point where plain untruths are being told; at best a fascinating and convoluted story has been reduced to the minimum, for easy, uncritical consumption.

Here, I wish to present an alternative, altogether more complex, view of the early history of microbiology. Although a number of beloved icons will be toppled on the way, I hope the end result provides a more realistic account of this fascinatingly diverse story.

I. From the Ancients and Fracastorius to van Leeuwenhoek

When did microbiology and our awareness of the germ theory begin, and how much did the ancients know about disease? Even before the

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birth of Christ, Marcus Terentius Varro (116–27 BC) made one of the first notable comments on the origin of disease when he stated: ''Attention should also be paid to any marshy place . . . certain minute animals grow there which the eye, cannot detect, and which get inside the nostrils, and give rise to stubborn distempers.''

The idea that disease is contagious and transmitted from person to person is usually credited to the Veronese physician, Geronimo Fracastorius (also referred to as Fracastoro) (Fig. 1). Fracastorius was born in 1484 and died in 1553 (Garrison, 1910a). He produced his seminal work, *De contagionibus et contagiosis morbis et eorum curatione*, in Venice in 1546. Fracastorius recognized that infection could be contagious, but more importantly he suggested that disease is transmitted by ''particles.'' These he claimed are too small to be comprehended by our senses, but with the appropriate media can grow and reproduce to infect surrounding tissues. Amazingly, he believed that his particles were viscous or glutinous, and because they are transmitted over long distance, must be capable of living in air. Such particles, he claimed, can survive reasonably well, but cannot resist agencies such as extreme cold or heat. He also maintained that in order to produce infection, these particles do not have to undergo dissolution, but only metabolic change.

II. Was van Leeuwenhoek the First Person to See Bacteria?

The first observation of microbes using a microscope is generally credited to the Dutch draper and haberdasher, Antonie van Leeuwenhoek, who lived for most of his life in Delft, Holland. It has, however, been suggested that a Jesuit priest called Athanasius Kircher (Fig. 2) was the first to observe microorganisms (Garrison, 1910a,b). Kircher was born in Geisa in 1602. He was among the first to design magic lanterns for projection purposes, so he must have been well acquainted with the properties of lenses (Winslow, 1943). One of his books contains a chapter in Latin, which reads in translation—Concerning the wonderful structure of things in nature, investigated by the Microscope. Here, he wrote ''who would believe that vinegar and milk abound with an innumerable multitude of worms.'' He also noted that putrid material is full of innumerable creeping animalculae. These observations antedate Robert Hooke's *Micrographia* by nearly 20 years and were published some 29 years before van Leeuwenhoek saw protozoa and 37 years before he described having seen bacteria. There seems no doubt that in 1656, Kircher explicitly stated the doctrine of ''contagium animatum.'' He stated that ''worms'' were present in various putrefying material thus:

FIG. 1. Girolamo Fracastorius (Fracastoro).

> It is known to all that decaying bodies abound in worms, but not until the wonderful invention of the microscope was it found that all putrid substances swarm with innumerable broods of worms which are imperceptible to the naked eye, and I would never have believed it if I had not proved it by frequent experiments, during many years.

The obvious question is do ''worms'' equate to bacteria or other microorganisms? It is generally accepted that Kircher could not possibly

FIG. 2. Athanasius Kircher.

have seen microorganisms using his primitive microscope. Hendrickson, however, concluded that ''it does not matter much what Kircher saw; it was sufficient that he saw organisms below the threshold of unaided vision and made the inference of still more minute manifestations of life beyond what he saw'' (Winslow, 1943). Unfortunately, Kircher clearly believed in spontaneous generation and also never drew the

organisms that he observed. Leeuwenhoek, on the other hand, provided accurate drawings showing chains of bacilli as well as individual spirilla and bacilli; such drawings give van Leeuwenhoek priority over Kircher. However, just to confuse things, Fielding Garrison of the U.S. Army Medical Museum once claimed that the microscopes of both Kircher and van Leewenhoek were insufficiently powerful to allow them to see bacteria (Garrison, 1910b).

Although Kircher is credited with the so-called vermicle theory, another Jesuit priest called Jon Baptista van Helmont (1577–1644) may have got there before him. van Helmont was appointed Chair of Surgery in Louvain, Belgium, and sometime after 1599 he suggested that Semnalia or seminaria ''hath in it a hidden power to produce its own like; and so they beget a disease of the same kind.'' Helmont, by the way, is better known for his discovery of carbon dioxide.

III. Lancisi and Miasma

Many accounts of the germ theory state that our forebears believed that diseases were caused by miasma, i.e., a mixture of gaseous poisons, often emitted from marshes. However, we find that a number of early scientists clearly concluded that disease was caused by living entities, or that miasma could be a combination of a living entity and a gaseous poison. In 1665, the Jesuit, Vircherus, published a book in which he discusses the existence of infectious bodies that carry with them ferments that ''convert blood and humours into their own'' (Elliot, 1872). Lancisi, a celebrated Italian physician, came to some remarkable conclusions in 1695 (although his views were not published until 1718), published in an essay entitled *De Noxiis Paludum Effluviis* (Dundas, 1852). Here he states that the causes of disease are (1) not everywhere and not constantly the same; (2) carried in the air as various kinds of corpuscles or particles held in solution, which by accident enter the body and cause disease; and (3) comprised of two kinds of material released from waters undergoing putrefaction. The first is entirely inorganic, whereas the other is distinctly organic. The former has an unpleasant smell, often of impure sulfur, whereas the second is composed of ''multitude of worms and ova, which float about in the atmosphere—a distinct host of animalcula.'' Lancisi then goes on to maintain that the ''animal effluvia'' from marshes affects the body by (1) the wounds and irritation they inflict, (2) mingling their corrupt juices with the fluids of our bodies, and (3) nourishing intestinal worms. He also refers to the work of Varro who described a host of animalculae, unseen with the naked eye, which ''insinuate themselves

into the body by the mouth and nostrils and occasion disease.'' Of particular note is that he states that ''the inorganic portion can scarcely be held by itself to be the cause of disease.''

IV. Piscatory Entities: The Forgotten Microbiology of the Georgian Period

According to the standard account van Leewenhoek's observations were essentially ignored for about 200 years. In reality, however, not only were microorganisms being studied during the Georgian period (1714–1830), the fact of the existence and role of microorganisms in disease was already entering the popular and artistic imagination. The following quotes taken from a comedy called *The Devil on Two Sticks* written in 1798 by Samuel Foote illustrate the point. In the play, the President of the College of Physicians gives a lecture in which he states:

> Brethren and students, I am going to open to you some notable discoveries that I have made respecting the source or primary cause of all distempers incidental on the human machine, and these brethren, I attribute to certain animalculae or piscatory entities, that insinuate themselves through the pores into the blood and in the fluid, sport, toss and tumble about like mackerel or cod fish in the great deep and to convince you that this is not a mere gratis dictum—an hypothesis only, I will give you demonstrative proof. Bring hither the microscope.

Although Foote seems to be satirizing those who believe in such views, it is clear that someone of his day must have expressed the idea that animaculae enter the body through pores into blood and body fluids and here become piscatory, i.e., behave like fish and toss and tumble about, all this apparently being observable with the aid of a microscope. This remarkable, if fictional and somewhat flowery, description of the germ theory suggests that such ideas were in general circulation when Foote wrote his play.

Another example of how aware people were of microbial disease during this period is provided by a remarkable pamphlet that appeared in 1721. Called *A Hypothetical Notion of the Plague*, it was written by a certain Mr Place; in it he states the following:

1. Permanent chemical compounds, gases or otherwise, are not capable of acting as infection; sulfuretted hydrogen, for example, is not miasma. Therefore in looking for miasma or infection, we are not to look for chemical compounds.
2. Miasmas do not, at least always, possess a smell, or we are not able to perceive it.

3. The infectious matter of fever, plague, and cholera is not a true gas, as it does not diffuse.
4. The similarity of decomposition produced in one person by contact with another is so analogous to fermentation or putrefactive change that we have no stronger mode of producing such identity of action.
5. Fermentation is the splitting up of a body into several parts, by a power within itself, or imparted to it by another body in contact with it. Sugar may give out alcohol and carbonic acid, but sugar may also give mucic acid and lactic acid.

Williamson (1955) provides yet more sixteenth-century references to the germ theory. Of particular note is the work of the English scientist, Benjamin Marten. Little is known about Marten except that in 1720, he wrote a book on tuberculosis entitled *A New Theory of Contagion*. Here, he makes it clear that he does not believe that spontaneous generation exists, and that various kinds of animalculae are responsible for a variety of diseases. He also comments on the importance of a patient's resistance, stating that some persons have ''a happy Constitution,'' so that ''if the animalcules get into their bodies they are soon forced out.''

By 1726 the idea that animalculae cause disease was so prevalent that it was made the subject of a French satire (*Systeme d'un medicin anglasi sur la cause de toutes* etc). Then, in 1762, a physician from Vienna called Marcus Antonius Plenciz maintained that not only do living, infectious agents cause disease but they also multiply in the body and are carried by the air. Finally the Italian, Agostino Bassi, conducted important studies from the late 1700s to the early 1800s (although his work was not published until 1835). He is usually remembered only for having determined the cause of muscardine, a disease of silkworms on which Pasteur later worked, but his work covered human disease as well. He suggested that variola, syphilis, and skin diseases are all caused by vegetable or animal parasites and that gonorrhea is contagious by virtue of it being caused by parasitic entities; such parasites could be killed, he suggests, by injections of corrosive sublimate. Bassi also recommended that needles used in smallpox inoculation should be sterilized (by using a portable flame) between each patient; this he said killed all contagion (Monti, 1900). Otto Frederick Muller made yet another great, largely unsung, contribution to early microbiology when he wrote his *Animalia Infusoria* in 1786, in which he described the genus *Vibrio* (Lankaster, 1922).

By the early 1800s, the increasing awareness of microorganisms was used to explain a long-observed phenomenon, often referred to as the ''blood of Christ.'' Blood-red spots had, from the middle ages, been

seen contaminating vegetables, bread, and polenta. When these spots were observed on confessional wafers they were proclaimed to be the blood of Christ. This conclusion may have been reassuring to some, but its darker side was revealed when it sparked off a murderous anti-Jewish pogrom. The realization that the spots were in fact living organisms is usually credited to the Italian, Bartholomeo Bizio, who, in 1823, concluded that the spots were caused by an organism, which he named *Serratia marcescens* (Bennett and Bentley, 2000). Bizio observed that the spots needed warmth to develop, could be transferred, and were of a ''botanical nature''; he even went on to extract the red dye, no doubt having in mind some practical use for his observations.

V. Did Jesty Beat Jenner to Vaccination?

In 1774, some 20 years before Jenner first vaccinated James Phipps in 1796 at Berkley in Gloucestershire, a farmer, called Benjamin Jesty, vaccinated his wife and two children in Yetminster, Dorset (Anonymous, 1858). In common with many country folk, Jesty was aware of the old tradition that those who caught the milder cowpox did not succumb to smallpox. He observed that his two dairymaids, who suffered from cowpox, safely nursed his family during an epidemic. From these observations, Jesty reasoned that cowpox would protect against smallpox. He took his family to the farm of Mr. Elford at nearby Chetnole and, in an open field, removed infected puss from the udder of a cow. Using a stocking needle he then transferred this to a scratch he made on the arm of his wife and two sons. The local people heard of his work and began persecuting Jesty. Perhaps because of this abuse, the Jesty family moved, in 1797, to Worth Maltravers on the Isle of Purbeck. Here, Jesty began vaccinating parishioners. A tablet in the parish church refers to the fact that the mother of a certain Mary Brown was vaccinated by Jesty. Despite his vigorous protestations, Jesty never received credit for being the first to employ smallpox vaccination, credit that of course is generally given to Edward Jenner (Razzell, 1977). Jenner may indeed have been the first to confer scientific status on vaccination and to popularize it widely; however, there seems little doubt that Jesty got there before him.

