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VOLUME **67**

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CHAPTER 1

Phage Evolution and Ecology

Stephen T. Abedon

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Abstract

Bacteriophages (phages) are the viruses of bacteria and the study of phage biology can be differentiated, roughly, into molecular, environmental, evolutionary, ecological, and applied aspects. While for much of the past fifty-plus years molecular and then applied aspects

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have dominated the field, more recently environmental concerns, especially the phage impact on biogeochemical cycles, have driven an increase in the appreciation of phage ecology. Over approximately the same time frame, decreasing sequencing costs have combined with phage molecular characterization to give rise to an inescapable consideration of phage comparative genomics. That, along with environmental metagenomics, has stimulated, especially among molecular biologists, a more general interest in phage evolutionary biology. However, while reviews of phage ecology have become exceedingly common, overviews of phage evolutionary biology are comparatively rare, and broad considerations of phage evolutionary biology drawn from an ecological perspective rarer still. In this chapter I jump into this latter void, providing an overview of phage evolutionary biology as viewed from the perspective of phage-environment interactions, that is, from the perspective of phage ecology. This I do over five sections constituting (1) an introduction to phages and how, phenotypically, they can be differentiated into three basics types that correlate, more or less, with genomic size, that is, tailed (generally larger genomes), lipid-containing (medium-sized genomes), and single-stranded (small genomes); (2) a brief introduction to phage ecology as considered particularly from a classical ecological perspective; (3) an extended introduction to evolutionary biology as viewed from a phage and phage-ecological standpoint; (4) phage evolutionary ecology, that is, consideration of phage adaptations from the vantage of why, in terms of phage fitness, those adaptations may have evolved; and (5) phage evolutionary biology, including evolutionary ecology, as viewed from the perspective of phage genomics.

I. INTRODUCTION

Bacteriophages (phages), the viruses of bacteria, were formally discovered over 90 years ago (d'Hérelle, 1917; Twort, 1915) and their initial study focused especially on organismal, population, and community aspects, for example, virion survival, broth or plaque growth, and impact on bacteria, respectively. Early phage biology, in other words, was inherently ecological (Abedon, 2008c). Phage studies were also evolutionary undertakings through efforts toward adaptation to new conditions and host bacteria (e.g., d'Hérelle, 1924). Furthermore, phagology was an applied activity in which phages were harnessed as antibacterial agents (e.g., Kutter, 2008).

With time, starting especially in the 1960s, more ecological or evolutionary approaches to the study of phage biology were overtaken by the analysis of phage genetics and related biochemistry (Abedon, 2000; Summers, 1999).

Increasingly powerful techniques, many of which were first appreciated or invented in the course of the analysis of phages, were applied with great enthusiasm toward bettering our understanding of phage molecular biology and, by extrapolation, the rest of biology as well. Morse (1994), emphasizing these trends as they impacted virology as a whole, wrote that ''Early work. . ., of necessity emphasized phenotype and phenotypic variation. . ., whereas more recent work. . ., has emphasized genotypic variation, with little reference to phenotype or the role of host or ecological factors, or to evolutionary constraints'' (pp. 1–2). Contrasting ecological approaches, he also noted that, with molecular approaches, ''The ability to accumulate meaningful experimental data with relative facility was an overwhelming advantage'' (p. 16). In the course of this ''molecular temptation'' (Ackermann and DuBow, 1987a, p. 87), especially as the study of genotype came to increasingly dominate molecular biology, seemingly the idea of phages as organisms—or at least as something more than exquisitely organized macromolecular machines (Villarreal, 2005)—was generally forgotten.

Over approximately the previous two decades—starting with seminal reporting of unexpectedly high viral total counts in marine waters by Bergh *et al.* (1989), the phage diversity overview presented in the *Viruses of Prokaryotes* volumes of Ackermann and DuBow (1987b,c), the explicitly *Phage Ecology* monograph edited by Goyal *et al.* (1987), and the phage population biology work summarized by Lenski (1988)—the study of phage biology has been returning to its more integrated, ecological, and organismal roots. This reawakening of the more ''holistic'' side of phage biology has occurred for a number of reasons including interest in the role of phages in aquatic nutrient cycling (Suttle, 2007), the increasing availability of whole phage genome sequences and consequent study of phage genomics (Brüssow, 2006), a reconsideration of phage therapy as an alternative to antibiotics (Kutter, 2008), and the employment of phages as evolutionary and ecological models (Bull, 2008; Duffy and Turner, 2008; Kerr *et al.*, 2008). In short, phage biology today may be differentiated into five general categories which increasingly can be viewed as related components of a well-integrated, phage-biological whole: phage molecular biology, phage evolutionary biology, phage ecology, phage environmental microbiology, and applied phagology.

Recently, over approximately the past decade (e.g., Hendrix *et al.*, 1999), it is the potential for phages to evolve, that is, to display changes in genome sequence over time, that has become perhaps the most exciting facet of their biology (''. . .there is perhaps now more interest in evolution among molecular biologists than among workers of any other nonstrictly evolutionary branch of biology,'' Mayr, 1994, p. 41). The phage ecological impact (Abedon, 2008a), their molecular sophistication (Calendar and Abedon, 2006), their laboratory and medical significance (Waldor *et al.*, 2005), and

their ability to kill bacterial pathogens (Kutter and Sulakvelidze, 2005), thus all may be viewed in light of the evolutionary history embodied within phage genomes, the evolutionary relationships between phages, and the phage potential to evolve. This evolutionary drama is captured explicitly by determinations of phage diversity, as elucidated using both comparative genomics (Brüssow and Desiere, 2006) and metagenomics (Casas and Rohwer, 2007; Edwards and Rohwer, 2005), which suggest that phages are both genetic mosaics (Casjens *et al.*, 1992; Hendrix, 2002, 2003, 2008; Hendrix and Casjens, 2008) and remarkably diverse (Pedulla *et al.*, 2003; Weinbauer *et al.*, 2007). Phage evolution is also observed via the evolutionary ecological characterization of phage phenotypic adaptation (e.g., Abedon, 2006, 2008d; Breitbart *et al.*, 2005; Bull, 2008; Kerr *et al.*, 2008).

The aim of this chapter is to explore the evolutionary biology that underlies the totality of phage biology, all as viewed, where possible, from a phage-centered, ecological perspective (see Fig. 1.1 for a summary of the general disciplines, subdisciplines, and interconnections as conceivably could be covered by such an approach). Given space limitations, my aim is to provide fresh looks at a subset of associated phenomena while avoiding excessive duplication of previous reviewing efforts (most notably those found in Abedon, 2008a). This I present within a context of my take on a general overview of key aspects of the field.

II. BACTERIOPHAGE TYPES

Phages may be broadly defined as the viruses of prokaryotes. Workers who study the viruses of members of the cellular domain Archaea, however, typically prefer to describe their research subjects as viruses rather than as phages (e.g., Stedman *et al.*, 2006). Consistently, I will concentrate here on reviewing the evolution and ecology of the viruses of members of domain Bacteria. Phages therefore may be defined, narrowly, as obligately intracellular parasites of domain Bacteria which, as viruses, pass at some point in their life cycles through an acellular and extracellular state. During this extracellular passage phages are described as ''free.''

A free phage, minimally, consists of nucleic acid found within a proteinaceous capsid, though capsids in some phages may be supplemented with various lipids. The dominant phage types have dsDNA, monopartite genomes which are packaged within protein-only capsids that display a complex morphology known as tailed (by contrast, most viruses of animals and plants have RNA genomes and none are tailed; Domingo and Holland, 1994). These tailed phages more formally are members of order *Caudovirales*, within which there are three families: *Myoviridae*

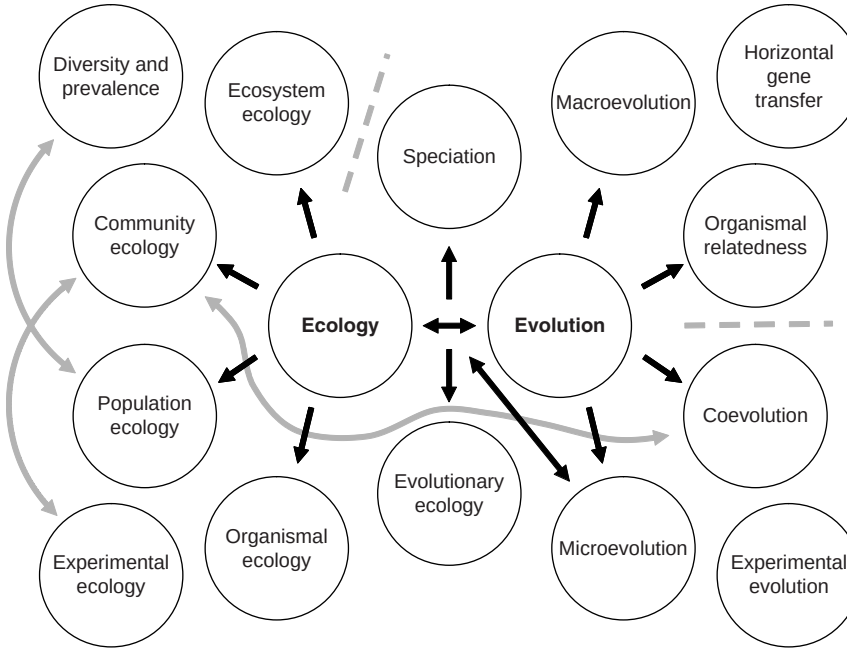

FIGURE 1.1 Phenomena and approaches associated generally with ecological or evolutionary research. Single-headed arrows indicate hierarchical connections between disciplines and subdisciplines. Generally circles found closer to each other, or with arrows between, indicates greater scientific relatedness. Alternatively, dashed walls have been placed between concepts which are closely located only coincidentally. The longer double-headed arrow angling up from "Microevolution" is intended to convey the role of both evolution and ecology in microevolutionary processes.

(phages with contractile tails such as phages T4 and Mu), *Siphoviridae* (phages with long, noncontractile tails such as phages T1 and λ), and *Podoviridae* (phages with short, noncontractile tails such as phages P22 and T7). Most tailed phages have genomes of 16 kb or larger. For example, at the lower end among fully sequenced phages, *Staphylococus* phage C1, a podovirus, has a genome which is 16,687 bp. Exceptional, though, is *Mycoplasma* phage P1, a podovirus whose genome is only 11,660 bp, as well as the satellite (hyperparasitic) myovirus, phage P4, which is only 11,624 bp. Tailed phage genomes, however, can range up to over 500 kb (Seaman and Day, 2007; Serwer *et al.*, 2007). By contrast, the genomes of tailless phages appear to be less than 15 kb (this less-than-15-kb ''rule'' does not extend to tailless Archaeal viruses, however; Fauquet *et al.*, 2005).

A second category of phage types, though one not representing a formal taxonomic category, have medium-sized, double-stranded genomes (though not necessarily DNA genomes), and lipid-containing capsids. Included in this category are the dsDNA phage families of

Corticoviridae, *Tectiviridae*, and *Plasmaviridae*. In addition, there is one family of dsRNA phages, the *Cystoviridae*, whose type species/strain is the *Pseudomonas* phage ϕ6. The latter is additionally unusual among phages in that members have genomes which are tripartite (three dissimilar segments) rather than monopartite. Genome sizes for fully sequenced, double-stranded tailless phages range from 9 to just under 15 kb.

A third category of phages, also not representing a formal taxonomic category, display single-stranded, small-sized genomes. These include the ssDNA phage families of *Microviridae* (which have isometric capsids and include phage ϕX174) and *Inoviridae* (which have helical and filamentous capids and include phages f1, fd, M13, and CTXΦ). The ssRNA phages, found in family *Leviviridae*, are also isometric and include phages MS2, F2, and Qβ. Genome sizes, of those phages which have been fully sequenced, range from 4.4 to 6.3 kb for the *Microviridae*, 4.5 to 9.0 kb for the *Inoviridae*, and 3.5 to 4.3 kb for the *Leviviridae*. Additional virion characteristic details can be found in Fauquet *et al.* (2005).