VI. Microbiology Just before Pasteur

Pasteur is often portrayed as the father of microbiology. Although there is now doubt that he did important, groundbreaking work, much of what has been credited to Pasteur had in fact already been observed

and published some years before he even began to work on microorganisms (Wainwright, 2001a).

The French microbiologist Antoine Bechamp worked at roughly the same time as Louis Pasteur. Bechamp was often first to discover fundamental concepts that have since been credited to Pasteur; perhaps not surprisingly the two Frenchmen were bitter rivals. Bechamp was born at Basing in Lorraine, France in 1816, and died in 1908. His microbiological work began when he moved to occupy the Chair of Medical Chemistry and Pharmacy at Montpellier University. It was there that he performed what he termed his ''Beacon Experiment.''

In 1854, while studying the formation of invert sugar (equal amounts of fructose and glucose) Bechamp noticed moulds growing in some of his solutions. He also observed that invert sugar was formed only when moulds were present and not when solutions were added that prevented their growth, e.g., zinc chloride and creosote. Bechamp concluded that moulds act as ''ferments'' and that they are necessary for the inversion of sugar to occur. The results were first published in *Comptes Rendu* of the French Academy of Science on February 19, 1855, and again on January 4, 1858. Bechamp next concerned himself with the origin of these moulds. At the time, Pasteur did not believe that fungi could arise in sugar solutions lacking a source of nitrogen and phosphorus and criticized Bechamp's work. Bechamp replied by showing that although moulds can develop in only sugar solutions they grew much better if nitrate and phosphates were added; such additions also clearly speeded up the inversion of sugar. By heating pure sugar candy with soda lime Bechamp showed that it was indeed free of ammonia. He then went on to suggest that moulds gain nutrients by attacking glass. In these experiments Bechamp became the first to observe the ability of filamentous fungi to grow oligotrophically in carbon-free medium (presumably by scavenging carbon and ammonia from the air) and trace nutrients from the glass or water.

Bechamp next compared the action of the moulds on sugar to that exercised by diastase on starch and suggested that the yeast cell is like a closed vesicle that is limited in space by a membrane that normally allows none of its contents to escape except excretory products. He suggested that when growing in contact with sugar, the yeast becomes ''irritated'' and releases a liquid that acts on the sugar. He also showed that boiling destroyed the activity of the ferment released by yeasts, much in the same way that it destroys diastase in boiled barley. Remarkably, he then went on to extract the fermenting agent from the yeast in the form of a powder that he then showed could invert sugar when incubated together at 40°C. Such action, he showed, was very

rapid at the ordinary temperature, but ''slower in proportion to a lesser amount of the active product.'' Bechamp named the active substance ''zymase'' and gave the name ''zythozymase'' to the zymase of moulds and yeasts. Later, he showed that similar zymases could be found in flowers, the fruit of the white mulberry, and animal kidneys. This work was read at a meeting of Academy of Science and on April 4, 1864, and it was once again published in *Compte Rendue*. Our textbooks, however, erroneously tell us that Buchner was the first to extract an enzyme from yeast, and call it zymase, a ''breakthrough'' that was achieved in 1897, some 35 years after Bechamp's experiments!

In 1865, an epidemic, referred to as pebrine, was decimating the French silkworm industry. As early as 1865, Bechamp suggested that pebrine was a parasitic disease and could be cured using creosote. In June 1885 Pasteur investigated the problem and by September had concluded that ''corpuscles that are neither animal nor vegetable and should be regarded like globules of blood and starch granules, rather than infusoria or moulds'' caused the disease. By 1886, Bechamp was again emphasizing that pebrine was a parasitic disease attacking the worm from outside and that the germs come from the air. He then went on to show how creosote could be used to prevent the disease, yet Pasteur continued to labor under the impression that the disease was caused ''neither by animacules nor cryptogamic plants.'' By 1867, Bechamp had shown that the organism involved could invert sugar and then ferment it, first to alcohol and then acetic acid. Bechamp also recognized a second disease of the silkworm that he called flacherie. At this point Pasteur was beginning to change his views on the silkworm disease and remarkably was gaining the credit for determining its cause.

On October 10, 1864, Bechamp also communicated to the Academy of Sciences that vinous fermentation was due to organisms present on the surface of grapes and leaves; the same conclusion was reported by Pasteur to this august body on October 7, 1872, yet Pasteur continues to be credited with this discovery!

VII. The First Bacterial Pathogen

In 1842, a 19-year-old boy, under the care of the famous Scottish pathologist John Goodsir (Fig. 3), complained of suffering from uncontrollable vomiting. On waking, he would involuntarily vomit from two-thirds to a whole wash-hand basin-full of liquid that smelled of ''fermenting wort.'' On standing, the vomit became covered with a mass of froth that looked like ''the head of a pot of porter.'' Goodsir took

FIG. 3. Sir John Goodsir.

some of the frothy liquid and examined it under the microscope. He observed a mass of small organisms that he described as consisting of ''small or slightly oblong plates the thickness of which is about one eighth of the length of one of the sides, divided into four equal squares by lines, which join the middle points of opposite sides which cross at right angles in the center of the face, so as to resemble a packet bound with cords which cross at right angles.'' Goodsir suggested that the

organism belonged to the Bacillariae, and he gave it the name *Sarcina* (Goodsir, 1868). Although this organism, at first, bore Goodsir's name (*Sarcina* Goodsir) it was later renamed *Sarcina ventriculi*. Remarkably, Goodsir claimed that his organisms caused diseases that could be cured by feeding his patients carbolic acid and sodium hyposulfite. Goodsir's *Sarcina* was later found in cases of cardiac murmur and a fatal case of fatty degeneration of the liver, and was also frequently observed in urine (Ferrier, 1872).

In 1854 (3 years before Pasteur published on fermentation), the English pathologist, George Budd, while studying Goodsir's *Sarcina*, made some important observations on the nature of fermentations. He concluded that torulae (i.e., yeasts) were also present in the vomit of patients suffering from *Sarcina* infections, and that not only was carbonic acid evolved, but also the ''common alcoholic fermentation'' was occurring. Here, however, the alcohol was rapidly transformed to acetic acid. Budd also observed that such acidification of alcohol ''would seem to be much more favorable when the matter is exposed to the air than when it is shut up in the body,'' and that the condition caused by *Sarcina* often coexists with chronic stomach ulcer, an observation that is echoed in our recent awareness of the role of *Helicobacter pylori* in this disease.

The fact that Victorian English pathologists possessed a clear appreciation of the role of Goodsir's *Sarcina*, and other germs that are associated with disease, is obvious from the following quote, from 1859, by Tilbury Fox:

> The importance of the subject indeed is one which cannot be overestimated; for if we reflect that myriads of these minute objects are constantly floating about in the atmosphere, that they are capable of entering through the finest conceivable aperture; that their agency is purely zymotic, that bodies very closely resembling these; if not identical with them, have been found in the blood and the kidney of patients affected with typhus.

In 1859, a fascinating paper appeared in the *Lancet*, describing bacteria in urine, written by Arthur Hill Hassall (Fig. 4), a physician at the Royal Free Hospital in London. Hassall describes how he isolated vibriones (notably *Vibrio lineola*) from urine (Hassall, 1859). He observed that the vibriones in urine were minute, linear, and of different lengths; some were very short, others were two, three, or even many times longer, and still others were filamentous, like fungi; vibriones, he observed, were also capable of movement that could be inhibited by adding iodine. He further noted that the vibriones formed a pellicle on the surface of stale urine that falls to the bottom of the tube on

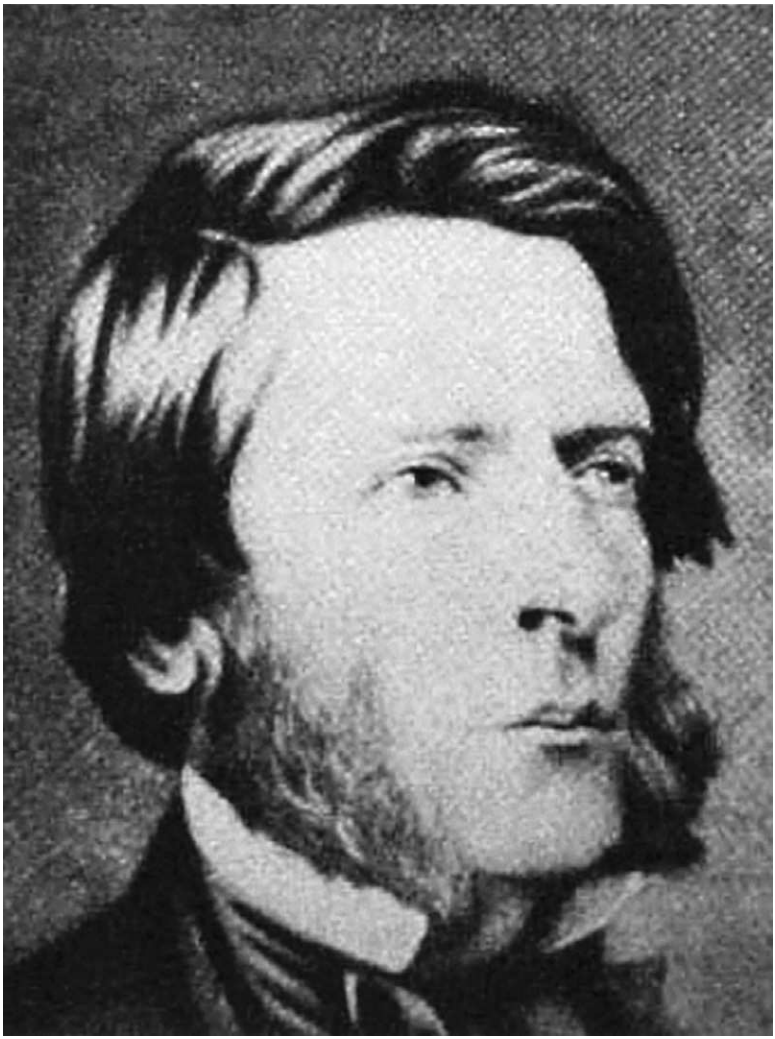

FIG. 4. Arthur Hill Hassall.

storage. Hassall continued by showing first that the type and number of vibriones present in urine varied in relation to acidity and depended upon the presence of air, and second that if the nutrient conditions of any liquid were sufficient then vibriones would develop. He demonstrated the truth of this last statement by growing vibriones aerobically in water containing egg white and two or three drops of liquid ''potasse of ammonia.'' Hassall then described a second organism found in

urine, which he called *Bodo urinarius*. He clearly describes how this organism possesses flagellae and points out that the organisms ''appear to fasten themselves to the surface of the plate of glass, their bodies swaying and oscillating like an inflated balloon kept down by its cords; this organism divides by so-called ''fissiparous reproduction'' (i.e., binary fission) (Gray, 1988).

In the mid-1800s, three English physicians, J. G. Swayne, Brittan, and Budd, reported some of the most remarkable pre-Pasteurean contributions to microbiology. As early as 1849, they described what is almost certainly the comma bacillus of cholera and showed that these comma-shaped ''fungoid bodies'' occurred in large numbers in cholera stools and in the condensed air of rooms inhabited by cholera victims. Amazingly, they also reported that the organism was present in every water sample taken from cholera districts, but was absent from uninfected districts, and concluded that the organism was taken into the human body in small numbers, where it then increased. The organism was described as having the appearance of small segments of circles, their diagrams clearly illustrating a comma-shaped organism. An editorial in the *Lancet* in October 1849 expressed the firm belief that an important series of discoveries had been made by these physicians concerning the cause of cholera. Nonetheless, a cholera subcommittee led by Drs. William Baly and William Gull, although suggesting that a ''virus'' may occasionally cause the disease, unfortunately concluded that miasma (or bad air) was the main cause.