A further means of differentiating phage types is in terms of their infection characteristics, for example, lytic versus chronic or obligately productive versus temperate (Abedon, 2008c, 2009a; Abedon *et al.*, 2009; Hyman and Abedon, 2009b): All nondefective phages of all families except *Inoviridae* and *Plasmaviridae* are lytic and only temperate phages display lysogenic cycles. Only a few of the many described temperate phages are known to release progeny chronically (Yamada *et al.*, 2007) plus at least one temperate phage, a tectivirus, is lytic but tailless (Verheust *et al.*, 2003). Phages also may be distinguished in terms of the types of bacteria that they infect, in terms of their more-specific infection (especially molecular) characteristics, or as a function of the ecosystems in which they are found (e.g., Calendar and Abedon, 2006).

III. PHAGE ECOLOGY

Ecology is the study of the interactions between organisms and their environments. Environments can consist of both biotic and abiotic aspects, which are living and nonliving components, respectively. I and collaborators have published a number of overviews in which we differentiate phage ecology especially into organismal, population, community, and ecosystem ecologies (Abedon, 2006, 2008b,d; Abedon *et al.*, 2009; Breitbart *et al.*, 2005; Hyman and Abedon, 2009b). These approaches can be summarized as representing the study of the impact of the abiotic environment on organisms (organismal ecology), the impact that individuals of the same species have upon one another (population ecology), the interactions between different species (community ecology), and the interaction and impact biotic entities have on abiotic aspects of

environments (ecosystem ecology). See Fig. 1.2 for illustration of these connections. Additional, general reviews of phage ecology can be found elsewhere (Abedon, 2008a; Gill and Abedon, 2003; Weinbauer, 2004; Weinbauer *et al.*, 2007; Wommack and Colwell, 2000).

Phage ecology also may be viewed using a more phage-inspired rather than ecology-inspired organization. Thus, for example, phage ecology may be described in terms of six core emphases: (1) the survival of individual phages, (2) the phage potential to reproduce, (3) the phage impact on bacterial fitness, (4) the phage impact on bacterial diversity, (5) the phage impact on nonhost organisms including on eukaryotes,

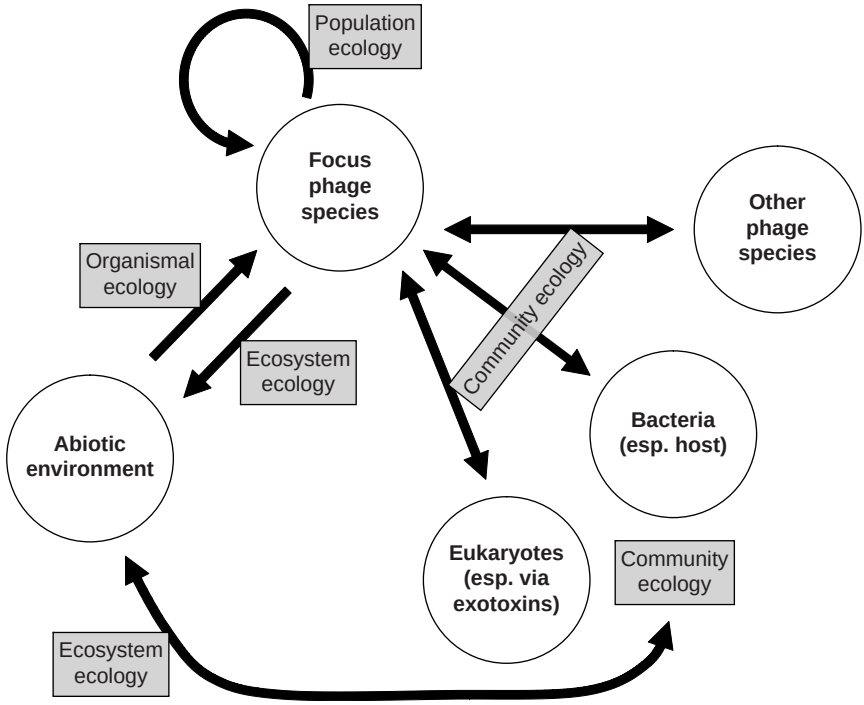

FIGURE 1.2 Phage ecological connections. Abiotic impact on phages may be described as the province of phage organismal ecology. Population ecology considers intraspecfic interactions. Community ecology, for phages, can be between heterologous phage types, phages and bacteria (especially host bacteria), and phages and eukaryotes (e.g., as a consequence of phage encoding of eukaryote-impacting exotoxins). Ecosystem ecology considers the interactions between the abiotic and biotic. For example, phage virions can decay (thereby becoming part of the abiotic environment) and bacteria can be lysed or, alternatively, absorb abiotic nutrients. Eukaryotes also may be manipulated by phages to contribute to the abiotic environment, again as a consequence of phage encoding of exotoxins.

and (6) the phage impact on the abiotic world, where the latter typically is formulated in biogeochemical terms. In addition to phage ecological concerns, there also exists a related emphasis, which I call phage environmental microbiology (Abedon, 2008b; Abedon *et al*., 2009), that in addition to being concerned with the phage impact on bacterial populations and biogeochemistry also emphasizes issues of phage number and diversity within natural habitats.

A. Phage organismal and population ecology

Phage organismal ecology is concerned with phage reproduction (infection) and survival, the latter especially as virions but also as infections. Phage population ecology can be distinguished into three aspects: phage–phage competition under conditions of (effectively) unlimited resource availability (which for phages occurs given low multiplicities of infection; Abedon, 2008d), phage–phage competition given limited resources (i.e., where phage populations are reaching or exceeding their environmental ''carrying capacity''; Abedon, 2008d, 2009a), and phage–phage competition (and interaction) at the level of individual bacteria. The latter occurs following the coadsorption and potentially subsequent phage coinfection of a bacterium (Abedon *et al.*, 2009; Turner and Duffy, 2008).

B. Phage community ecology

Phage community ecology traditionally has been studied from the perspective of the interaction between phage populations and populations of host bacteria including in terms of phage–host coevolution (Brockhurst *et al.*, 2007; Forde *et al.*, 2008; Gill, 2008; Kerr *et al.*, 2008; Thingstad *et al.*, 2008). However, phage community interactions also may be legitimately studied in the guise of phage interactions with other phage species (Abedon *et al.*, 2009) including, but not limited to, phage superinfection of bacterial lysogens (Turner and Duffy, 2008). Phages also interact with eukaryotes (Abedon *et al.*, 2009; Bettarel *et al.*, 2005; Górski *et al.*, 2006; Merril, 2008).

C. Phage ecosystem ecology

Phage ecosystem ecology typically considers the phage impact on nutrient cycling within ecosystems as well as energy flow through ecosystems (Suttle, 2007; Weinbauer, 2004; Wommack and Colwell, 2000). Phages can impact nutrient and energy movement via bacterial lysis (which releases nutrients into the extracellular environment that otherwise are locked within bacteria), by producing bacterial exotoxins (during bacterial infection) which serve to disrupt eukaryotic tissues (or, more generally, which

modify the environments surrounding phage-infected bacteria), or by releasing, from bacteria, normally cell-associated, especially hydrolytic enzymes into the extracellular environment (so-called ectoenzymes) (Abedon and LeJeune, 2005). Though considerations of phage community and ecosystem ecology, especially as they occur within aquatic ecosystems, today dominate the study of phage ecology (e.g., Suttle, 2007; Thingstad *et al.*, 2008; Weinbauer *et al.*, 2007), this perspective will not be an emphasis of this chapter.

IV. PHAGE EVOLUTIONARY BIOLOGY

Phage evolutionary biology—consideration of heritable changes in phage genetic material over time—is increasingly becoming the basis for understanding phage biology in general. In this section I provide an overview of microevolution that is written, where appropriate, from phage-centered perspective. For additional review, see Campbell (1988), Bull (2008), Duffy and Turner (2008), and Hendrix (2008).

A. Microevolution versus macroevolution

Generally the evolutionary forces impacting organisms consist of the standard Hardy–Weinberg violations (Hardy, 1908; Weinberg, 1908)—mutation, migration, genetic drift, natural selection, and nonrandom mating (plus recombination) (Fig. 1.3). To summarize: Mutation and migration move genetic alleles both into and out of populations. Genetic drift and natural selection are unbiased (stochastic) and biased (deterministic) changes, respectively, in the frequency of existing alleles. Recombination, in the course of sexual processes, changes allele combinations as they are found within a single genome, including by bringing together alleles and loci that had evolved within different lineages (horizontal/lateral gene transfer). Finally, nonrandom mating gives rise to biases in opportunities for generating combinations of either alleles or loci. All of these forces have the effect of affecting either allele frequencies within populations (mutation, selection, drift, migration) or allele combinations within individuals (mutation, nonrandom mating, recombination). Important endpoints for changes in allele frequencies are extinction and fixation, that is, allele frequencies of 0 and 1, respectively. Brüssow and Dessier (2006) describe all of these aspects except recombination (and perhaps migration) as ''Vertical'' evolution (versus ''Horizontal'' evolution).

The previous paragraph describes microevolutionary processes, that is, factors affecting the genetic structure of individual populations, where a population consists of potentially interbreeding individuals hailing from the same ''species.'' Macroevolution, by contrast, is a description

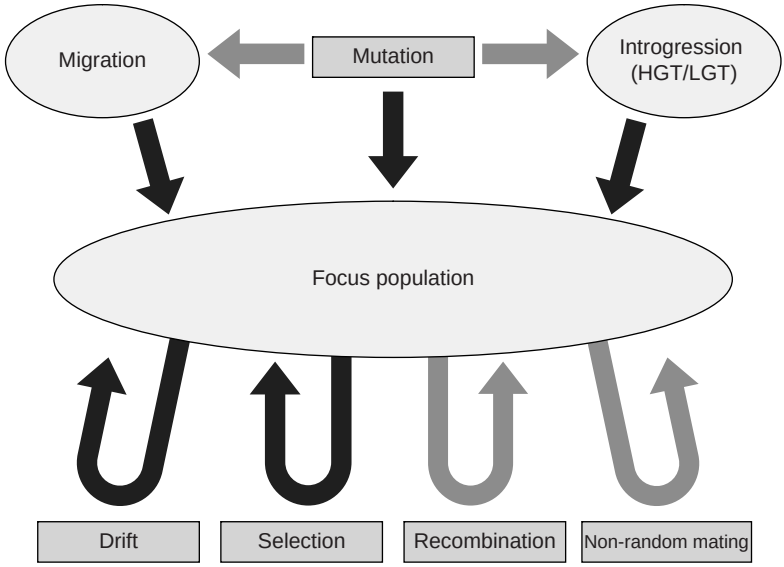

FIGURE 1.3 Basic mechanisms of evolution. Evolution is defined as changes in allele frequencies within populations. Mechanisms impacting the allele frequencies of a focus population are shown using darker gray (i.e., nearly black) arrows. Mechanisms not directly impacting the focus population or not directly giving rise to changes in allele frequencies are indicated using lighter gray arrows. New or additional copies of alleles are introduced into populations by mutation, migration from nonlocal (allopatric) conspecifics, or via low-levels of hybridization with local (sympatric) populations of different species (introgression, here a.k.a. horizontal gene transfer or lateral gene transfer, which in terms of violations of Hardy–Weinberg equilibrium can be defined as a kind of migration). Populations (including those effecting migration or introgression) have their allele frequencies also impacted by genetic drift and natural selection. Recombination (e.g., as with conspecifics) gives rise to variation in allele combinations found within individual organisms. Nonrandom mating can give rise to or can be a consequence of nonrandom distributions of alleles within populations.

of evolution as it occurs above the level of species such as differential rates of speciation and species extinctions as well as the divergence of genetically isolated lineages. Macroevolution, per se, is not well understood among phages, and this stems in part from difficulties in defining what exactly a phage species is (Campbell, 1988; Lawrence *et al.*, 2002; Silander *et al.*, 2005) (see, however, Campbell, 1994; Duffy and Turner, 2008; Duffy *et al.*, 2007; Villarreal, 2005). In general I concentrate in this chapter on exploring phage microevolution, rather than on concepts or trends in either phage speciation or species extinction (macroevolution). See also Campbell and Botstein (1983), Morse (1994), and Hendrix *et al.* (2000, 2003) for discussion of how phages may have originated.