It would be another 10 years before John Snow would report his epidemiological studies showing that cholera was spread in polluted drinking water. Snow also believed that cholera was caused by a contagium vivum and attributed to it ''the property of reproducing its own kind'' in the intestine of those suffering the infection; there is, however, a debate as to whether William Budd or Snow should be given credit for recognizing the infectious nature of cholera (Budd certainly deserves such credit for recognizing the infectious nature of typhoid fever). Moreover, 5 years after the above-mentioned English observations, the Italian, Filippo Pacini, observed a comma-shaped organism in cholera discharges and named it *Vibrio cholerae*. It would take another 35 years before Robert Koch confirmed the part played by a comma-shaped bacterium in cholera.

Although most of what has been written about the germ theory relates to bacteria, the first microorganisms to be associated with disease were yeast and fungi, no doubt because they are visible to the naked eye or with the aid of low magnification. Such observations can be dated from 1844, when Gruby discovered that *Trichophyton* caused

ringworm. Around the same time, Alfred Donne discovered that the protozoan pathogen *Trichomonas vaginalis* was the cause of vaginitis.

Charles Cagniard Latour detailed the role of yeast in alcoholic fermentation as early as 1838. Latour summarized his findings on the beer yeast as follows: (1) it is composed of clusters of spheres that reproduce, (2) it does not swim and is therefore a plant not an animal, and (3) yeasts ferment only when alive and can grow in an atmosphere of carbon dioxide and survive drying and freezing. The German pathologist, Henle, provided one of the most underrated pre-Pasteurian contributions to the germ theory in two books of 1848 and 1853. He stated that (1) contagion belonged to the vegetable world, (2) currents of air carry it over long distances, (3) it retains its powers for years in the dry state, and (4) the formation of contagion is a reproductive process and the formation of disease is the result of the reproduction of the extraneous being in the organism and at its expense. Finally he equated miasma to infection (Flugge, 1890). It is noteworthy that what we now lazily refer to as Koch's postulates were originally referred to as the Koch–Henle postulates.

Further evidence that the germ theory was well established before the mid-point of the nineteenth century comes from a book written in 1849 by J. K. Mitchell, Professor of Practical Medicine at the Jefferson College, Philadelphia (Mitchell, 1849). In this book, *On the Cryptogamous Origin of Malarious and Epidemic Fevers*, Mitchell suggests that epidemic diseases are caused by germs, although he distances himself from the then long-held view that such organic germs equate to animalculae.

VIII. The Semmelweis Legend

In the early years of the nineteenth century the northern English city of Leeds was gripped with epidemics of puerperal fever. The Surgeon of the General Infirmary and House of Recovery, William Hey, commented as follows on his approach to avoiding the disease: ''It was an invariable rule with me never to attend a patient in childbirth, in any article of clothing which had been in the presence of one affected with the puerperal fever, nor without washing repeatedly such parts of my person as could be exposed to infection.'' These words were written in 1815, some 30 years before Semmelweis began his work.

Was then Ignatz Semmelweis really the first to show that puerperal fever is contagious, or is the famous story just a satisfying myth? Before the introduction of prontosil and then penicillin, childbed (puerperal) fever was the scourge of childbirth, leaving many babies without a

mother. (It should not be forgotten moreover that, worldwide, puerperal sepsis still kills some 100,000 women a year.)

We are taught that the Hungarian Ignaz Philip Semmelweis solved the problem of puerperal fever only to face persecution and a premature death. As long ago as 1905, C. J. Cullingworth had his doubts that Semmelweis was the first to recognize the contagiousness of puerperal fever when he stated that (Cullingworth, 1905) ''We English speaking people on both sides of the Atlantic while giving abundant honour to Semmelweis have been in danger of forgetting the earlier and equally remarkable contributions to our knowledge of puerperal fever.'' J. P. Greenhill similarly pointed out (in 1936) that the contagiousness of puerperal fever had been recognized long before Semmelweis even thought about the disease. One such pioneer was the Manchester-based physician Charles White, who, in the late 1700s, showed that the incidence of childbed fever could be radically reduced by isolating victims and insisting on cleanliness. By 1795, Alexander Gordon, of Aberdeen, had come to the radical conclusion that, like many other doctors, he had accidentally spread the disease and had caused the death of many women in his care (Wainwright, 2001b).

Semmelweis' work on puerperal fever began in 1846 and was first published in 1848. Although an English version of his work was published in 1849 (by C. H. F. Routh), its full appreciation had to await the publication of Semmelweis' book in 1860. Semmelweis became aware of puerperal fever following a change in hospital practice. The Vienna hospitals where he worked had originally followed Charles White's methods. These had produced very low death rates from puerperal fever, but when they were abandoned, and cadavers were again used to demonstrate midwifery techniques, a dramatic increase in mortality resulted (Burges, 1941). Semmelweis concluded that childbed fever was spread by a poisonous component of dead flesh (the so-called ''cadaveric principle''). He boldly stated that ''Puerperal fever is not a contagious diseases, but it is conveyable from a sick to a sound puerpera by means of decomposed organic matter.''

By insisting that the cadaveric principle alone caused childbed fever Semmelweis invoked the wrath of his critics. The Dublin-based physician John Denham, for example, pointed out (in 1862) that Semmelweis overlooked the fact that the childbed fever frequently appeared in towns where there were no lying-in hospitals or dissecting rooms, and in rural districts where medical practitioners were seldom called upon.

Many of Semmelweis' forebears and contemporary critics were also aware of the link between puerperal fever and erysipelas (scarlet fever). Such observations can now be readily explained by the fact that

puerperal fever is caused by the hemolytic streptococci that are spread on unwashed hands and on the breath of anyone carrying *Streptococcus pyogenes*.

To an American reader the name of Oliver Wendell Holmes is more usually associated with belle-lettres and poetry than with medicine. In 1843, however, Holmes wrote an article that appeared in the *New England Journal of Medicine and Surgery* entitled ''The Contagiousness of Puerperal Fever.'' Here he agrees with White and Gordon that the disease is both contagious and is often transmitted, via an unknown agent, by both physicians and nurses. He also describes how, in 1835, an unnamed doctor had the ''good sense to change his clothes after each maternity-related visit and wash his hands in chloride of lime''—a practice usually credited to Semmelweis (in 1848). Holmes also refers to the fact that in 1821, an Edinburgh doctor, called Campbell, assisted in a postmortem and then spread the disease to a woman while attending a delivery. The same doctor attended an autopsy in June 1823 and, because he was unable to wash his hands, transmitted puerperal fever to two pregnant women. On the basis of his observations, Holmes recommended that physicians should (1) never attend autopsies prior to examining a pregnant woman, (2) always change every article of dress after attending a delivery and allow 24 hours or more to elapse before conducting any further midwifery, (3) always leave a period of at least a month between attending a case of puerperal fever and any uninfected patients, (4) on finding three or more closely connected cases of puerperal fever in the practice (with no others existing in the neighborhood) assume that they are the prima facie vehicle of the infection, and, finally, (5) regard it as their duty to pass on these warnings to nurses and assistants. Like Semmelweis, Holmes was ridiculed for such views. These, it should be emphasized, were published in 1843, some 3 years before Semmelweis began his work.

Historians place great emphasis on the fact that Semmelweis used an essentially modern statistical approach to demonstrate the contagiousness of puerperal fever; however, it is not for such subtleties that he is generally eulogized. Instead, we are often told that he alone realized that puerperal fever was spread by doctors and could be prevented by antiseptic hand washing. As we have seen, this is simply not the case. It is noteworthy that when Semmelweis' work first became known, the famous Edinburgh surgeon James Young Simpson fired off vitriolic letters to the medical journals refuting the idea that the Hungarian doctor should receive any priority on his ideas. Despite this intervention, the Semmelweis myth grew, and continues to be uncritically propagated—essentially because it is such a good a story and helps

satisfy our need to elevate the underdog to near mythical status (Wainwright, 2001b). In so doing, we ignore the many pioneers who went before Semmelweis and miss out on a more complex and far more fascinating story.

IX. The Lister Legend

In the summer of 1859, the medical world was greatly interested in the introduction of a new and remarkable application for dressing wounds. The French surgeon Velpeau, who instigated this approach, treated infected breast ulcers with the new substance, reporting an immediate decrease in odor and the production of puss (Nickles, 1859). He then went on to treat a young man who, following a scalded hand, had developed an infected finger, which emitted a disgusting odor. Velpeau dressed the wound with his powder, three parts coal tar and 100 parts plaster of Paris, and the ''mortification ceased.'' The active ingredient was known to be phenic acid, also known as phenol or carbolic acid.

The story of how Lister came to use carbolic acid is usually portrayed as follows (Guthrie, 1949). During the early 1860s, on the advice of Dr. Thomas Anderson, the then Professor of Chemistry at Glasgow University, Lister read about Pasteur's work and concluded that germs were the cause of wound infection. He then realized that to prevent infection all one needed to do was find a substance that would kill these harmful germs. As it happens, the authorities in Carlisle in England were using a substance called carbolic acid to treat sewage, where it reduced the smell and appeared to prevent putrefaction. According to the usual story, Lister realized that because carbolic prevented the putrefaction of sewage, it should also inhibit this process in wounds. Many accounts of the story say that it was Anderson who gave Lister the carbolic or, as it was sometimes called, German creosote. Lister first used carbolic in March 1865, but this proved a failure. On August 12 of that year an 11-year-old boy, James Greenlees, was carried into Lister's ward with a compound fracture of the left leg caused when he was run over by an empty cart. Lister applied undiluted, impure carbolic acid onto the wound and then covered it with a cloth soaked in carbolic that, in turn, was covered with tinfoil to prevent the liquid from evaporating. A scab formed and there were no signs of putrefaction. In the following weeks, Lister treated 11 cases, 9 of which were a success, an unheard-of survival rate at the time.

Lister published his work in the *Lancet* in early 1867 and presented his work at The British Medical Association's annual meeting in

Dublin (Lister, 1867). Lister was so confident of his new technique that on June 17, 1867, he used carbolic on his own sister, Isabella Sophie Lister, who had developed cancer of the breast. The breast was removed, together with the glands of the armpit. The operation was a success and no putrefaction followed; this operation was to be regarded as the first successful breast amputation–armpit removal in medical history. Strangely, this story is not mentioned in the authoritative accounts of Lister's life and work (e.g., Guthrie, 1949).

To kill germs, Lister also sprayed carbolic acid into the air of the operating theater to kill airborne germs; not surprisingly, many surgeons objected to breathing in corrosive carbolic, and this approach was soon dropped. The *Lancet* published a very appreciative article describing Lister's work as if it had been the first occasion on which carbolic had been employed in medicine to treat wounds. Lister was well aware that this was not the case and stated instead that rather than merely claiming priority on the use of carbolic acid, he had invented a new principle of treatment, i.e., the antiseptic principle, which could employ any antiseptic.

Lister also developed the idea of using dissolvable, carbolic-soaked catgut to close wounds and thereby upset one of the leading surgeons of the day, Sir James Simpson. Simpson was at the Glasgow meeting and was apparently visibly distressed by the applause that greeted Lister's lecture. Simpson attempted to initiate a response to Lister's talk, but was upstaged by a certain Dr. Hingston of Montreal, who commented that Lister's use of carbolic was far from unique and that on his last trip to Europe he had observed that although it had previously been widely used, it had been abandoned because of so many failures. Simpson then rose and with something approaching fury, confirmed that his own methods were preferred in Europe and then attacked Lister's use of carbolic-treated ligatures. Simpson regarded this as a retrograde step, especially as he had recently introduced the use of metal clamps to close bleeding blood vessels without the use of ligatures.