B. Fitness and natural selection

One way of differentiating among the various mechanisms that can violate the conditions which are necessary for the establishment of Hardy–Weinberg equilibrium (= absence of evolution) is into those mechanisms which are biased towards the retention of some (specific) alleles but not others (Darwinian evolution, that is, as mediated by natural selection) versus those mechanisms which are essentially random in their effects vis-à-vis the retention of specific alleles (nonDarwinian evolution, that is, as mediated by mutation, drift, or migration). NonDarwinian evolutionary forces are those responsible for creating, or at least modifying, the variation upon which natural selection can act. See Mayr (1994) for a philosophical discussion of natural selection as loosely written from the perspective of viruses.

Biases in reproductive success can be characterized in terms of an organism's Darwinian fitness (or, simply, fitness). Specifically, the number of offspring a genotype on average can produce is its ''absolute'' fitness (or ''mean effective reproduction rate,'' p. 64; Garnett and Antia, 1994), which needs to be equal to a value of at least one—each individual producing a single replacement organism—for a genotype to not be in decline. Typically ''relative'' fitness is employed instead of absolute fitness in experimental studies; relative fitness is simply a measure of change in the frequency of one genotype in comparison to the change in frequency of one or more competitor genotypes (e.g., Duffy and Turner, 2008).

Natural selection occurs as a consequence of factors, often environmental, that give rise to fitness differences between different organisms, that is, differences in genotype reproductive success. Given the key role played by the environment in natural selection, natural selection often may be profitably viewed not just in evolutionary biological terms but also from the perspective of ecology. Thus, nonDarwinian mechanisms provide genotypic variation within populations and it is differences in genotype fitness following organism interaction with environments (ecology) that gives rise to evolutionary adaptation (selective retention of alleles associated with greater organismal fitness). Manipulate an environment, change a population's density, or even modify an organism's genetic background, however, and the fitness impact associated with a given allele may change.

Because natural selection acts on genotypes rather than directly on specific alleles or, indeed, specific nucleotides (i.e., ''the individual (organism) is the target of selection,'' Mayr, 1994, p. 35), it is possible for alleles to increase in frequency, as products of natural selection, even if other alleles within the same population actually can confer greater fitness. This seeming paradox, called genetic hitchhiking, occurs due to genetic linkage of not-selected alleles with selected alleles (Barton, 2000).

With asexual populations, genetic hitchhiking can combine with a phenomenon known as periodic selection (Holder and Bull, 2001), which can be viewed as selective sweeps of populations by clones carrying specific alleles or as the clonal expansion of these lineages at the expense of other lineages. The result can be not only fixation of deleterious alleles but also reduction in population genetic diversity in general (Koch, 1974).

An additional aspect of natural selection is worth highlighting due to its potential importance to both phage evolution and ecology, and that is frequency-dependent selection. As discussed by Levin (1988), frequency-dependent selection can take on two general forms: disruptive frequency-dependent selection in which greater fitness is associated with being a member of a genotype already existing at a high frequency and stabilizing frequency-dependent selection in which greater fitness is associated with having a rare (low-frequency) genotype (where ''disruptive'' versus ''stabilizing'' refer to effects on retention of polymorphisms, that is, more than one allele within a population found at a give genetic locus). For example, one way that phages may attain an advantage of being a dominant type, in this case as prophages, is in terms of immunity to superinfection expressed by related phages. If the majority of phages found within an environment have the same immunity type as the prophage, it means that there are fewer phages which are capable of coopting a lysogenic infection towards lytic ends.

Stabilizing frequency-dependent selection, for phages as well as for bacteria in terms of their interactions with phages, likely is more common than disrupting frequency-dependent selection. For instance, in the above example potentially superinfecting temperate phages are better off possessing a *rare* immunity type. This advantage occurs because more bacteria, even lysogenic bacteria, should be available to phages possessing rare immunity types than to phages with immunity types that are so common that a substantial fraction of bacteria will have been already lysogenized by prophages displaying the same immunity type (Campbell, 1994; Campbell and Botstein, 1983). Indeed, such stabilizing frequency-dependent selection should be especially strong, given that not only are lysogens unavailable for infection by a phage possessing the same immunity type but adsorption by these phages will result in their inactivation (via superinfection immunity, a.k.a., homoimmunity).

Similar to the situation with superinfection immunity, more-common phage host-range types may be particularly well adapted for a host population that is in the process of being decimated by that phage population. However, as a consequence of this decimation, a phage displaying a rarer host range type may at least temporarily have more bacteria available to it (Campbell, 1994; Campbell and Botstein, 1983; Lenski, 1988). Another important example of stabilizing frequency dependent selection has been hypothesized to occur with bacteria displaying rare phage-susceptibility types. These

rarer susceptibility types should be less vulnerable to catastrophic phage attack due to their lower population densities than so-called bacterial ''winners'' (Thingstad *et al.*, 2008). Such stabilizing frequency dependent selection, as mediated by phages, also has been invoked to explain why there exists such a diversity of anti-phage bacterial restriction-modification systems (Levin and Lenski, 1985). A given bacterial community consequently may only temporarily sustain substantial densities of either specific phage host-range types or specific bacterial phage-susceptibility types.

In a related but more complex form of stabilizing frequency-dependent selection, too-common phage types can be *hyper*parasitized by other phage types. One example of such hyperparasitism gives rise to the formation of defective interfering particles which cannot successfully infect without a coinfecting wild-type helper virus (Villarreal, 2005). A second form of phage hyperparasitism is seen with phage ϕ6 cheater strains, which are able to functionally infect singly and therefore are not strictly ''defective.'' Nevertheless, these mutants display a lower relative fitness in comparison to wild type when singly infecting versus a greater relative fitness—again relative to wild type and therefore at the expense of wild type—when coinfecting with a wild-type virus (Turner and Duffy, 2008). A third form of phage hyperparasitism is seen with temperate satellite phages, such as phage P4. Absent its helper phage, which for phage P4 is an otherwise unrelated P2-like phage, phage P4 can display lysogeny but not a lytic cycle (Dehò and Ghisotti, 2006; Duffy and Turner, 2008).

In all of these cases of phage–phage hyperparatism, coinfection by a wild-type phage with a parasitic phage reduces the fitness of the wild-type phage. This implies a fitness cost to phage types for being sufficiently common that hyperparasitism by other phages can evolve (or, for the hyperparasites, there is a fitness cost for being so common that the helper phage is driven to lower densities). Thus, except for prophage immunity types, frequency-dependent selection involving phages appears to typically favor both rarer phage types and rarer bacterial types. Such selection is thought to be generally stabilizing (i.e., tending towards retaining) both phage and bacterial diversity.

For additional, especially phage-specific considerations of natural selection, including in terms of evolutionary optimization, multilevel selection theory, adaptive landscapes, intracellular competition, epistasis, experimental adaptation, etc., see Abedon (2008d), Bull (2008), Duffy and Turner (2008), Kerr *et al.* (2008), and Turner and Duffy (2008).

C. Mutation

Mutations are changes to genomes that are heritable, that is, which are recognizable by nucleic acid polymerases as templates for the production of nucleic acid daughter strands. This is in contrast to damages to nucleic

acid, such as strand breaks, which prevent the replication of genomes, at least prior to repair (Bernstein and Bernstein, 1998). Genotypically, one can consider mutations in terms of per-nucleotide rates, per-genome rates, their types (i.e., transversions vs. transitions vs. deletions vs. insertions vs. inversions), and whether they reach fixation (Domingo and Holland, 1994; Duffy *et al.*, 2008). Mutations also are routinely considered in terms of their impact on phenotype, especially on fitness. Thus, mutations can be classified as beneficial, neutral, or detrimental.

Because mutations occur randomly, it is necessary, on average, for a lineage to endure additional detrimental mutations in order to gain more beneficial mutations. Whether a given mutation will have an impact on phenotype (or organismal fitness), however, can be a function of genotype and environmental variables (Duffy and Turner, 2008). All else held constant, a phage with a high mutation rate will produce both more detrimental and more beneficial mutations, per unit time, than a phage with a low mutation rate.

Typically higher mutation rates are considered to be more beneficial given organism exposure to either novel or rapidly changing environments. This can occur if the cost of harboring greater numbers of detrimental mutations can be made up for by the benefits of producing beneficial mutations in greater numbers. Technically, this is an argument that alleles giving rise to higher mutation rates may hitchhike, with greater likelihood, on genomes containing beneficial mutations than will alleles that don't give rise to higher mutation rates (Denamur and Matic, 2006). The catch, though, is that typically mutations that are detrimental are thought to occur more frequently than mutations that confer a benefit (Drake *et al.*, 1998) (also, e.g., Domingo and Holland, 1994). As a consequence, alleles giving rise to higher mutation rates also produce an accumulation (higher load) of detrimental mutations within their genomes.

It is at this point that differences between per-nucleotide versus per-genome mutation rates become especially important. That is, a phage displaying a small genome may benefit from higher likelihoods of beneficial mutations on a per-nucleotide basis but simultaneously display a moderately low likelihood, due to their smaller genome size, that a given beneficial mutation will share a genome with a detrimental mutation. However, raise per-genome mutation rates too much more than one mutation per genome per round of replication (the so-called error threshold; Domingo and Holland, 1994; Duffy *et al.*, 2008) and the likelihood of producing a phage with a lone mutation, beneficial or otherwise, will be relatively low. In addition, even a rare advantaged single mutant, given high per-genome mutation rates, will subsequently pay a cost of producing mutationally defective progeny. Too-high mutation rates in fact result in what may be described as a lethal mutagenesis (Bebenek *et al.*, 2002) or, alternatively, as error catastrophe (Domingo and Holland, 1994; Villarreal, 2005).

Among phages, it is those displaying dsDNA genomes that have the lowest per-nucleotide mutation rates and, consequently, both the greatest genetic stability (at least at the level of individual nucleotides) and the potential to maintain larger genomes. On the other hand, it is the ssRNA phages (family *Leviviridae*) that have both the highest per-nucleotide mutations rates and smallest genomes (Duffy and Turner, 2008). For example, the typical ssRNA phage has a genome that is ~10-fold smaller than the ''typical'' dsDNA phage genome (i.e., phage Qβ versus phage λ), or even ~100-fold smaller than the myovirus, phage G (Serwer *et al.*, 1995). At a minimum, these higher mutation rates may bestow on RNA viruses an ''extreme adaptability'' (Domingo and Holland, 1994).

In Table 1.1 I present per-nucleotide and per-genome mutations rates as well as minimum population sizes required for the display of all possible point mutations, all for a variety of DNA and RNA as well as single-stranded and double-stranded phages. A key observation is that mutation rates for the dsDNA-based phages are consistently ~0.004 mutations per genome per round of replication (Drake *et al.*, 1998). This implies that no more than about 1 out of every 250 replication events will result in a potentially deleterious phage mutation. On the other hand, the RNA phages as well as the ssDNA phage ϕX174 display mutation rates that are ten-fold or more higher than those of the dsDNA phages, ranging from as few as 1 in 25 replication events resulting in mutation to as high as (or even higher than) nearly every replication event resulting in mutation. Phage mutator strains also exist, that is, phages displaying mutations that increase (or decrease) their rate of mutation relative to that of wild type (Mansky and Cunningham, 2000). Alternatively, mutation rates of prophages can be lower than those associated with productive infections (discussed in Abedon and LeJeune, 2005).

A facile but not terribly satisfactory explanation for why differences in mutation rates between phages should occur is biochemical, that is, that DNA polymerases, with their proofreading ability, can display higher fidelities than RNA polymerases (Drake *et al.*, 1998), or that single-stranded molecules are inherently less stable (Duffy *et al.*, 2008) (more generally, one can speak of conflicts between the metabolic costs of reducing mutation rates versus the genetic costs of increasing mutation rates; Drake *et al.*, 1998). Alternative considerations are more ecological, that is, that phages employ higher mutation rates as an adaptation that otherwise is less efficaciously exploited by dsDNA phages or that phages with higher mutation rates can more readily compensate for higher per-genome mutation rates such as by also displaying very large burst sizes (see also arguments that viruses with higher mutation rates may also have phenotypes that are less sensitive to mutational change than the phenotypes of organisms with lower mutation rates; Duffy *et al.*, 2008; Elena and Sanjuan, 2005).

TABLE 1.1 Phage mutation rates

Family	Phage	Genome	Size (kb)[a]	Rate per nucleotide[b] ($\times 10^{-8}$)	Rate per genome per replication	Minimum population size for full coverage[c]
Myoviridae	T4	dsDNA	170	2.0	0.0034	1.5×10^8
Myoviridae	T2	dsDNA	170[d]	2.7	0.0046	1.1×10^8
Siphoviridae	λ	dsDNA	48.5	7.7	0.0037	3.9×10^7
Cystoviridae	ϕ6	dsRNA	13.4	270	0.0362	1.1×10^6
				970[e]	0.13	3.1×10^5
Inoviridae	M13	ssDNA	6.4	72	0.0046	4.2×10^6
Microviridae	ϕX174	ssDNA	5.4	740	0.040	4.1×10^5
Lentiviridae	Qβ	ssRNA	4.2	35000[f]	1.5	8.6×10^3

[a] Derived from www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=10239&type=6&name=Phages.
[b] See Duffy and Turner (2008) for references.
[c] This is the minimum population size necessary to achieve full coverage of possible point mutations and is calculated by multiplying the inverse of the per-genome, per-replication mutation rate by the genome size by 3 (with the latter specifying all possible transversion and transition mutations). The actual population size necessary to achieve all possible mutations must be greater given combinations of stochasticity of mutations and lack of uniformity in mutation rates both for a given nucleotide position and toward a given change (Drake, 2007).
[d] Estimated.
[e] Second value is as presented by Drake and Holland (1999).
[f] This is the lower-end value presented by Duffy and Turner (2008).

The smaller genome sizes associated with phages displaying higher per-genome mutations rates are consistent with virions being relatively inexpensive to produce (i.e., they are small in size, have short genomes, and only half as many nucleotides per base since their genomes are single-stranded). Low production expense is consistent with producing larger burst sizes, especially if nutrients are limiting, as is also an argument that lower fidelity polymerases are able to polymerize at a faster per-nucleotide rate (Duffy *et al.*, 2008; Elena and Sanjuan, 2005) (see, however, Drake *et al.*, 1998). Indeed, a standard rule of thumb holds that in general one cannot simultaneously maximize speed, economy, and fidelity, so it is not an unreasonable default hypothesis to assume that replication fidelity may very well have been sacrificed to achieve both greater replication speed and economy.

Another perspective is that having a relatively small number of genes could allow phages to better take advantage of a higher per-genome mutation rate if mutations in only a fraction of an organism's genes (or, more generally, of an organism's nucleotides) tend to be adaptive at any one time. For example, nucleotides responsible for host range determination during phage adsorption are potentially similar in number going from phage ϕX174 to phage T4—such as consisting of only one or a few point mutations, for example, as seen with phage ϕ6 (Duffy *et al.*, 2007), although other than point mutations can be involved, for example, as seen with T2-like phages (Hashemolhosseini *et al.*, 1994). However, phage ϕX174 has $>$25-fold fewer nucleotides in its genome in comparison with phage T4. A single randomly occurring mutation consequently could be much more likely to result, for example, in adaptive host range change with phage ϕX174 than with phage T4. An additional argument is based on per-genome cost (and hence relative metabolic costs) of encoding mutation-reducing modifiers, where costs presumably would be relatively higher given smaller genome sizes (Drake *et al.*, 1998).

A phage population that is large will have a higher potential to contain beneficial mutations than a phage population that is small. This growth to higher population densities also can be ''purifying'' in the sense that it will be especially those phages that display the fewest detrimental mutations that will tend to come to dominate the population (i.e., as a consequence of natural selection; Elena and Sanjuan, 2005; Hahn *et al.*, 2002). Larger population sizes also can exacerbate tendencies toward clonal interference (Pepin and Wichman, 2008), where multiple beneficial alleles within finite populations mutually compete rather than recombine into the same individual. High per-nucleotide mutation rates, however, may alternatively help to overcome clonal interference if novel mutation combinations can be created by successive mutation rather than relying on recombination (Bollback and Huelsenbeck, 2007).

D. Genetic drift

Deleterious mutations will be efficiently lost from populations, as a consequence of natural selection, only if those populations are sufficiently large. Indeed, the lower the impact that a deleterious allele has on the fitness of individuals then the larger the population that is required for selection to efficiently remove the deleterious allele (Simpson, 1967). Taking the same idea but in the opposite direction, even somewhat deleterious alleles can persist within populations if those populations are small enough, potentially resulting in those alleles randomly drifting to fixation. Less obvious, though essentially identical to the idea of fixation of deleterious alleles, is that small population sizes will tend to facilitate the elimination of beneficial alleles. This does not occur in a directed way, but instead as a consequence of random effects, in this case due to sampling error overwhelming natural selection. These phenomena are termed genetic drift and the result is a potential for loss of even wild-type alleles from very small phage populations (Duffy and Turner, 2008). Indeed, in practical, experimental terms, even phage plaque purification can have the consequence of causing lineages to accumulate detrimental alleles, especially given high mutation rates (Chao, 1990).

Genetic drift probably plays important roles in evolution by providing a stochastic countering force to the deterministic evolution effected by natural selection. It may allow especially ''maladapted'' lineages to explore potentially taller ''adaptive peaks'' (i.e., as found on metaphorical adaptive landscapes; Bull, 2008; Duffy and Turner, 2008). Furthermore, phages, particularly phage ϕ6 (Poon and Chao, 2004, and references cited therein), have served as important experimental systems for exploring the basic evolutionary implications of genetic drift on organismal evolution and the perhaps-resulting evolution of sexual interactions (Duffy and Turner, 2008).

E. Migration (Introgression)

In migration, alleles move from one population to another. This migration may or may not involve physical displacement and, in a Hardy–Weinberg sense, need only be a genetic rather than a physical (or spatial) phenomenon. This distinction can be confusing and thus it is important to realize that my emphasis in this chapter is on the genetics of migration rather than on the physical displacement component. Most other phage considerations of migration, often by experimental necessity, highlight physical displacement (e.g., see last paragraph, this section).

Migrating alleles may already be found in the recipient population or they may be novel to that population. Within the multicellular species in which migration is typically considered, this migration occurs over spatial distances since two populations between which genes can readily

pass would typically have to be physically separated in order to avoid devolving, essentially, into a single population. Alternatively, and potentially more applicable to phages, is the concept of introgression, where alleles pass between otherwise sympatric (geographically overlapping) populations at a low rate (Dowling and Secor, 1997): ''By introgressive hybridization[,] elements of an entirely foreign genetic adaptive system can be carried over into a previously stabilized one, permitting the rapid reshuffling of varying adaptations and complex modifier systems. Natural selection is presented not with one or two new alleles but with segregating blocks of genic [*sic*] material belonging to entirely different adaptive systems'' (Anderson and Stebbins, 1954, pp. 378–379). Introgression may occur, for example, at a hybridization zone found between the ranges of two similar but mostly reproductively isolated species such as during coinfection of a single bacterium found at overlapping host-range limits of two otherwise reproductively isolated and heterologous phages.

Alternatively, introgression between phages might occur as a consequence of phage DNA incorporation into bacteria which is then passed to other bacteria before being picked up by a singly infecting phage (i.e., phage horizontal gene transfer to a bacterium followed by bacterium horizontal gene transfer to a phage). Further, once we incorporate diverse routes of movement of DNA (e.g., see Fig. 1.4), or phages accidentally entering ecosystems in which they otherwise are poorly equipped to compete (Sano *et al.*, 2004) (but still able to infect at least at some low level), then it may be that introgression plays a dramatic role in phage evolution, perhaps equivalent, literally, to the musings of ''All the world's a phage'' by Hendrix *et al.* (1999, 2002; Section VI).

Phage migration, especially in a physical or spatial sense, also may be analyzed in terms of biogeography (Abedon *et al.*, 2009; Breitbart and Rohwer, 2005; Breitbart *et al.*, 2004; Casas and Rohwer, 2007; Desnues *et al.*, 2008), carriage by animals (Dennehy *et al.*, 2006; Sisler, 1940), movement within aerosols (e.g., Tanner *et al.*, 2005), experimental transfer between environments (Sano *et al.*, 2004), employment as environmental tracers (e.g., Rheinbaben *et al.*, 2000; Urban and Broce, 2000; Vidales-Contreras *et al.*, 2006), movement into as well as out of biofilms (e.g., Skraber *et al.*, 2005, 2007), source tracking of fecal contamination (e.g., Stapleton *et al.*, 2007), and experimental evolution studies (Forde *et al.*, 2007; Kerr *et al.*, 2006, 2008; Morgan *et al.*, 2005; Wilke, 2004).