On September 1, 1867, a letter appeared in the *Edinburgh Daily Review*, signed anonymously by a certain ''Chirugicus.'' It reiterated the view that Lister's approach was not novel and in particular emphasized the work of a certain Dr. Lemaire of Paris, who had produced a second edition of a book, first published in 1865, that advocated the use of carbolic in medicine and surgery. Lemaire pointed out that carbolic was effective because it destroyed microscopic organisms, germs, or sporules, and that it could be used to arrest suppuration in surgery and as a dressing for both compound fractures and wounds. This anonymous letter has been credited with almost certainty to Simpson, who

widely circulated copies of it among the medical profession in Glasgow. One of these copies reached the *Lancet* and was published in 1867; the impression was clearly given that Lister had merely repeated a common Continental practice (Lister, 1867). Lister responded by stating that he had never heard of Lamaire, and in a subsequent letter to the *Lancet* stated that he was not impressed by Lemaire's work, and once again reiterated that he did not claim to have been the first to use carbolic acid in surgery. He then included a letter by a certain Phillip Hair, who claimed that although he had observed much carbolic in use on the Continent, he had seen nothing to match Lister's technique, nor results; a number of similar letters, defending Lister's claim to priority on the use of carbolic, were published in the medical literature (e.g., Dewar, 1867). The argument continued unabated, fuelled by a further letter, this time signed, by Simpson to the *Lancet*. It accused Lister of almost culpable ignorance of the medical literature and reiterated Simpson's belief that the Frenchman Lamaire should be given priority on the use of the carbolic method. Lister replied, stating that future successful reports of his method would make his point.

In a letter to his father (October 13, 1867) Lister states ''I told the story just as it happened; and it so happened that I had not heard of carbolic acid being used in surgery previously. I had only seen it mentioned as a deodoriser of sewage.'' Lister must therefore have been ignorant of the work of the Manchester chemist Frederick Crace Calvert (1819–1873), who had been largely educated in France. Although Lister can be excused for not having read of Lamaire's work, he certainly should have known of Crace Calvert's findings because they were published in the *Lancet* (Crace Calvert, 1863b). Crace Calvert was appointed Professor of Chemistry at the Royal Institution in Manchester. He actually manufactured carbolic acid on a small scale in 1865, and then on a large scale with his partners in 1865. In 1860, he wrote an article suggesting the use of coal tar (from which carbolic is purified) as a dressing for wounds. More importantly, he began to purify carbolic and offered it for sale. In 1863 a full paper appeared by him describing the work that was being done on the medical use of carbolic at Manchester Royal Infirmary (Crace Calvert, 1863b). Here, carbolic was being put to all kinds of medical uses; most importantly, the Manchester surgeon Thomas Turner had applied it to wounds. Manchester then seemed to have been a hotbed of carbolic use.

An article in the *Proceedings of the Manchester Literary and Philosophical Society* (1883, p. 72) refers to the fact that as early as 1852, a certain Alex McDougall was using carbolic and disinfecting powders

on infected wounds, apparently with success. Some of Crace Calvert's purified carbolic was also used by Lister. In 1869, Crace Calvert produced the first carbolic soap by combining 20% carbolic and an equal amount of glycerine. Crace Calvert had been educated in France and was aware that carbolic had been used, as early as 1851, to preserve cadavers, while during the 1870s he performed experiments on spontaneous generation and tested a wide range of disinfectants against bacteria.

The work of the eminent surgeon William Arbuthnot Lane provides an excellent example of how surgeons gave up Lister's antiseptic surgery in favor of aseptic surgery. Although Lane was brought up to use Lister's antiseptic method, he soon gave up using the carbolic spray. Instead, he wore long mackintoshes that were first boiled and soaked in lysol, with all surgical instruments being immersed in carbolic. At the beginning of the twentieth century he also abandoned the application of antiseptics to wounds and instead exposed everything to steam sterilization. By 1902, Arbuthnot Lane was wearing sterile, rubber gloves and going to incredibly great lengths to avoid his hands approaching the wound, designing long instruments with which he could tie ligatures and sutures; any instrument he touched during the operation was then reboiled before being used again.

X. Conclusions

I hope I have succeeded in fulfilling my original intention of showing that the history of the germ theory and microbiology is far more convoluted than is generally portrayed. It is often argued that this complexity has been simplified, with the inclusion of standard heroes, to satisfy the needs of students. This is clearly not the case, however, as the ''standard version'' is also repeated time after time by historians and established microbiologists (myself included, see Wainwright and Lederberg, 1992). The standard account has clearly too often been uncritically passed on without any attempt being made to critically refer to the original literature, a practice that would be correctly frowned upon in general scientific research. Personally, I am not against ascribing hero status to those scientists who make major discoveries; the problem, of course, comes in allotting credit to those who actually made the discoveries in question.

Of course, a large body of scholarship exists on the history and sociology of science, which speaks to many of the issues raised here (for example, see Coulter, 1994; Strick, 2000). It is probably true to say that it is mostly scientists and those who popularize science who

perpetuate oversimplified ''myths''; in contrast, many of the professionals who study the development of science are far more aware of the convulsions and social-political influences that shape our hero and (more rarely) heroine worship.

Finally, the historical literature is also worth reading not only from the viewpoint of who did what and how, but also because it contains much forgotten information, particularly in relation to diseases caused by bacteria. For example, there is a wealth of literature suggesting that microorganisms play an important role in the etiology of cancer, and the literature on bacterial pleomorphism and filterable bacteria is as relevant today as when it was first published during the late 1800s and into the last century (Wainwright, 2000).

Hopefully this review will help correct some of the falsehoods found in the ''standard version'' of the history of our science, and encourage microbiologists to take an interest in the fascinating history of their subject.

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The Delft School of Microbiology, from the Nineteenth to the Twenty-first Century

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I. Introduction

Just over 50 years ago, Cornelis van Niel (1949) was the first to coin the phrase ''Delft School of Microbiology.'' I rather suspect that he would be surprised today to see how this description has survived. Five years ago, when we were organizing a symposium to celebrate the 100th anniversary of Martinus Beijerinck's professorship, we used the Internet, microbiology journals, and local newspapers to ask whether people felt that they were members of ''the Delft School.'' We were stunned at the number of replies from people all over the world, and it became clear that even people who have never visited Delft feel strong links to the city as one of the homes of modern microbiology (and then biotechnology). The resulting book (Robertson, 1996) contained around 7000 names, and was clearly only the tip of the iceberg. We still get requests for inclusion in the next edition.

What was and is the Delft School of Microbiology? For the purposes of the Beijerinck Centennial, we defined a member very simply as one who had studied or carried out research within the Microbiology Department in Delft, or who had worked under the supervision of a Member. It could be an undergraduate, technician, Ph.D. student, Post-Doc, or other research partner. This, of course, was (deliberately) a fairly light-hearted definition, and not really of much use in any scientific evaluation. To establish any potential scientific credentials of the Delft School, it is first necessary to briefly consider the people involved

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in its birth. Many of the papers referred to here are very old and are now difficult to find. I have therefore also included a few more modern references to publications in which the relevant work is discussed more extensively than here in English. In addition, Beijerinck's publications are often more easily found in the six-volume ''Collected Works'' (Beijerinck, 1921) than in the original journals.

When ''Delft'' and ''microbiology'' are mentioned in the same sentence, it is inevitable that Antonie van Leeuwenhoek comes first to mind, but sadly he was not a teacher. Despite the fact that he was born and buried in Delft (Fig. 1) we can claim no closer links to him. The history of the Delft School of Microbiology goes back only just over 100 years, to the appointment of Martinus Willem Beijerinck as the first professor of microbiology at what was then the Delft Technical College.

II. Martinus Willem Beijerinck, 1851–1931

Beijerinck (Fig. 2) was a botanist whose interest in plant life was encouraged from an early age by his parents. The description of him revealed in the pages of his sister's diary (Henriëtte Beijerinck, unpublished diaries) is that of a rather shy, retiring man who achieved much despite poor health and chronic depression. Throughout his childhood, the family was usually in financial difficulties, and very little came easily to him. His closest friend appears to have been the eventual Nobel Prize winner, Jacobus van 't Hoff, with whom Beijerinck shared a room as a student. The two young men must have been a nightmare to their landlady as they saw no reason to confine their experimentation to the laboratory. In his article to mark Beijerinck's 70th birthday, van Iterson (1921) describes how the two of them damaged the furniture and wallpaper in their room while heating ox gall and HCl over the stove! Their friendship extended to their families—when Henriëtte Beijerinck studied art in Amsterdam, for example, she lodged with the van 't Hoff family. van 't Hoff is one of the unsung heroes of the Delft School. A continuous theme running through the diaries of Henriëtte Beijerinck is the emotional support and encouragement that van 't Hoff gave his chronically depressed friend, right until van 't Hoff's death in 1911. On several occasions, Beijerinck announced his intention of giving up in Delft (he would often claim that he would have been more successful had he stayed in Wageningen), and van 't Hoff would come and visit (even from Germany), and convince him that he should stay.

Beijerinck's doctoral thesis was on the galls of plants (Beijerinck, 1877). In it, he commented that he had been unable to find the insect that causes the ''galls'' on the roots of plants in the pea family, among

FIG. 1. A. J. Kluyver visiting the grave of Anthonie van Leeuwenhoek in the Old Church in Delft.

FIG. 2. M. W. Beijerinck and his sister, Henriëtte, in the garden of their retirement residence at Gorssel.

others. Paintings by his sister, Henriëtte, clearly show the strong superficial resemblance between galls and root nodules (Fig. 3A and B, see color insert). Later, of course, he showed that these ''root galls'' were in fact nodules full of symbiotic, nitrogen-fixing bacteria, a subject he returned to in his laboratory journals again and again (Fig. 4). That the subject of galls was close to his heart can be seen from the extensive collection of dried and bottled (in alcohol) plant galls, gall wasps, and caterpillars still to be seen in the collection at the Kluyver Laboratory today. The samples date from the time of his thesis, right up to the year of his retirement from Delft. Among them are four samples of root nodules stored in alcohol (Fig. 5).

During his time as a teacher at the Agricultural School in Wageningen, Beijerinck spent much of his time working on the hybridization of agricultural grain plants, especially those from the genera *Triticum* and *Hordeum*. This work involved classic crosses to make F1 and F2

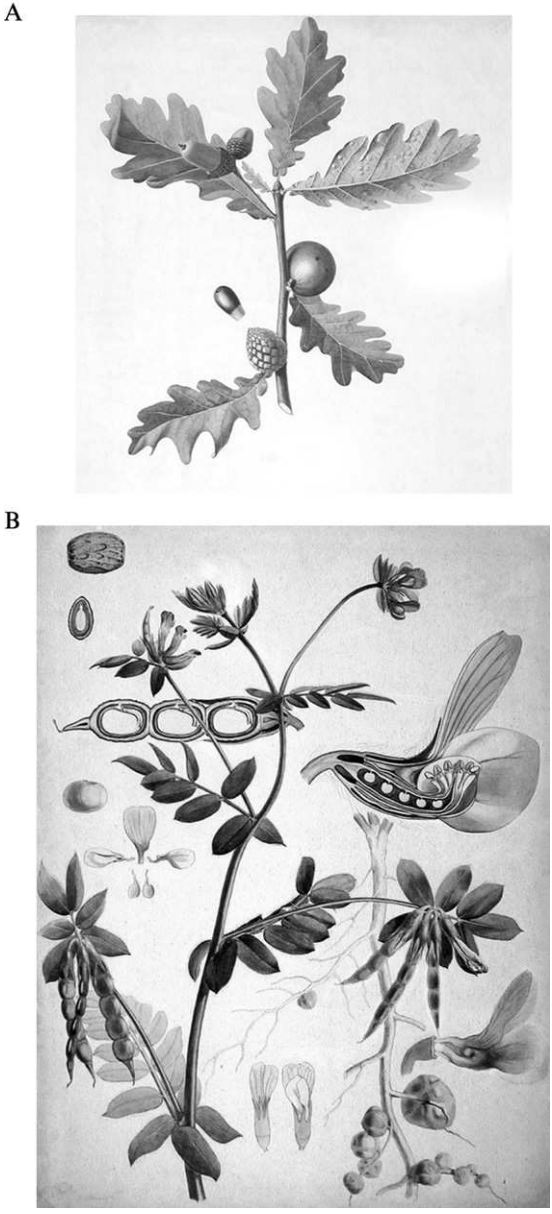

FIG. 3. (A) The oak apple or gall. Watercolor painting by H. W. Berijerinck. (B) Vetch. Watercolor painting by H. W. Beijerinck.