F. Recombination

Recombination can be differentiated into genetic, molecular, homologous, nonhomologous (or illegitimate), heterologous, and site-specific forms (e.g., Casjens *et al.*, 1992; Duffy and Turner, 2008; Lawrence *et al.*, 2002). Genetic recombination is the more general term, consisting of either

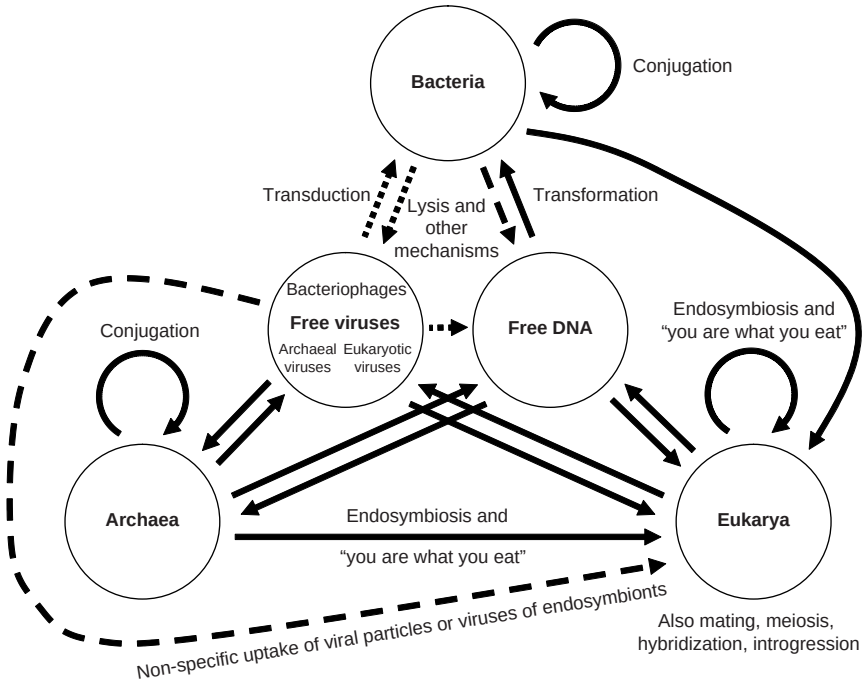

FIGURE 1.4 Pathways of horizontal gene transfer especially between cellular organisms. Phage-dominated processes are indicted with dotted arrows. Phage-influenced processes are indicated with dashed arrows. Free viruses consist of three more-or-less distinct pools, which are further subdivided according to individual host ranges. Free DNA pools are less distinct though the competence (for transformation) of certain bacteria are biased toward uptake of DNA of certain types (especially that of their own species). In addition, the integration, utility, and evolutionary fixation of acquired DNA likely is biased (Lawrence and Hendrickson, 2003). Acquisition of DNA from domains Archaea or Eukarya by phages likely occurs mostly following availability rendered by bacterial transformation events. For references of at least some of these pathways see (Doolittle, 1998; Hotopp *et al.*, 2007; Jordan and Koonin, 2004; Merril, 2008; Prangishvili *et al.*, 1998).

molecular recombination or reassortment. Most molecular recombination is homologous, that is, that occurring between nucleotide sequences which are similar (or even identical). Nonhomologous (or illegitimate) recombination requires little or (essentially by definition) no homology between recombining sequences (Casjens *et al.*, 1992 and Campbell, 2000, by contrast, distinguish between homologous, micro-homologous, illegitimate, and site-specific recombination). Heterologous recombination is asymmetric in the sense that gains or losses in total nucleotides occur. Site-specific recombination is employed by temperate phages to integrate into the bacterial genome (Campbell, 2006, 2007).

Homologous recombination shuffles existing combinations of genetic loci, potentially resulting in the generation of novel combinations of alleles. Nonhomologous recombination can result in novel combinations of genetic loci that otherwise would not be found together within the same gene pool, including the recombining of bacterial genetic material into phage genomes (and *vice versa*). The segmented genome of phage ϕ6 and related cystoviruses can reassort, giving rise to a genetic recombination between individual segments rather than between individual genes or individual nucleotides (Mindich, 2006; Silander *et al.*, 2005). The most striking evolutionary consequence of genetic recombination, in association with introgression, is its potential to give rise to mosaic genomes (Campbell, 1988; Hendrix, 2003) as seen especially among tailed phages (Casjens *et al.*, 1992; Hendrix, 2002, 2008; Hendrix and Casjens, 2008; Hendrix *et al.*, 2003; Lawrence *et al.*, 2002), that is, genomes in which different genes have different evolutionary histories (Section VI).

V. PHAGE EVOLUTIONARY ECOLOGY

All evolution occurs within the context of ecological interactions while ecological interactions are constrained by organismal evolutionary histories. One approach toward studying these evolutionary-ecological interactions is in terms of evolutionary adaptations, which is the province of the field of evolutionary ecology. Here I consider ''rules of thumb'' of some basic aspects of phage evolutionary adaptation, as viewed especially from a perspective of phage life history evolution. For additional considerations of phage evolutionary ecology, see Bull *et al.*, (2004b), Breitbart *et al.*, (2005), Abedon (2006, 2008d), Bull (2008), Kerr *et al.*, (2008), Turner and Duffy (2008), and Abedon *et al.*, (2009).

A. Life history evolution

Adaptations can be studied, in a manner that is comparable across the diversity of life, within the context of life history characters. These are descriptions of organism survivorship, age of reproduction, number of lifetime reproductive episodes, number of progeny produced per reproductive episode, etc. (Stearns and Krebs, 1992). One typically asks why, from the perspective of evolutionary biology, do organisms display the growth, survival, and reproductive strategies that they do.

For phages, as relatively simple organisms, life history characters may be differentiated into those associated with adsorption (a prereproductive portion of the phage life cycle), infection (which encompasses the reproductive portion of the phage life cycle as well as some of the prereproductive

portion), or total number of progeny produced (which is a phage's fecundity or clutch size). That is, the majority of phage adaptations, and therefore the majority of molecular details associated with the phage life cycle, may be conveniently differentiated, phenotypically, in terms of their impact on these three characters. A fourth character might be described as an extended phenotype (Dawkins, 1982; Lambrechts *et al.*, 2006), which arguably is represented in phages by the environment-modifying role displayed by at least some of the exotoxins they can encode (Abedon and LeJeune, 2005; Hyman and Abedon, 2008a) (see perhaps also Hendrix, 2003).

The impact of phages on their environments (their ecology) are, almost exclusively, byproducts of phage attempts to adsorb bacteria (including their movement as free phages), their infection of these cells, and their production (and release) of phage progeny (Abedon, 2009c). Environments, in turn, can impact phage molecular functions, resulting in what can be described as an organism's phenotypic plasticity (=variation in phenotype as a function of environmental variables), and much of this plasticity in phages can be assessed in terms of changes in life history characters. For example, phage burst sizes may decline in response to nutrient deficiencies (Hadas *et al.*, 1997) or phages may be unable to adsorb bacteria in the absence of sufficient quantities of environmental adsorption cofactors (Hyman and Abedon, 2009a). Through natural selection, environments also impact phage molecular adaptations and thereby phage life history characters. The resulting interplay between adaptations and environments, highlighting the centrality of phage life history characters, is presented in Fig. 1.5. Additional, complementary discussion of phage life history evolution, constraints, and evolutionary optimization can be found elsewhere (Abedon, 2006, 2008d; Abedon and Culler, 2007a, b; Breitbart *et al.*, 2005; Bull *et al.*, 2004a, 2006; de Paepe and Taddei, 2006; Heineman and Bull, 2007; Kerr *et al.*, 2008; Villarreal, 2005; Weinbauer, 2004; Weinbauer *et al.*, 2007; You and Yin, 2006).

B. Adsorption

To a first approximation we can expect evolution to favor phages that adsorb to suitable hosts as rapidly as possible. Factors impacting this rapidity include (1) the rate of phage encounter with bacteria—which is a function of (i) phage diffusion rates, (ii) phage (and bacterial) movement other than by diffusion, and (iii) the density of phage-susceptible bacteria within an environment—and (2) the rate as well as likelihood of infection given phage-bacterial encounter. If adsorption rates are currently less than maximal then phage rapidity of adsorption might increase by switching to a more prevalent bacterial receptor (or receptors) or adherence to its receptor with greater affinity, resulting in a lower likelihood of

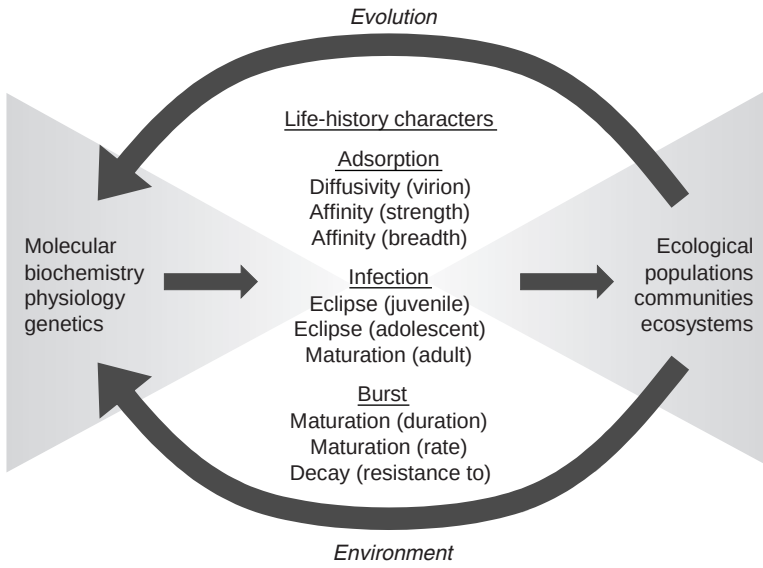

FIGURE 1.5 Bacteriophage life history characters. Flowing from left to right, molecular aspects underlie a limited number of life history characters which, in turn, underlie phage interactions with their environments (ecology; in addition to adsorption, infection, and burst, see text for discussion of a possible fourth category of phage life history characters: extended phenotypes). At the same time as molecular aspects underlie life history characters, environments feedback on molecular aspects both evolutionarily (especially via natural selection but also as a consequence of environmental mutagens) and by nonevolutionary phenotype modification effected by the environment (e.g., burst size reductions given low-nutrient conditions; Hadas *et al.*, 1997) (in both cases these are represented by arrows flowing from right to left). "Affinity" refers to phage adsorption affinity to bacteria (i.e., strength of affinity to specific bacteria or, alternatively, breadth of host range as determined by virion adsorption affinity to a variety of bacteria). "Infection" is differentiated into three stages which, analogous to the human life cycle, I describe as a prerecombinative "juvenile" stage (traditionally a part of the eclipse period), a sexual but preadult (and therefore "adolescent") stage (also traditionally a part of the eclipse period), and, after Kerr *et al.* (2008), an "adult" stage during which phage-progeny maturation takes place. "Burst," a product of the rate and duration of the "adult" stage of phage-progeny maturation, is presented in terms of an "effective" burst size which is a function not just of the number of phage progeny produced but also of their reproductive survival (more generally this may be described as a reproductive ratio; Villarreal, 2005).

detachment following encounter. Phage evolution presumably also selects for phages that more rapidly transition from reversibly adsorbed to fully delivering their genome into the bacterial cytoplasm (infection).

It is uncertain, by contrast, what the overall impact of phage host range—as determined by phage adsorption ability—might be on phage

adsorption rapidity. On the one hand, having a broader host range could result in faster phage adsorption within a given environment due to greater host availability. On the other hand, it is conceivable that evolving such ''generalism'' (Elena and Lenski, 2003; Weinbauer, 2004) could adversely impact phage adsorption rates to specific hosts. Consistently, a specialist phage, with a narrower host range, might obtain a benefit by evolving more effective (e.g., faster) phage-adsorption abilities to (or, more generally, higher fitness on) specific hosts, though to fewer potential hosts overall (Duffy and Turner, 2008; Poullain *et al.*, 2008). This is an argument for the evolution of specialization as a consequence of antagonistic pleiotropy—that mutations which improve performance on one host (the preferred host), may simultaneously decrease performance on other hosts (Duffy *et al.*, 2006; Heineman *et al.*, 2008). This kind of antagonistic pleiotropy explains how live attenuated vaccines can be developed via passage of pathogens through alternative species and/or tissue culture.

An alternative view is that phage evolution could result in reduced adsorption rates under conditions where host adsorption alone is not the primary or at least the sole ecological ''objective.'' This lack of primacy of maximizing adsorption could be for reasons of overall infection avoidance, long-term survivability, or discrimination against adsorption to certain bacterial types. For example, some enteric phages (e.g., phage T4) can exist in an adsorption-capable state while within the colon versus an adsorption-resistant state while within the extracolonic environment. The latter state could represent either an avoidance of physiologically unsuitable hosts or a bid towards increased survivability as virions (Conley and Wood, 1975; Kutter *et al.*, 1994a), which is a plasticity in behavior consistent with many considerations of optimal foraging theory (Heineman *et al.*, 2008). Alternatively, it is conceivable that phage attempts to narrow their adsorbable range, such as to avoid adsorbing to various kinds of debris (Daniels and Wais, 1998) or to a certain sub-optimal hosts (Bull, 2006; Heineman *et al.*, 2008), could result in reduced rates of adsorption to preferred infection targets. These latter arguments essentially are descriptions of the evolution of specialization as may be viewed in terms of a less-flexible optimal foraging strategy. That is, mutations decrease affinity for suboptimal hosts or materials but with a pleiotropic consequence of decreased affinity for the preferred host (Heineman *et al.*, 2008).