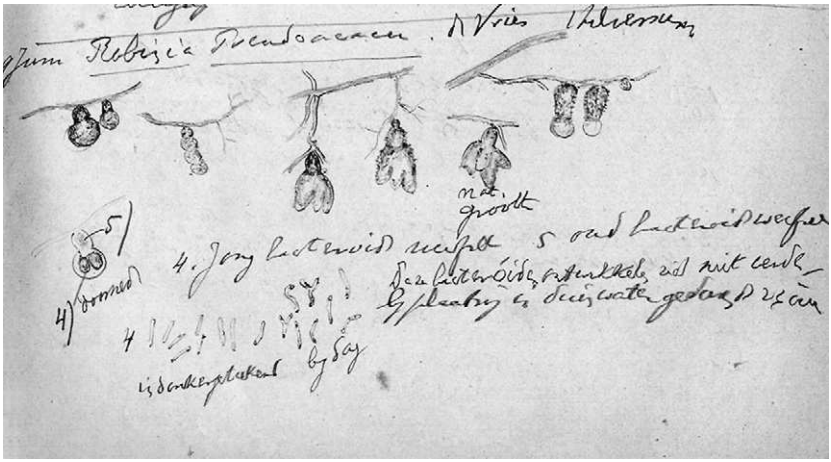

FIG. 4. Root nodules and the bacteria isolated from them. Pencil drawings from the laboratory journal of M. W. Beijerinck.

hybrids, and he used the results as a basis for speculating about the origins of cultivated wheat strains (Zeven, 1970). This work may form the basis of his complaint, late in his life, that had he stayed in Wageningen, he would have rediscovered Mendel's Laws before Hugo de Vries (van Itallie-van Embden, 1928).

In some ways, Jacob Cornelis van Marken could be regarded as another of the founding fathers of the Delft School, for he was the man who brought Beijerinck from his teaching post in Wageningen to Delft. van Marken was the somewhat idealistic owner of the ''Nederlandsche Gist-en Spiritusfabriek'' (Dutch Yeast and Spirit Factory) in Delft. There is a short note about him, together with photographs of his factory, online (Fabre, 1879). He was determined to use the newly developing field of microbiology to improve his products and control contamination problems. Persuading Beijerinck to come to Delft and set up an industrial microbiology laboratory in his factory had not been easy. In 1884, van Marken offered Beijerinck a salary of F4500, and gave him a couple of months to think about it. Beijerinck was hoping to be able to stay in Wageningen (with a lower salary of F3500 and a laboratory in the garden of the Agricultural School), but these plans were not approved by the Government before van Marken's deadline was up, and Beijerinck decided to move to Delft.

It cannot be claimed that Beijerinck was particularly happy during his time as an industrial microbiologist. His wide range of interests, especially in microbial ecology, did not really suit the more focused

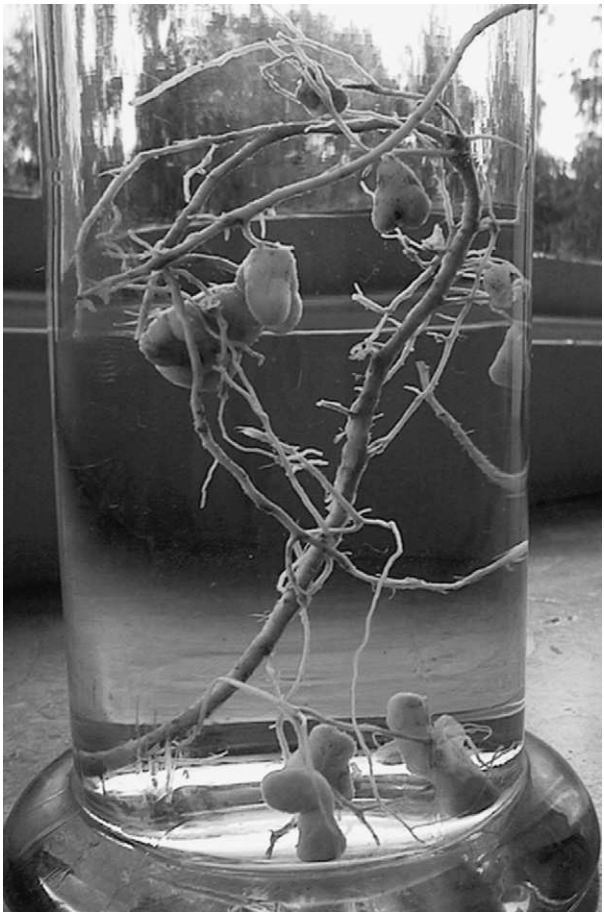

FIG. 5. Root nodules preserved in alcohol, and dated 1886.

requirements of an industrial life, despite the broad freedom that van Marken gave him. This is reflected in the range of subjects on which he published during his industrial days (Table I). Henriëtte's diary tells us that he rapidly became depressed at having given up his chances of promotion in Wageningen. When his sisters visited him in his laboratory, he ''sat there, surrounded by a mass of retorts, bottles and glasses, boxes, corks and heating apparatus, so that it looked like the workshop of an alchemist.'' He received offers of assorted positions ranging as far afield as Java and Naples, as well as a professorship in Groningen.

TABLE I

TOPICS OF PUBLICATIONS THAT APPEARED DURING BEIJERINCK'S TIME IN INDUSTRY[a]

Sunsets (Were the spectacular sunsets of the time due to dust from Krakatoa?)
Root nodules and their bacteria
Plant galls
Grasses, carrots, gardenias, barley
Algae, protozoa in drinking water, hydrogen peroxide in living organisms
Fermentation, butanol fermentation, *Saccharomyces* associated with beer, *Schizosaccharomyces octosporus*
Lactase, maltase, blue cheese bacteria, kefir
Photobacteria, sulfate reduction
Methods: auxanograms, gelatine plates, Chamberland filters, sampling stratified cultures, microbiochemical analysis

[a]For further details, see volumes 1, 2, and 3 of the ''Collected Works'' (Kamp *et al.*, 1959).

Beijerinck himself proposed a Laboratory of Agricultural Microbiology in either Wageningen or Utrecht. This proposal is in the Kluyver Laboratory Archive, and reveals an ambitious and surprisingly modern research program (Table II). The idea of seeking bacteria to combat insect pests, for example, is still current. His proposal apparently tipped the balance in Delft, where van Marken, together with several members of Parliament and a few others, managed to convince the Government that a Laboratory of Microbiology was needed within the Polytechnic College in Delft. Beijerinck was characteristically indecisive about accepting the Professorship at the Polytechnic, but when offered an unusually high salary, new laboratory and house, agreed. The original plans included only a relatively modest laboratory with an apartment upstairs for the professor, and were rejected by the Governing Board of the Polytechnic. A much grander design, including larger laboratories on several floors, a greenhouse, and a large house for the Professor (and a tiny one for the Concierge), was adopted. The floor plans, together with photographs taken in Beijerinck and Kluyver's times, are in the Kluyver Laboratory Archive. This building still stands on the bank on the main canal in Delft but has been converted into apartments. It is interesting to speculate that with the much smaller facilities and lack of a greenhouse in the initial version, Beijerinck might never have done his work on tobacco mosaic virus (TMV), and coined the term ''virus''!

TABLE II

BEIJERINCK'S PROPOSED RESEARCH PLANS FOR A LABORATORY OF AGRICULTURAL MICROBIOLOGY

1. The role of microorganisms in the various reactions in soil.
2. The role of microorganisms in the digestion of domesticated animals.
3. Infectious agents and vaccines.
4. Microbiology of different agricultural activities (e.g., dairying, silage, tobacco production, storage and preparation of fertilizer and compost, fermentation technology).
5. Microbiology of putrefaction and rotting, with special reference to the quality of food and drinking water for domesticated animals.
6. The microbiology of insects and other animals that can be damaging to agriculture, with attention to be given to the use of microorganisms in controlling pests.
7. Plant pathogens.

It was Adolf Mayer, Director of the Agricultural Experimental Station in Wageningen, who introduced Beijerinck to the problem of tobacco mosaic disease. Mayer had been working on the problem and in 1882 described the causative agent as ''a soluble, possibly enzyme-like contagium'' (Mayer, 1882; Bos, 1995). By 1886, he had decided that the disease was probably due to an undiscovered bacterium (Mayer, 1886; Bos, 1999). Beijerinck used Chamberland filters (Fig. 6) to show that something much smaller than a bacterium was present in the sap of infected leaves, and that whatever it was did not lose its potency with subsequent ''subcultures'' onto fresh plants, i.e., it could not be diluted out. Moreover, the active agent was inactivated by heating to 90°C, but not by drying and storage (Fig. 7). In 1898, he published his conclusion that the infectious agent is not a *contagium fixum* or bacterium, but rather a *contagium vivum fluidum*, or virus (Beijerinck, 1898; Scholthof *et al.*, 1999). Sadly, Beijerinck did not live long enough to see the crystals of his virus or electron micrographs of the individual virus particles. Indeed, he seems to have become frustrated by his inability to culture TMV and lost interest in the work. There are only two pages in his laboratory journals describing these experiments (Fig. 8), possibly because Beijerinck was in the habit of keeping notes on odd bits of paper (which went into the incubators with the relevant

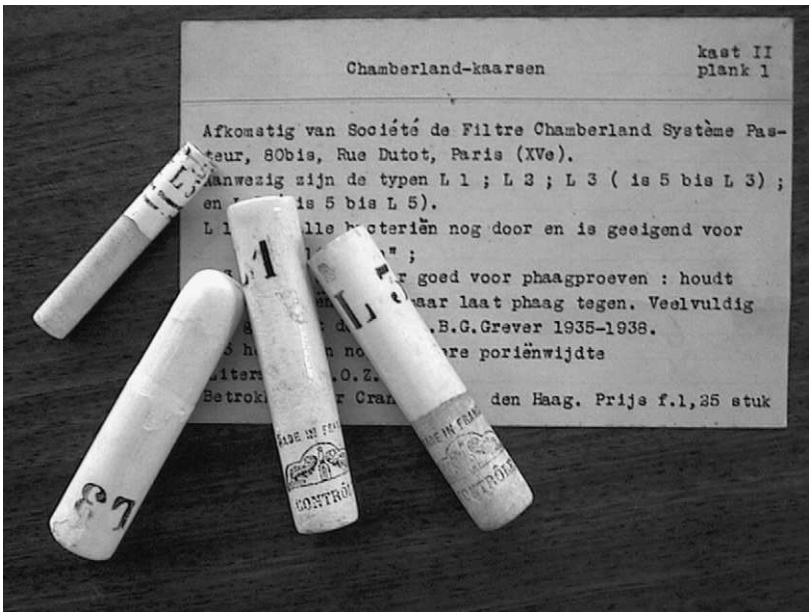

FIG. 6. Chamberland filters.

vessels) and then writing things up at the end of an experiment. The Kluyver Laboratory Archive contains some of these papers, but not those relating to the TMV work. The text may be translated as follows (comments in brackets and italics are mine):

30 Nov. 1898

Tobacco sickness

1. Sap from sick plants pressed in August—rapidly full of *anglomerans* [*this may be* Phytomonas anglulata, *causative agent of leaf spot on tobacco*] (almost only two sorts).
2. Sap from healthy plants pressed in November (plant from the garden). Sub-cultured on malt extract gel:
   a. Many fluorescent liquifiers. [*This may be* Pseudomonas fluorescens, *previously known as* Bacterium fluorescens liquifaciens]
   b. Many anglomerans
   c. *Escherichia coli*
   d. Odd other types in lower numbers

FIG. 7. Tobacco leaves infected with TMV. This is the original photograph on which the painting in Beijerinck's original paper was based (Beijerinck, 1898).