C. Infection, prereproductive period

The eclipse period is prereproductive portion of the phage latent period, that is, which occurs prior to the maturation of the first phage virion (Abedon, 2008c; Doermann, 1952). Since the eclipse period is a portion of the overall phage latent period, any shortening should result in faster phage population growth, whether in environments that are well mixed

(Stopar and Abedon, 2008) or poorly mixed (Abedon and Culler, 2007a). Thus, for example, it may be that an observed eclipse period has already evolved to be as short as possible or that a phage, in the course of adapting to a new host or conditions, may evolve toward a shorter eclipse (Heineman and Bull, 2007).

There may exist situations in which evolution either favors or does not actively select against longer prereproductive periods. For example, low nutrient densities may favor avoidance of virion production, such as during pseudolysogeny* (Abedon, 2009b), which essentially is an extended prereproductive infection stage that occurs in response to host starvation (Miller and Day, 2008). Thus, phages may avoid producing phage progeny under circumstance where hosts are least physiologically equipped to support a fecund phage infection (see Pedulla *et al.*, 2003, for speculation on an alternative phage approach towards dealing with dormant hosts).

Like pseudolysogeny*, lysogeny probably occurs as an adaptation to conditions that are less supportive of immediate virion-mediated phage population growth (Abedon, 2008b; Miller and Day, 2008; Stewart and Levin, 1984). With lysogeny a phage is able to delay virion maturation essentially indefinitely and therefore until a time, especially, when phage population growth is more effectively accomplished via productive infection rather than lysogen growth (Abedon, 2008b). Note that while pseudolysogeny* is clearly an extension of the prereproductive state, lysogeny actually represents more of an extended reproduction period (though not maturation period) since prophages replicate along with their host bacteria.

D. Infection, reproductive period

With longer reproductive periods in the course of extended phage latent periods come larger burst sizes, though at the expense of longer phage generation times. Given this tradeoff, there are a number of different ways that one can describe how and why the duration of phage latent periods may evolve, especially in response to host availability. The basic prediction (Abedon, 1989; Abedon *et al.*, 2001; Heineman and Bull, 2007; Wang *et al.*, 1996) and observation (Abedon *et al.*, 2003; Heineman and Bull, 2007) is that shorter optimal latent periods are adaptations to higher bacteria availability, whereas longer latent periods should evolve in response to lower bacteria availability. Alternative but otherwise compatible ways of describing why this should be include: (1) When bacteria are rarer, then an adsorbed bacterium is more valuable, resulting in greater payoffs associated with larger burst sizes, despite the costs associated with latent period extension (Abedon, 2006; Abedon *et al.*, 2003); (2) when bacteria are common, then the phage latent period makes up a much larger proportion of the phage generation time resulting in greater payoffs associated with shorter latent periods which thereby offset the

associated burst size decrease (Abedon, 2008d); (3) when search distance for a resource is greater, then it pays to continue exploiting that resource for longer durations before embarking on a search for new resource (which is a statement of what is otherwise known as the marginal value theorem; Wang *et al.*, 1996); and (4) a longer preadult stage (dissemination plus eclipse) should select for a longer adult stage (Abedon, 2006). Abedon and Culler (2007a,b) consider phage latent-period evolution in plaques.

We can extend these ideas of tradeoffs between phage generation time and burst size beyond the simple lytic infections considered above. One example is the lysis-inhibition phenotype displayed by T-even phages such as phage T4—a latent period extension and burst size increase that together are induced by the adsorption of T-even phages to already infected bacteria. Lysis inhibition can be viewed, essentially, as a conditional lengthening of the phage maturation period (Abedon, 1990, 2008d). The resulting burst size increase should be especially beneficial when host densities are low, and lysis inhibition is induced in phages precisely when densities of uninfected phage-susceptible bacteria would be expected to be in decline, that is, following multiple phage adsorption to individual bacteria (Abedon, 1990).

Similarly, temperate phage reduction to lysogenic cycles, as conditionally occurs upon infection, can also be a response to higher phage multiplicities (see references cited in Abedon, 2008d). Lysogeny itself has been explicitly described as a phage adaptation to conditions in which phage virions are a less effective growth and survival strategy relative to ongoing infection of a bacterium (Abedon, 2008b; Miller and Day, 2008; Stewart and Levin, 1984). Thus, the extended latent period of lysogeny would appear to have some utility even without a simultaneous burst size enhancement. For lysogeny, nevertheless, the latent period extension does likely result in burst enhancement. This occurs because lysogens replicate, via binary fission, and thereby potentially increase, per each phage adsorption, the number of bacteria that end up being phage infected. Each of these lysogens can potentially produce a phage burst following induction. Thus, again, there exists a tradeoff between infection period duration (here, time until induction) and virion yield per bacterium infected (this time in the form of burst number). Pseudolysogeny*, too, has been explicitly described as a means by which phages delay lysis in order to at least potentially increase their subsequent burst size (Miller and Day, 2008).

E. Burst

It is difficult to imagine a situation where a greater burst size, without tradeoff, could be costly to an individual displaying phage. Therefore, it seems reasonable to assume that selection generally would favor faster

virion maturation. For example, the attainment of more-rapid phage production (and/or shorter eclipse periods) may be attained via phage encoding of tRNA genes which are supportive of their own codon biases (Bailly-Bechet *et al.*, 2007; Hacker *et al.*, 1999). Phage ''physiological ecology'' is one means of describing the general study of such phage-host interactions (Breitbart *et al.*, 2005) as well as such things as the variance in phage life history characteristics as a consequence of environmental variation (e.g., Hadas *et al.*, 1997). Alternatively, greater virion size, or sophistication (de Paepe and Taddei, 2006), could come at a burst-size cost while rapidity of virion maturation could conflict with the ongoing maintenance of a phage infection, a tradeoff which may be especially relevant with chronic phage release.

VI. PHAGE GENOME EVOLUTION

Full-genome sequencing has allowed unambiguous determinations of degrees of phage sequence homology and thereby presumptive evolutionary relatedness. In this Section I consider phage genome evolution and relatedness from a variety of evolutionary as well as ecological perspectives.

A. Horizontal gene transfer

Horizontal gene transfer (HGT, a.k.a., lateral gene transfer) is the movement of DNA (or RNA for RNA-based viruses) from one lineage to another and can also be the result homologous recombination involving only a subset of genes that happen to be shared among coinfecting viruses. The likelihood of such exchange declines with reduced genetic homology until it is only via comparatively rare illegitimate (or microhomologous) recombination processes that novel genetic material may be introduced into phage genomes (Campbell, 1988; Hendrix, 2008). Upon arrival, as with any changes in nucleotide sequence, newly formed combinations of genes are subject to natural selection (see, e.g., ''zone of paralogy'', as applied to HGT in bacteria; Lawrence and Hendrickson, 2003). The pathways of HGT as they may occur among all organisms are summarized in Fig. 1.4.

An alternative view of horizontal gene exchange is that which occurs between bacteria as mediated by phages, that is, transduction. Such movement can occur in various ways that differ in the degree to which the carried DNA interferes with subsequent phage functioning—that is, successful bacterial infection and production of phage progeny. This interference is a consequence of the degree to which essential phage genes are displaced to make room for new DNA, to what degree phage genes

are disrupted by recombinational processes, and to what extent the products of these genes affect phage functioning. Since not all of these effects are necessarily negative, we can describe the phage products variously as inviable (i.e., unable to initiate a new infection), viable but with reduced fitness, viable with unmodified fitness, or, even, viable with enhanced fitness. Otherwise intact phages which have lost gene functions as a consequence of carrying host DNA may be propagated, in the laboratory, by coinfecting with what are known as helper phages (Weisberg, 1996).

Looking at actual transduction-related phenomena, at one end of a spectrum is lysogenic conversion where a nondefective phage carries lysogen-expressed genes that otherwise are integrated components of the phage genome (Hendrix and Casjens, 2008). The expression of these genes presumably contributes to lysogen and thereby prophage fitness, and in some cases perhaps even contributes to phage fitness independent of the lysogenic cycle (Abedon and LeJeune, 2005). Next along the spectrum of phage functionality lie the so-called morons. Morons are various genes that phages have picked up, presumably from bacteria via nonhomologous recombination, which, especially prior to selection, may or may not contribute positively to phage fitness (Hendrix, 2008; Hendrix *et al.*, 2000, 2003) (see also Bailly-Bechet *et al.*, 2007 and Campbell, 1988). Specialized transduction comes next (Weisberg, 1996), where specific bacterial genes integrate into the phage genome as a consequence of imprecise genome excision following prophage induction. The inclusion of this extra genetic material can result in phage inviability upon subsequent infection, especially when larger amounts of bacterial DNA are carried. This DNA is also limited in its spectrum to that flanking inserted prophage. Alternatively, ''the term 'specialized transduction' has broadened to encompass any high-efficiency virus-mediated replication and packaging of a nonviral gene no matter how the nonviral gene is incorporated into a virus chromosome'' (Weisberg, 1996, p. 2442). By this latter definition, bacterial genes moved by specialized transduction versus as phage morons presumably could be similar if not identical phenomena. Finally, generalized transduction is the accidental packaging of exclusively bacterial or plasmid DNA into a phage capsid. Phage genetic viability in this latter case is completely lost. Furthermore, the amount of bacterial DNA that can be transferred is equal (approximately) to the normal size of the phage genome (Masters, 1996).

Note that morons in a sense represent an intermediate state between generalized and specialized transduction (the latter *sensu stricto*). On the one hand, as with DNA subject to specialized transduction, morons are relatively small fragments that, once acquired, are flanked by significant amounts of phage DNA (though, alternatively, as also with specialized transduction, acquired morons can presumably be larger though this largeness could be at the expense of phage fitness). On the other hand,

what DNA is included in a phage genome as a moron, upon acquisition, presumably is less biased as to where it comes from in the bacterial genome than with specialized transduction and instead may better resemble the less-biased genome packaging seen with generalized transduction. However, in moron acquisition, as also seen with generalized transduction (Masters, 1996), there presumably are biases such as, in the case of morons, towards material that possesses at least some sequence homology with phage DNA. Given these ideas, one can envisage a continuum where at one end lies generalized transduction which does not involve recombination with phage DNA and is relatively unbiased in what DNA is acquired while at the other end is specialized transduction which does require recombination with phage DNA (even if only site-specific) and is highly biased in terms of what DNA is included. With morons there are perhaps fewer biases than with specialized transduction in terms of what genes are acquired, but still a requirement for recombination. However, as with generalized transduction, there is less dependence on lysogen formation for moron acquisition versus the absolute dependence seen with specialized transduction (Paul and Jiang, 2001). How morons fit into these various ''spectra'' is illustrated in Fig. 1.6.