The same sap on . . . gel with 2% cane sugar [*no one can make out the missing word, but I think this refers to a medium he was fond of for the nitrogen-fixing root nodule bacteria where he pulverized plant material and then added cane sugar*].
Only the very active colony of fluorescens general

4 Dec. 1898

New experiments on tobacco sickness

26. Inspection of plants from 4 Dec. infected with sap from healthy plants grown 20 October.

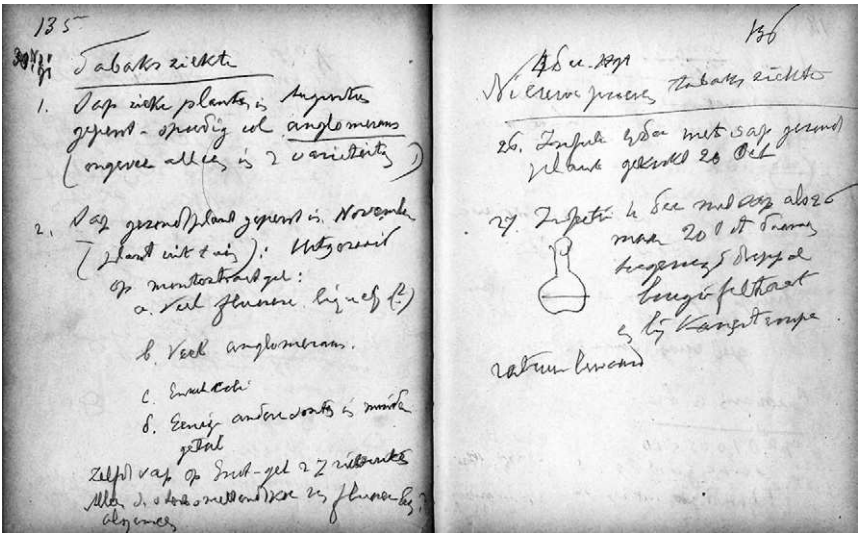

FIG. 8. Pages from the laboratory journal of M. W. Beijerinck, describing experiments with ''tabaks ziekte''—tobacco disease (translation in the text).

27. Inspection of plants from 4 Dec. treated with sap as 26, but with a drop of bougie filtrate added 20 Oct., and kept at room temperature.

There is no real point in trying to review Beijerinck's entire research career here—that was extensively done by van Iterson *et al.* (1940) and expanded by Bos and Theunissen (1995) when they published his collected works, and since by other authors (e.g., van Niel, 1949; Bos, 1999; Bennett and Phaff, 1993; Theunissen, 1996). Suffice it to say that his interests ranged over a wide area including sulfur-oxidizing bacteria, denitrifiers and nitrifiers, yeasts, the production of indigo, enzymes, and food microbiology. In the Kluyver Laboratory Collection are a few samples of his ''ferments,'' at least one of which is still active (Rouwenhorst *et al.*, 1989). However, in addition to his work on the virus, it is for the development of the elective or enrichment culture that he is perhaps most recognized. It may seem very obvious to us now that to find microorganisms of a particular metabolic type, it is necessary to provide cultural conditions that will favor that metabolic type, but in the very early days of microbiology, this was a new idea. One of Beijerinck's famous isolations, that of *Azotobacter croöcoccum*, was due to the fact that he recognized that to isolate N_2-fixing bacteria, it is a good idea to omit a source of fixed nitrogen in the medium.

Simplicity in experimental design was very important to Beijerinck, and an experiment cannot be more elegantly simple than his isolation of N_2-fixing bacteria from soil (Beijerinck, 1908).

In his ''Oratie'' (the first formal lecture a new professor gave to mark the appointment), Beijerinck talked extensively about his belief that microbiology holds a central place in the study of life, and pointed out that its complexity and apparent ability to change mean that progress would necessarily be slower than that in other fields. He also made an appeal for what we would call today good, quantitative data backed up by experiment and lamented the standard of some publications and editors of journals (illustrating his point by claiming that if someone submitted a paper stating that fermentation in bread was not due to the yeast cells, but to the bacteria present in bakers' yeast preparations, it would be accepted by quite serious journals!). This careful search for hard data defines his career—he was not patient with students who did not wring every possible bit of information from an experiment. In the same ''Oratie,'' he pinpointed research priorities as growth, reproduction, inheritance, and variation (interests that he passed to at least one of his pupils, van Iterson). His interest in variation between apparently similar strains of plants and microorganisms continued to the end of his life. The letters between Beijerinck and Kluyver after Kluyver took over the Chair of Microbiology on Beijerinck's retirement are full of discussion about the differences Beijerinck observed between plants and soil isolates in Gorssel (his retirement home) and in Delft. Kluyver often provided chemicals, reprints, and cultures to keep his ''illustrious predecessor's'' research going. Indeed, on more than one occaision Beijerinck even asked Kluyver to cycle along a particular canal and look for a particular plant, or seek out something (often a weed) from a specified plot in the garden of the microbiology laboratory.

Nine years after he began his professorship, Beijerinck contributed a short chapter in a book brought out to mark the promotion of the Delft Polytechnic School to a ''Technische Hogeschool'' (literally Technical High School, but in fact more equivalent to the Polytechic Colleges of the UK). In this chapter, he again discussed the importance of microbiological research in the fundamental and applied sciences, but also emphasized the importance of microbiology in education, listing students from the dairy industry, research stations, pharmacy and medicine, and various factories. He also mentioned the people from all over the world who visited to Delft to learn, and take their new knowledge home to be used there. It is easy to see how the influence of the ''Delft School'' began to spread.

Among his many distinguished research assistants were two who took his teaching back to Wageningen where they became Professors of Microbiology in their own right, thereby establishing the first branches on the Delft School Tree—Nicholaas Söhngen and Jan Smit. Another of his pupils, Gerrit van Iterson, remained in Delft after Beijerinck persuaded the College to establish a Professorial Chair for him.

III. Gerrit van Iterson, Jr., 1878–1972

At first, Professor of Microscopical Anatomy and then of Applied Botany, van Iterson (Fig. 9) is frequently ignored when the Delft School of Microbiology is discussed. However, he deserves more recognition in the microbiological community for a number of reasons, not least because he was both a Ph.D. student of Beijerinck and the Ph.D. supervisor of Kluyver. Somewhat surprisingly, there are only a couple of short biographies of van Iterson (Kluyver and van Iterson, 1932; Lambert-Avis, 1990). His nomination for an honorary degree at the University of British Columbia June 13, 1933, sums his career up.

> The Senate of this University recommends, Mr. Chancellor, that you confer the honorary degree of Doctor of Laws upon Professor Doctor Gerrit van Iterson, Junior, of the University of Delft and official representative of the Netherlands; one of the most eminent living authorities on plant organisms; discoverer and disseminator of knowledge; founder and director of institutions of research; a scientist who brings to bear upon his special interest the resources of chemistry, physics, bacteriology and economics; a citizen of the world traversing the seven seas and equally at home upon them all.

van Iterson did his first degree in the Faculty of Chemistry in Delft, studying microchemistry and microbiology with Professors Behrens and Beijerinck, respectively. On graduation, he became one of Beijerinck's research assistants and his first publications were on denitrifying and cellulose-degrading bacteria. His thesis title, however, reveals his lifelong interest in the combination of mathematics and biology—''*Mathematische und mikroskopisch anatomische Studien über Blattstellungen*.'' Although much of his work involved the higher plants and animals, van Iterson continued Beijerinck's close contacts with the Yeast and Spirits Factory. His teaching ranged from Mendelian genetics to fiber recognition, when the students would be presented with a box of fibers ranging from hemp and wool from different sorts of sheep to nylon and other synthetic fibers. The annual photos of his practicals in the Kluyver Laboratory Archive reveal a formal world with the

FIG. 9. G. van Iterson, Jr.

students all dressed in suits, with not a white coat to be seen (Fig. 10). van Iterson shared Beijerinck's great interest in biological variation in shape and color, and strongly emphasized the theoretical aspects of research. Nevertheless, much of his work reflected the second title of his Chair—Applied Botany—and he was heavily involved in research into tropical products including tea. He was one of the founders of the Dutch Rubber Institute, and was also one of the driving forces behind the formation of T.N.O. (The Netherlands Organisation for Applied Scientific Research). Together with Beijerinck, he founded the Botanic Garden in Delft as a source of materials for study and research.

van Iterson is best known today for his work on rubber, other tropical products such as tea, and on paper making, but among his great

FIG. 10. The ''Paper and Paper Constituents'' course of 1947. van Iterson is fifth from the left, in the background. On the extreme left is Piet Nieuwdorp, formerly electron microscopist in the Delft laboratory. Third from the right is Lex Scheffers, currently Chief Editor of FEMS Yeast Research.

interests were the mathematics and physics underlying biological structure and function. Some of his drawings strongly resemble fractals, and it seems likely that he would have been very much at home among the mathematical modelers of modern biotechnology laboratories.

van Iterson's branches of the Delft School Tree include Bastiann Meeuse, Professor of Botany of the University of Washington, USA, and A. van Rossum, Professor of Rubber Technology in Delft, but there was at least one microbiologist among them. Albert Jan Kluyver became Professor of Microbiology on the retirement of Martinus Beijerinck.

IV. Albert Jan Kluyver, 1888–1956

Like his predecessor, Kluyver (Fig. 11) was not a microbiologist by training. He had qualified in Delft as a chemical engineer (as microbiology graduates do to this day), and then had taken up a position as

FIG. 11. A. J. Kluyver.

assistant to Professor van Iterson. His doctoral thesis was on biochemical sugar determinations, and this interest in (bio)chemical reactions was to stay with him throughout his life. However, he felt his lack of microbiological knowledge keenly—writing after Kluyver's death, van Niel (1957) described how he spent the weeks between his appointment as professor and his ''Oratie'' frantically reading everything he could lay his hands on that would give him a better grasp of microbiological fundamentals. Kluyver used a great deal of his ''Oratie'' (Kluyver, 1959) to establish the importance of microbiology in the applied sciences, especially with regard to possible microbiological contributions to the

energy shortage that Kluyver predicted. He talked of the biological production of fuel alcohol as well as other products, including fats, proteins, and the simpler organic acids. van Niel commented that a great deal of the lecture was a justification for the presence of a microbiology laboratory within a technological college. He did, toward the end of his lecture, emphasize that his focus on applied microbiology did not mean that general or theoretical microbiology would be neglected—that one could not do good applied science without an understanding of the fundamentals. Kluyver practiced what he preached, and it is impossible in the space allowed here to review all of the work that was done during Kluyver's time in Delft; the reader is referred to the excellent biography of Kluyver (Kamp *et al.*, 1959) and the extensive obituary by van Niel (1957).

Unlike Beijerinck, Kluyver preferred a very informal atmosphere, which at first caused a certain amount of difficulty among some of the staff he ''inherited'' from his predecessor. However, this relaxed attitude began to pay off when increasing numbers of undergraduate and graduate students joined the laboratory, and the results began flowing in. The discovery that cultures of bacteria from beer were producing crystals of calcium 5-ketogluconate when streaked on yeast extract, glucose, and calcium carbonate medium sparked an interest in the metabolism of these and other acetic acid bacteria. In turn, the comparisons between the various cultures led to one of the subjects for which Kluyver is most famous, comparative biochemistry. Extending the research to other groups of bacteria and yeast, they found that even if a culture was started with the same medium, substrate, and cultural conditions, the outcome of the experiment was defined by the type of microorganism involved. As Kluyver wrote of a collection of such cultures:

> After some time it will be evident that the yeast has converted the sugar largely into ethanol and carbon dioxide; *L. delbrückii* into lactic acid; *L. fermentum* into lactic and acetic acids, ethanol and carbon dioxide; *B. coli* into lactic, acetic, and succinic acids, carbon dioxide, and hydrogen; *B. aerogenes* into the same products with, in addition, 2,3-butylene glycol; *B. typhosum* into formic, acetic, and lactic acids, and ethanol; *G. saccharobutyricum* into butyric and acetic acids, carbon dioxide, and hydrogen; *G. butylicum* into butanol, acetone, carbon dioxide, and hydrogen. Thus a remarkable diversity emerges.

Ultimately, these studies led to the formulation of the concept of ''Unity in Diversity'' (Kluyver and Donker, 1926). It is hard for someone who was born long after these events to capture the excitement of the time, and so I'll leave it to van Niel (1949) to explain the concept:

> Any biochemical process, whether oxidation, fermentation, or synthetic reaction, was considered as a chain of step reactions, each one of which represented a simple mechanism in which hydrogen is transferred from one molecule, the H-donor, to another, the H-acceptor. The only apparent exception to this principle was exhibited in the metabolism of complex molecules, composed of a number of simple entities, for example the polysaccharides . . . , proteins . . . , and fats . . . . Such complexes would first be converted to their constituent units by hydrolytic cleavages, with the products subsequently under-going the various hydrogen-transfer reactions.

Of course we now know a great deal more about the various enzymes and reactions and, as van Niel (1949) pointed out, some of the intermediates suggested by Kluyver are now known not to be involved. New principles have been worked out, to quote van Niel (1949) again, writing of the progress that had been made after only 20 years:

> Furthermore, new principles have been introduced. Among the most important ones must be mentioned: Michaelis' theory of the single-electron shifts; Lipmann's concept of the high-energy phosphate bond and its significance for the preservation and storage of energy; and the ideas concerning the transfer of whole blocks of atoms, as in trans-aminations, trans-methylations, trans-acetylations, trans-glucosidations and trans-phosphorylations.

These concepts and many other developments of the past 50 years are refinements of the original model. Moreover, Kluyver's team had established the value of using microorganisms for fundamental biochemical studies.

Like most scientists in those less-specialized times, Kluyver's interests were wide-ranging, and his interest in comparative biochemistry meant that all sorts of microorganisms were studied by his undergraduate and postgraduate students. Together with one of his assistants, Cornelis van Niel, Kluyver eventually proposed a system of bacterial classification that combined biochemical and morphological properties in an attempt to organize the different taxonomic systems in use by other researchers (Kluyver and van Niel, 1936). In this system (Table III), they separated the bacteria into Families, and then subdivided the Families into Tribes on morphological grounds, and then Genera on physiological grounds (Table IV). Obviously the numbers of known genera and species have massively increased, and we can no longer show all bacteria taxonomy in a single table. Moreover, as more information has become available, our understanding of the relationships between different microorgnisms has altered, but Kluyver's basic idea

TABLE III

THE DIVISION OF BACTERIA INTO FAMILIES AND TRIBES AS PROPOSED BY KLUYVER AND VAN NIEL (1936)

Family	Tribe
Micrococcaceae	Micrococceae
	Streptococceae
	Sarcineae
	Sporosarcineae
Pseudomonadaceae	Pseudomonadeae
	Vibrioneae
	Spirilleae
Bacteriaceae	Bacterieae
	Bacilleae
Mycobacteriaceae	Corynebactieae
	Mycobacterieae

underlies modern taxonomic methods and structures. Even the introduction of nucleic acid analysis to taxonomy is providing only the fine tuning of the system, and not replacing it.

The affection of his staff for Kluyver can clearly be seen in a lighthearted poster produced by the laboratory staff to mark the 25th anniversary of his appointment as a professor. As well as illustrating the scientific high points of his career, there are several comments about his smoking (including a gift token for a canary to check air quality)! Overseas researchers were coming in increasing numbers to work as PostDocs or on sabbatical, among them C. E. Clifton, H. A. Barker, R. L. Starkey, T. O Wikén (who was to follow Kluyver as professor in Delft), B. Volcani, J. De Ley, J. Senez, and S. Soriano, to name but a few. Many of Kluyver's Ph.D. students followed van Niel's example and took up their own professorial chairs in the Netherlands and all over the world, each creating a new branch of the Delft Tree. When, in 1951, Delft decided to award HRH Prince Bernhard, the husband of Queen Juliana, an honorary doctorate, Kluyver was selected to be his ''Promotor'' (the sponsoring professor). According to Kluyver's children, the Prince proved to be a good friend to Kluyver and the laboratory, taking an active interest in discoveries, and providing encouragement and support that were highly valued.

TABLE IV

PHYSIOLOGICAL SUBDIVISION OF ONE TRIBE SHOWN IN TABLE IV, THE PSEUDOMONADEAE OR STRAIGHT RODS[a]

Physiological characteristic	Genus
Photoautotrophic	
Green pigment complex (Chlorobacteria)	None
Purple pigment complex (Thiorhodaceae)	*Thiothece*
Photoheterotrophic	
Brown pigment complex (Phaeobacteria)	*Phaeomonas*
Purple pigment complex (Athiorhodaceae)	*Rhodomonas*
Chemoautotrophic	
Oxidize inorganic sulfur compounds (Leucothiobacteria)	*Sulfomonas*
Oxidize ferrous iron (and manganese)	*Sideromonas*
Oxidize ammonia	*Nitrosomonas*
Oxidize nitrite	*Nitrobacter*
Chemoheterotrophic	
Obligatory oxidative catabolism	*Acetobacter*
	Pseudomonas
	Rhizobium
	Azotobacter
Fermentative	
Mixed acid fermentation	None
Symmetric dimethylglycol fermentation	*Aeromonas?*
Alcoholic fermentation	*Zymomonas*
Butyric fermentation	None
Protein fermentation	None
Propionic acid fermentation	None
Homofermentative lactic acid fermentation	None
Heterofermentative lactic acid fermentation	None
Sulfate reduction	None
Methane fermentation	*Methanobacterium?*

[a]Extracted from Kluyver and van Niel (1936). Some characteristics were not possessed by genera within this Tribe but have been retained in the table to illustrate the range of tests in use.

In 1922, Kluyver arranged with the Centraal Bureau voor Schimmelcultures (CBS; now known as the Fungal Biodiversity Centre) that their Yeast Division would be housed in the Microbiology Laboratory in Delft—in fact, in the small room originally used by Beijerinck as his private laboratory. When the department moved into the current building, now the Kluyver laboratory, the CBS moved as well, and remained until 2000, when it was transferred to Utrecht. During its time in Delft, the CBS became an international center for yeast taxonomy, and the monograph published by N. J. W. Kreger-van Rij and J. Lodder (1952), entitled ''The Yeasts, a Taxonomic Study,'' now in a fourth edition (Kurtzman and Fell, 1998), is still the standard work on the subject.

In addition to the work on bacteria and yeasts, the comparative biochemistry research demonstrated that it was very difficult to get reproducible results from filamentous fungal cultures. At that time, the most common way of growing molds for fermentation was as floating colonies on liquid media in stationary vessels. The metabolites observed depended greatly on the age of the culture, and other, less obvious factors. Kluyver's team discovered that if a fungus was grown submerged, and aerated from below, it grew as small balls of mycelia, did not form spores, and gave far more easily reproducible results (Kluyver and Perquin, 1933). From this work came the Kluyver flask (Fig. 12). The efficiency of mixing obtained with these flasks is such that they are still routinely used for many types of aerobic batch culture, and with argon replacing the air as the sparging gas if anaerobic cultures are required. They can even serve as simple continuous culture systems, with an overflow tube inserted in the side and electrodes in rubber plugs set into the ''shoulders.''

Kluyver had maintained the ''Beijerinckian'' tradition of close contacts with the ''Nederlandsche Gist-en Spiritusfabriek,'' by this time known as Gist brocades, and during World War II, collaborated with them in the secret cultivation of *Penicillium* and subsequent production of penicillin of a degree of purity sufficient to impress Alexander Fleming when the war ended. The use of submerged cultures based on Kluyver and Perquin's work was an important part of the scale-up of the penicillin production process (Burns and van Dijke, 2002).

In 1935, Kluyver joined forces with the professor of physics at the University of Utrecht, L. S. Ornstein, to form the Biophysical Group Utrecht-Delft, a consortium funded by the Rockefeller Foundation to investigate biophysics. The Biophysical Group mainly concentrated on the study of bioluminescence and photosynthesis and was, over the 20 years it existed, highly successful. In many ways, this was

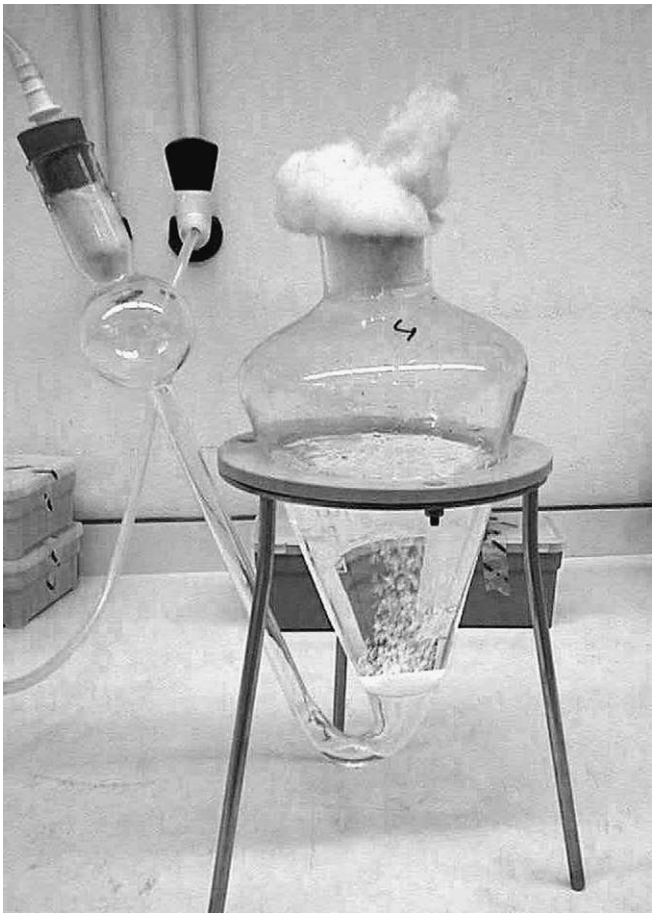

FIG. 12. A Kluyver flask. Air (or another gas) is pumped through the side-arm, passing through a sintered glass filter to create a stream of small bubbles.

the forerunner of the multidisciplinary teaching that is so strongly emphasized in Delft today.

V. Cornelis Bernardus van Niel, 1897–1985

During his years as professor of microbiology in Delft, Kluyver had a number of very talented students and postgraduates. The first, and probably one of the most influential of these pupils, Cornelis van Niel (Fig. 13), remained a lifelong friend and colleague, despite the fact that

FIG. 13. C. B. van Niel in his office in Delft.

he moved to the United States very early in his career and meetings between the two were, of necessity, limited after that. Indeed, Beijerinck was so impressed when he met van Niel that he wrote to Kluyver to urge that the young man should be kept in Delft! Beijerinck's letter is reproduced as Fig. 14, and translated below:

Gorssel, 3 Nov. 1928

Dear Mr. Kluyver,

last Wednesday v. Niel explained to me his splendid discovery concerning H_2S assimilation. I will not neglect to heartily congratulate the instigator of it, you his teacher.

I do not really think that he is very suitable for America whereas he is in the right place with you. Would it be profitable if you, you and I together, or still better [Professor] Went and you went together to warn the Minister of Education not to dismiss him but to keep him for your laboratory?

van Niel would very much like to have my copy of Winogradsky's Sulphur Bacteria. I told him that I had promised it to you; I shall send it to you and you can then either keep it yourself or give it to him.

I am just now sending it to the book binder.