B. Genomics and mosaicism

The first genomes sequenced were those of phages MS2 (Fiers *et al.*, 1976) and ϕX174 (Sanger *et al.*, 1977). In all, more than thirty phage full genomic sequences were available (Table 1.2) prior to the publication of the first bacterial (and cellular) full genome sequence, that of *Haemophilus influenzae* (Fleischmann *et al.*, 1995). Today, literally hundreds of phage isolates have been sequenced, including as prophages found within sequenced bacterial genomes (though presumably there exists a bias against the sequencing of prophages which exist instead as plasmids; Campbell, 2007). Through comparative genomics, it has become inescapably obvious that not only are phage genomes incredibly diverse (with individual phage isolates often possessing numerous unique regions; Rohwer, 2003), but that nonunique regions do not display consistent homology, across the entire genome, with other individual phage isolates. In other words, phages, especially tailed phages, are genetic mosaics with different genes possessing different evolutionary histories (Botstein, 1980; Brüssow and Desiere, 2006; Campbell, 1988; Hendrix, 2002, 2003, 2008; Hendrix and Casjens, 2006, 2008; Hendrix *et al.*, 2003; Lawrence *et al.*, 2002). Why is it that collective genomes of especially tailed phages are both highly diverse and apparently mosaic? Presumably this is a consequence of the interaction of numerous aspects of phage biology. These I list here in no particular order:

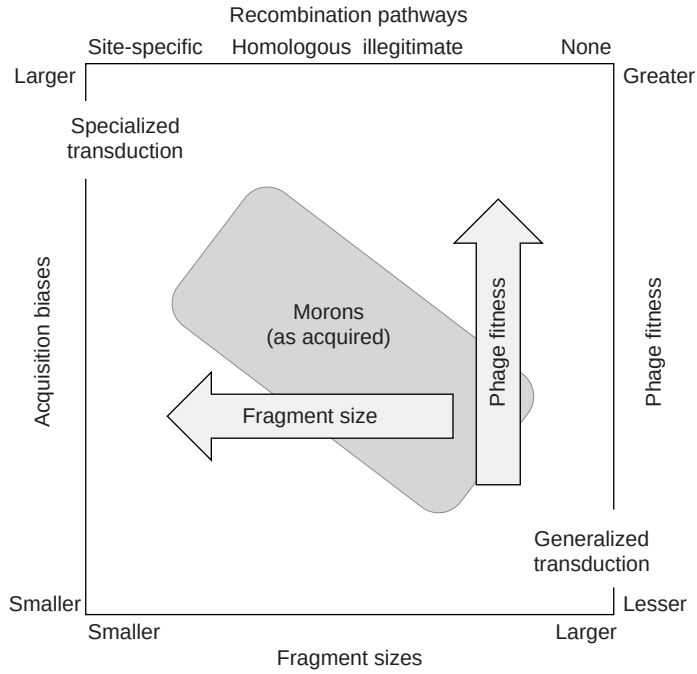

FIGURE 1.6 Bacterial transduction as viewed from a phage evolutionary ecological perspective. The "shadow" indicates moron acquisition biases versus recombination pathways whereas arrows indicate biases in selective retention. Note that if defined less strictly, such that it refers to any packaged product of recombination between phage and nonphage DNA (Weisberg, 1996), then specialized transduction would occupy all of the space present on the graph except for those areas occupied instead by generalized transduction.

(1) Phage per-nucleotide mutations rates are relatively high, which phages can tolerate due to their small genome sizes, but which otherwise means that individual genes are relatively likely to be mutated during a given round of replication (Table 1.1). (2) Phage population sizes, for example, those of individual clones, likely can be quite large, which should allow a fairly thorough exploration by phage populations of phage sequence space (Villarreal, 2005). (3) Limitations on phage diffusion and mixing within many environments may obstruct gene flow, resulting in large numbers of somewhat independently evolving and thereby potentially diverging phage subpopulations. (4) Phage generation times can be relatively short such that blocks to gene flow need not be especially long lasting to nevertheless allow for significant genetic diversification as a consequence of either genetic drift or selection. (5) Bacterial hosts are highly diverse, again resulting in a potential for significant

TABLE 1.2 Early fully sequenced phage genomes

Phage	Year[a]	Phage family	Host	Accession #	Reference
MS2	1976	*Leviviridae*	Enterobacteria	NC 001417	Fiers *et al.* (1976)
ϕX174	1977	*Microviridae*	Enterobacteria	NC 001422	Sanger *et al.* (1977)
fd	1978	*Inoviridae*	Enterobacteria	J02451	Beck *et al.* (1978)
G4	1978	*Microviridae*	Enterobacteria	NC 001420	Godson *et al.* (1978)
M13	1980	*Inoviridae*	Enterobacteria	NC 003287	van Wezenbeek *et al.* (1980)
f1	1981	*Inoviridae*	Enterobacteria	V00606	Beck and Zink (1981)
λ	1982	*Siphoviridae*	Enterobacteria	NC 001416	Sanger *et al.* (1982)
T7	1983	*Podoviridae*	Enterobacteria	NC 001604	Dunn and Studier (1983)
Ike	1985	*Inoviridae*	Enterobacteria	NC 002014	Peeters *et al.* (1985)
Pf3	1985	*Inoviridae*	*Pseudomonas*	NC 001418	Luiten *et al.* (1985)
S13	1985	*Microviridae*	Enterobacteria	M14428	Lau and Spencer (1985)
ϕ29	1986	*Podoviridae*	*Bacillus*	NC 011048	Vlcek and Paces (1986)
GA	1986	*Leviviridae*	Enterobacteria	NC 001426	Inokuchi *et al.* (1986)
PZA	1986	*Podoviridae*	*Bacillus*	M11813	Paces *et al.* (1986)
4	1987	*Microviridae*	*Spiroplasma*	NC 003438	Renaudin *et al.* (1987)
ϕ6	1988	*Cystoviridae*	*Pseudomonas*	NC 003714, 15, 16	Mindich *et al.* (1988)
SP (FI)	1988	*Leviviridae*	Enterobacteria	NC 004301	Inokuchi *et al.* (1988)
Chp1	1989	*Microviridae*	*Chlamydia*	NC 001741	Storey *et al.* (1989)
fr	1990	*Leviviridae*	Enterobacteria	NC 001333	Adhin *et al.* (1990)
I2–2	1990	*Inoviridae*	Enterobacteria	NC 001332	Stassen *et al.* (1992)
P4	1990	*Myoviridae*	Enterobacteria	NC 001609	Halling *et al.* (1990)
SpV1-R8A2 B	1990	*Inoviridae*	*Spiroplasma*	NC 001365	Renaudin *et al.* (1990)

(*continued*)

TABLE 1.2 (*continued*)

Phage	Year[a]	Phage family	Host	Accession #	Reference
Cf1 c	1991	*Inoviridae*	*Xanthomonas*	NC 001396	Kuo *et al.* (1991)
MV-L1	1991	*Inoviridae*	*Acholeplasma*	NC 001341	
Pf1	1991	*Inoviridae*	*Pseudomonas*	NC 001331	Hill *et al.* (1991)
PRD1	1991	*Tectiviridae*	Enterobacteria	NC 001421	Bamford *et al.* (1991)
α3	1992	*Microviridae*	Enterobacteria	NC 001330	Kodaira *et al.* (1992)
If1	1993	*Inoviridae*	Enterobacteria	NC 001954	
L2	1993	*Plasmaviridae*	*Acholeplasma*	NC 001447	Maniloff *et al.* (1994)
L5	1993	*Siphoviridae*	*Mycobacterium*	NC 001335	Hatfull and Sarkis (1993)
PP7	1994	*Leviviridae*	*Pseudomonas*	NC 001628	Olsthoorn *et al.* (1995)
T4	1994	*Myoviridae*	Enterobacteria	NC 000866	Kutter *et al.* (1994b)

[a] Dates are as published or, if unavailable, then as created on www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=10239&type = 6&name=Phages.

evolutionary divergence among phages, though in this case the mechanism of divergence would be explicitly due to natural selection (arguably representing coevolution; Weitz *et al.*, 2005) rather than due to genetic drift. (6) Stabilizing frequency dependent selection, too, may favor the retention of considerable genetic diversity at least at certain phage loci (Campbell, 1994; Campbell and Botstein, 1983). (7) Bacterial hosts are not so diverse that the potential for phages to display relatively broad host ranges, if only in terms of rare adsorption and subsequent DNA uptake (rather than successful infection), that recombination between different phage types infecting diverse bacterial hosts is relatively likely.

(8) Because of the dsDNA nature of most phages, they can readily recombine with and incorporate host DNA (see ''Morons,'' above and below), including prophage DNA (Campbell, 1988, 1994; Campbell and Botstein, 1983; Labrie and Moineau, 2007). (9) Prophages are very commonly found within bacteria (Hendrix, 2003) resulting in a potential for phage–phage recombination during a large fraction of lytic infections (Lawrence *et al.*, 2002), and even if inactive, prophage DNA should still be available for recombination, including illegitimate recombination (Campbell and Botstein, 1983; Casjens *et al.*, 1992). (10) Prophage genes presumably are relative free to explore sequence space without functional constraint since the survival of prophage sequence within a specific bacterial lineage is not dependent, at least over the short term, on its potential to produce functional virions (thus, for example, a mutation that would result in a defective phenotype could then be followed by a compensating mutation). (11) Homologous recombination during phage infection commonly occurs (Hendrix *et al.*, 2002), with some phages possessing recombination-promoting genes (Hendrix and Casjens, 2006) while for other phages recombination plays integral roles in the phage replication process (Mosig and Eiserling, 2006). (12) Temperate phages, if lysogenizing the same bacterium, can potentially recombine among themselves (Hendrix, 2003; Hendrix and Casjens, 2008; Ohnishi *et al.*, 2001).

(13) The relatively large size of especially tailed-phage genomes (i.e., many 10s or even 100s of kilobases) lowers barriers to incorporation of novel genes/DNA such as morons. (14) Certain nonessential, often deletable regions of phages can be ''less tightly packed'' with genes (Campbell and Botstein, 1983; Hendrix, 2002), perhaps either allowing greater flexibility in acquiring novel gene sequences via illegitimate recombination or reflecting a relatively recent acquisition of such genes. (15) Often seemingly unrelated phage genes possessing equivalent functions can be swapped, for example, lysozymes and integrases, a phenomenon known as orthologous replacement (Casjens *et al.*, 1992; Hendrix *et al.*, 2003). (16) Even entire regions, making up substantial fractions of the entire phage genome, may in some cases be orthologously swapped and gene clustering into potentially coevolved modules increases the

potential for region utility within recipient phages following these swaps (Campbell and Botstein, 1983; Casjens *et al.*, 1992; Hendrix *et al.*, 2003). (17) Consistent gene orders among related phages may increase likelihoods of viability among recombinants even if genes themselves do not display close sequence homology (Campbell and Botstein, 1983; Casjens *et al.*, 1992); this may explain why phage gene orders persist, though an additional, complimentary explanation is that retaining gene order retains patterns of regulation of gene expression (Campbell, 1994; Springman *et al.*, 2005).

(18) Bacteria are not very morphologically complex plus often live in wet environments, which together may reduce barriers to effective phage transmission, that is, transmission can be mostly limited to diffusion across aqueous gaps found between individual cells, which could allow for greater phage evolutionary experimentation in the presence of fewer adaptive constraints (e.g., as compared with terrestrial-animal viruses which must disseminate within immunologically hostile bodies or span otherwise desiccating gaps between hosts). (19) A major means of bacterial protection from phage attack, restriction endonucleases, may actually contribute to the phage potential to recombine (Kobayashi, 1998; Milkman, 2004). (20) The vast numbers of phages and phage infections allows even extremely rare events to occur in significant absolute numbers (Hendrix, 2002, 2008). And (21), all of the above has been going on potentially for billions of years. The consequence is a combination of exceptional genetic diversity among phages, a large potential for recombination between phages as well as with other genetic entities, and the occurrence of large absolute numbers of even statistically rare viable recombinants.