With best wishes, yours, M. W. Beijerinck

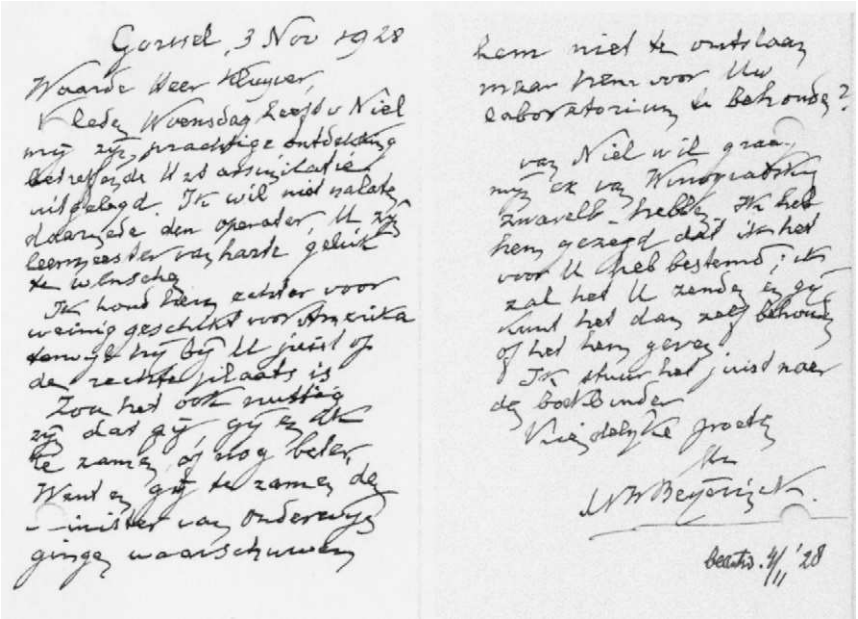

Fig. 14. A letter from Beijerinck to Kluyver complaining about van Niel's plans to move to the United States (translation in the text).

Many of van Niel's letters to Kluyver begin ''My esteemed Master,'' and the affection between the two shines clearly throughout their extensive correspondence, which lasted until Kluyver's death. Unlike the very formal letters between Kluyver and Beijerinck, the letters between Kluyver and van Niel are relaxed and include comments on people and places as well as work.

van Niel was, of course, the man who coined the phrase ''Delft School of Microbiology.'' He is, today, frequently remembered as a charismatic teacher, especially his summer schools at Pacific Grove, and many eminent scientists trace their ''Delft roots'' back through studies with him. Of course, as Norbert Pfennig (1987) reminded us, he also spent a great deal of his time studying the phototrophic sulfur bacteria—research that began in Delft and continued when he moved to the New World to become a professor at the Hopkins Marine Station in California. Using the principles of comparative biochemistry, he was eventually able to unite hydrogen transport during the various types of photosynthesis in the generalized formula:

$$2H_2A + CO_2 \rightarrow 2A + (CH_2O) + H_2O$$

where A can be oxygen, but can also be something else such as sulfur.

There are excellent biographies of van Niel by Barker and Hungate (1990) and Susan Spath (1999).

VI. The Delft School?

Now that we have briefly considered the founding fathers of the Delft School, it is time to return to the questions asked in the Introduction: What was, and is, the ''Delft School of Microbiology''?

It is certainly a term that has been used a great deal over the years since van Niel first coined it (van Niel, 1949). Indeed, it is clear that it is an important concept to many—a brief search of the internet reveals a number of great microbiologists who felt their links to Delft so strongly that it is mentioned in their obituaries—these include Michael Douderoff (1911–1975), Marvin Bryant (1925–2000), Jozef De Ley (1924–1997), Sidney Rittenberg (1915–1995), Benjamin Volcani (1915–1999), Horace Barker (1908–2001), Lourens Baas-Becking (1895–1963), Holger Jannasch (1927–1998), and Herman Phaff (1913–2001). Add to this the numbers of people who enthusiastically came forward for inclusion in the Family Tree (Robertson, 1996) and those who have paid tribute to the Delft School in autobiographical memoirs in different publications, and it becomes clear that we are dealing with something valued by many. However, if membership is limited to those who gained degrees or otherwise studied in Delft, or who studied with someone who could trace their own microbiological ''heritage'' to Delft, many great microbiologists are omitted, whereas others who would not describe themselves as microbiologists are included. Another definition is necessary.

van Niel (1949), when he first mentioned the ''Delft School of Microbiology,'' never actually defined what he meant by the term. He mentioned the discoveries of van Leeuwenhoek and, while reminding the reader that van Leeuwenhoek was not a teacher, left no students, and could not therefore have been said to have founded a ''School,'' nevertheless described Beijerinck as the *second* great Delft microbiologist, thereby retaining the shadow of van Leeuwenhoek in the background. He described the enrichment culture as Beijerinck's greatest achievement, and the principle of comparative biochemistry as Kluyver's.

Bulder *et al.* (1989) defined the main elements of the Delft School as ''the metabolic versatility uncovered by Beijerinck with his enrichment technique and Kluyver's concept of Unity in Biochemistry.''

In their 1977 tribute to Michael Doudoroff, Barker, Snell, and Wilson described the Delft School thus: ''the presentation of bacteria and other microorganisms as biological systems worthy of study for their own structures, metabolic activities and roles in nature, agriculture and industry, as well as for their relations to infection and disease.''

Another description suggests that it was almost a public relations campaign for microorganisms. ''Members of the Delft School of Microbiology, in the early part of this century, did bridge the gap between bacteriology and genetics. Clearly separating themselves from the medical bacteriologists who maligned bacteria, they believed that progress in fundamental microbiology depended on people who 'loved' microbes'' (Zuckerman and Lederberg, 1986).

At the Beijerinck Centennial, Maurits la Rivière (1997), another pupil of Kluyver's, summarized his vision of the Delft School approach in four points:

1. General microbiology is a distinct subject within biology while remaining a part of it because of unity in biochemistry.
2. General microbiology seeks comprehensive understanding of the microbial world and its significance for man.
3. The study of microbiology comprises a humanizing factor and a fascination of its own through the shared delight of acquaintance with an invisible world full of treasure and surprise.
4. The practical laboratory course in which students perform their own isolations is a time-honoured, proven teaching instrument.

These various definitions are not necessarily mutually exclusive. In fact, they all fit within the ideas expressed by Martinus Beijerinck during his ''Oratie'' (see above). The most important factors that seem to link them are an absolute zest for practical microbiology, a need to know how biosystems fit and work together, and an almost ''holistic'' approach to microbiology, especially microbial ecology and physiology. These qualities were possessed by all four of the Delft School founding fathers, and by most of those who could be considered their scientific heirs, whether or not they have ''academic links'' to Delft.

In many ways, the four ''Founding Fathers'' of the Delft School embody the multiple disciplines of modern applied microbiology and biotechnology. Beijerinck was a taxonomist, physiologist, and ecologist. van Iterson was fascinated by genetics and biomathematics. Kluyver represents the biochemists and van Niel the ecologists. All of them had links to industry, particularly the Gist & Spiritus Fabriek (later Gist brocades and now a subsidiary of DSM), and used their

various skills in putting microbial systems to work in the service of humans. It is possibly this combination of fundamental and applied science that is their greatest legacy. Kluyver, himself, put this into words when accepting the Emil Christian Hansen medal in Copenhagen (1947):

> the coming generations of microbiologists, far from being pitiable, will experience joys from both an increased insight into the mysteries of microbial life and from an increased mastery of its manifestations. And the industrial microbiologist will add one key industry to those already existing: the industry in which microbes with desired qualities will be fabricated.

Perhaps we should add the ability to predict the future to the list of qualities desirable in the member of the Delft School?

VII. Whence the Delft School in the Twenty-first Century?

Beijerinck was obsessed with simplicity in experimental design, but one might hope that he would approve of, and even enjoy using some of the equipment in use today. For example, his major contribution to the discovery of the virus was confirmed only with the development of the electron microscope, and consequent photographs of TMV particles some years after his death. His enrichment cultures are now best done in continuous cultures with computer control and monitoring of environmental parameters, and he would surely appreciate the level of control this confers. The great domestic and industrial wastewater treatment systems in use today owe their specific microbial communities to enrichment processes.

Johanna Verhoop, van Iterson's widow, during a visit to Delft, expressed her regret that her husband had not lived long enough to have had his own personal computer. Evidence of the long evenings of calculations she remembered can still be seen in the Kluyver Laboratory Archives in the form of increasingly complicated graphs and drawings that, to my eye, strongly resemble fractals. He was fascinated by biological variation and genetics, and one can only suppose that he would have been more than happy to pick up many of the tools of modern molecular biology.

Molecular biology would also have been a major asset to Kluyver—the ability to insert, remove, or disrupt the genetic coding for specific enzymes, for example, would have greatly assisted his work in comparative biochemistry. Many modern techniques are built on the foundations he laid in his concept of Unity in Biochemistry. The BioPhysics group he formed with Utrecht University led the way to

the multidisciplinary research of today, where the Laboratory of Microbiology is an integral part of the Kluyver Laboratory for Biotechnology. We share a building, facilities, and often students with enzymologists, molecular biologists, mathematical modelers, chemists of all types, and (bio)chemical engineers. The physicists are still involved as well. We have formal agreements for collaboration in research and teaching with the Universities of Leiden and Wageningen. van Niel, not only a great microbiologist but also a great communicator, would surely have approved. One of his academic descendants, Wolf Vishniac, employed the Delft School principles of microbial selection in designing experiments to try and detect life on Mars—it seems logical now to search for microbes that would have a selective advantage under Martian environmental conditions rather than those of Earth, which was still a new concept then. Sadly, the death of Vishniac meant that many of his ideas were not used in the early experiments.

Delft still has much that the four professors would recognize. We are still working on the sulfur and nitrogen cycles, and isolating very unusual microorganisms. We still cooperate with Gist brocades (now a subsidiary of DSM) and supply them with many of our graduates. Research into comparative biochemistry still proceeds, with an accent on industrial products, and we have groups working on bioreactor development—surely an extension of Kluyver's work on submerged fungal cultures.

It would be very easy to feel relaxed and comfortable about the future. General microbiology is popular again after a decade or two when it seemed that only *Escherichia coli* was of interest. Improved culture techniques are permitting the isolation and study of all sorts of extremophiles, and molecular biology, for the first time, is allowing us to be absolutely sure of what organisms we are isolating, and how their metabolic pathways work. However, there is a danger that both Kluyver and van Niel mentioned in different contexts. It is too easy to become overspecialized and to develop a sort of ''tunnel vision.'' Current examples of this can be found in the extreme focus of some of the molecular biology work being done today. In the same way that rRNA analysis is not sufficient on its own to identify a species or strain, but can provide valuable information on microbial relationships, it is also not enough to know that a given microbial community contains a collection of microorganisms identified only by their nucleic acids. We also need to know what they are doing in there, and how they interact. There is an obvious need to extract every last bit of information from any experiment—indeed, for the approach pioneered by Martinus Beijerinck over 100 years ago.

FIG. 15. Tribute to M. W. Beijerinck and his contribution to microbial ecology. Enamel on metal by A. W. Wijkniet, 1926.

The opportunities exist. Microbiology will continue to be essential—whether it is in the production of nutrients or fine chemicals, waste treatment or bioremediation, or any of the myriad fields of molecular biology and genetics, to name but a few. It seems likely that microbial ecology will become important in the space program, whether it is to maintain a healthy environment on the space station or during long-term space travel, or to understand what might have happened on other planets. We are not too far from new experiments to look for evidence of past or present life on Mars—and the indications are that should Martians exist, they'll be little, they may be green, but they certainly won't be men! Experimental equipment and methods seem to improve on a daily basis. It is thus still appropriate to echo the words of Martinus Beijerinck, spoken during his final lecture before he retired and now shown on a plaque on the wall of our entrance hall (Fig. 15, see color insert): ''Happy are they who are beginning now.''

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