C. "All the world's a phage"

Phage genomes seem to fall into a relatively small number of types, based upon genetic similarities (Rohwer and Edwards, 2002), and even when sequence similarities are negligible, gene orders can be remarkably consistent (Casjens *et al.*, 1992; Hendrix *et al.*, 2002). The consistency observed among phage genomes, as found within the seeming chaos of their sequence diversity, is suggestive of unknown constraints on phage evolution, significant (and constraining) gene exchange even between ostensibly reproductively isolated lineages, or both. Hendrix and colleagues champion the latter explanation, that in fact all the world's tailed phages, in particular, are more closely evolutionarily related than their presumptive billions of years of divergence should warrant; in other words, as they have pithily put it, ''All the world's a phage'' (Hendrix *et al.*, 1999, 2002).

The basics of the Hendrix *et al.* model, as applied especially to tailed phages, is that phage genomes are mosaic, that is, that disparate phages or at least their parts often are more closely related to each other than are their

host bacteria. Furthermore, the total tailed-phage gene pool may approach the size of the entirety of tailed phages but nevertheless no individual phage genotype likely has direct access to the entire global gene pool. They argue, for example, that relatively recent phylogenic connections exist between phages infecting the genera *Escherichia* (Gram negative), *Streptomyces* (Gram positive), and *Mycobacterium* (Acid fast) (see also Campbell and Botstein, 1983). Ultimately they ''suggest on the basis of the sequence similarities that many and probably most of the genes of contemporary phages derive from a common ancestral pool of genes'' (Hendrix *et al.*, 2002, p. 137). However, the paths connecting phages of distantly related bacteria likely are multi-stepped, with each step consisting of recombination between phages whose host ranges only partially overlap. It would be an intriguing ecological challenge to confirm the existence of such chains of host-range overlap, including especially the occurrence of presumably low-likelihood infection at host-range extremes. Alternatively, it could be that transformation of bacteria with prophage genes released as free DNA, from either bacteria or decaying virions into the extracellular (and extra-phage) environment, provides the evolutionary bridge between phages infecting otherwise disparate bacterial taxa (i.e., see Fig. 1.4).

VII. CONCLUDING REMARKS

We exist in an exciting time for the study of phage ecology and evolutionary biology, as well as for phage biology in general: The insights gleaned from comparative genomics have profoundly altered our understanding of how microorganisms evolve; experimentalists and modelers have provided intriguing insights into how phage ecology and evolutionary biology ''works'' (or ''should'' work) under well-controlled conditions; and environmental observations of phages, including especially using genomic and metagenomic techniques, have provided an unprecedented window on phage diversity in the wild. The challenge now before us is an integration of our laboratory and environmental knowledge of phage biology with well-considered principles of ecology and evolutionary biology as they apply to organisms in general. The payoff will be not just a better understanding of phage biology in particular but also a more robust understanding of ecology and evolutionary biology in general.

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CHAPTER 2

Nucleoid-Associated Proteins and Bacterial Physiology

Charles J. Dorman

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Abstract

Bacterial physiology is enjoying a renaissance in the postgenomic era as investigators struggle to interpret the wealth of new data that has emerged and continues to emerge from genome sequencing projects and from analyses of bacterial gene regulation patterns using whole-genome methods at the transcriptional and posttranscriptional levels. Information from model organisms such as the Gram-negative bacterium *Escherichia coli* is proving to be invaluable in providing points of reference for such studies. An important feature of this work concerns the nature of global mechanisms of gene regulation where a relatively small number of regulatory proteins affect the expression of scores of genes simultaneously. The nucleoid-associated proteins, especially Factor for Inversion Stimulation (Fis), IHF, H-NS, HU, and Lrp, represent a prominent

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group of global regulators and studies of these proteins and their roles in bacterial physiology are providing new insights into how the bacterium governs gene expression in ways that maximize its competitive advantage.

I. INTRODUCTION

For many years, studies of nucleoid-associated proteins (NAPs) and bacterial physiology rarely intersected. The former were pursued by investigators concerned with the physical organization of the genetic material in the bacterium and the latter was studied by those interested in the operation of the internal economy of the cell. Connections were discovered when mutations in the genes coding for NAPs were found to alter the expression of genes that contribute in obvious ways to metabolic processes. For example, the functioning of the promoters of the genes that express the tRNA and rRNA molecules that are essential for the process of translation is modified by the NAPs, Fis, H-NS, and Lrp (Hillebrand *et al.*, 2005; Hirvonen *et al.*, 2001; Pul *et al.*, 2007); adaptation to environmental stress frequently involves coregulation in which one or more NAPs participate with other regulators to fine-tune the expression of stress-response genes (Bouvier *et al.*, 1998; Grainger *et al.*, 2008; Hengge-Aronis, 1999), and one could cite many other examples, including that of genes involved in pathogenesis (Dove *et al.*, 1997; Falconi *et al.*, 1996, 2001; O'Byrne and Dorman, 1994).

Studies of gene regulation by NAPs have been hampered in some cases by the lack of a clear consensus DNA sequence for the binding sites used by the proteins. It is difficult to assemble a comprehensive picture of the regulatory ''wiring diagram'' of the cell when one cannot determine where in the genome many of the global regulators bind. The Integration Host Factor (IHF) has long been an exception because it has a well-defined consensus binding site sequence (Ussery *et al.*, 2001). In addition, compilations of data from pains-taking single-gene studies have over many years provided valuable information about the nature of the DNA elements that are bound by proteins such as Fis, H-NS, and Lrp. The situation has been assisted greatly by the application of chromatin immunoprecipitation-on-chip (ChIP-on-chip), a technique that has revealed much new information about the binding sites that are occupied by NAPs in the bacterial genome. Both ChIP-on-chip methods and transcriptomic studies with DNA microarrays have yielded valuable information about the locations of particular NAP binding sites and the genes whose expression is modulated by those NAPs (Arfin *et al.*, 2000; Grainger *et al.*, 2006; Hung *et al.*, 2002; Kelly *et al.*, 2004; Lucchini *et al.*, 2006; Mangan *et al.*, 2006; Navarre *et al.*, 2006; Oshima *et al.*, 2006;

Tani *et al.*, 2002). The results have advanced our understanding of the role of NAPs in influencing bacterial physiology, providing new insights into long-standing problems, and posing new questions for research in the future.

E. coli has been described as possessing up to 12 members of the NAP family (Azam and Ishihama, 1999) although the number is almost certainly greater than that when one takes into account proteins encoded by horizontally transferred genetic elements and chromosomally-encoded polypeptides such as Hha and its homologues (Doyle *et al.*, 2007; Paytubi *et al.*, 2004). Our knowledge of the roles of each NAP in physiology has advanced unevenly (Dorman and Deighan, 2003). Among the best understood is the Fis (Table 2.1), a protein that has been investigated

TABLE 2.1 The NAPs of E. coli

Protein	Structure	Monomer molecular mass (kDa)	Comments
Dps	α_{12}	19	Stationary phase expression; induced by oxidative stress (OxyR-dependent); Ferritin-like activity
Fis	α_2	11.2	Expression peaks in early exponential phase
H-NS	α_2 and higher-order oligomers	15.4	Approximately constant level of expression; forms heteromers with StpA, Sfh, and other full-length and partial paralogs and orthologs
HU	α_2, β_2, $\alpha\beta$	9.2 (α); 9.5 (β)	Composition varies with growth phase: early exponential (a_2), mid-exponential ($\alpha\beta$), stationary (b_2)
IHF	$\alpha\beta$	11.2 (α); 10.7 (β)	Bends DNA by up to 180°; α_2 and β_2 forms exist and transcriptomic data suggest that each has a regulon that overlaps that of the $\alpha\beta$ form
Lrp	α_2–α_8	19	Structure may become modified on binding DNA and/or leucine

thoroughly for about two decades by a steadily increasing number of research groups. Another intensively studied example is the H-NS protein, a global repressor of transcription in many Gram-negative bacteria (Dorman, 2004, 2007a). IHF and its close relative HU affect the expression of many genes and for this reason they have attracted the attention of a large number of investigators, extending far beyond the groups that originally studied them in the context of bacteriophage lambda site-specific recombination (IHF) or the organization of the *E. coli* nucleoid (HU). The leucine-responsive regulatory protein (Lrp) has a broad influence on gene expression and is especially important in the context of physiology due to its role in controlling the transcription of operons involved in branched chain amino acids and also a multitude of virulence factors (de los Rios and Perona, 2007; Hung *et al.*, 2002; Tani *et al.*, 2002). The Dps protein is a ferritin-like polypeptide that is expressed in late stationary phase when it forms a protective barrier on the chromosome (Grant *et al.*, 1998). Although Dps is often grouped with the NAPs (Azam and Ishihama, 1999), information about its role in gene regulation is largely lacking. Its characteristic expression pattern is the reciprocal of that displayed by Fis, a protein that is normally associated with the earliest stages of exponential growth (Azam and Ishihama, 1999). However, Dps expression is activated by the OxyR regulatory protein earlier in growth if the bacterium experiences oxidative stress; stationary phase expression of Dps involves a role for IHF as a coactivator with the RpoS sigma factor of the *dps* promoter (Altuvia *et al.*, 1994). Furthermore, the *dps* promoter is down regulated by the Fis and H-NS proteins, with the latter acting to repress transcription by RNA polymerase containing the RpoD housekeeping sigma factor but not the RpoS stationary phase and stress response sigma factor (Grainger *et al.*, 2008). These observations illustrate the complexity of NAP studies; the proteins (i) have distinct and overlapping functions in the cell, (ii) have individual expression patterns, (iii) are frequently found to cross-regulate each other's genes, and (iv) they often coregulate other target genes, either cooperatively or antagonistically. It will not be possible to do justice to such complexity in a short review so this article will lead with a discussion of Fis, referring to the other better-studied NAPs in the context of bacterial physiology to give a flavor of the range of activities with which these proteins are associated.

II. THE MULTIFUNCTIONAL Fis PROTEIN

The Fis was identified originally as an important accessory protein in site-specific recombination reactions that are catalyzed by members of the invertase family of recombinases (Johnson *et al.*, 1986; Koch and Kahmann, 1986). Fis is now recognized as playing a wide range of roles

in the cell, beyond its relationship with the invertase enzymes (Finkel and Johnson, 1992). It contributes to the lifecycle of bacteriophage lambda by acting as a cofactor for phage integration and excision from the chromosome *in vitro* and *in vivo* (Esposito and Gerard, 2003; Papagiannis *et al.*, 2007; Thompson *et al.*, 1987). In the case of bacteriophage Mu, Fis cooperates with the Mu repressor to inhibit transposition, thus helping to maintain Mu lysogeny (Bétermier *et al.*, 1993; van Drunen *et al.*, 1993). It is also an accessory factor in the transposition of Tn*5* and IS*50* (Weinreich and Reznikoff, 1992). Fis is involved in initiating the replication of the chromosome at *oriC* in *E. coli* (Filutowicz *et al.*, 1992; Gille *et al.*, 1991; Ryan *et al.*, 2004) and it regulates the transcription of a very large number of bacterial genes (Grainger *et al.*, 2006; Kelly *et al.*, 2004). In this latter role, Fis can both activate and repress promoters (Keane and Dorman, 2003; Ninnemann *et al.*, 1992). It functions in some cases as a conventional transcription activator, making physical contact with RNA polymerase (Bokal *et al.*, 1997; McLeod *et al.*, 2002). In other cases, its positive effect on transcription is indirect and involves modulation of local DNA supercoiling at the target promoter in ways that enhance transcription initiation (Auner *et al.*, 2003). These widespread effects on transcription underline the importance of Fis as a governor of cellular physiology. This point is further reinforced when one considers that Fis regulates the expression of genes such as *rpoS, gyrA, gyrB*, and *topA* that in turn encode global regulators playing pivotal parts in the physiology of the cell (Hirsch and Elliott, 2005; Travers *et al.*, 2001; Weinstein-Fischer and Altuvia, 2007). Fis is also thought to be a key genome-structuring element, possibly contributing to the formation of the looped domain architecture of the nucleoid, although the relationship to nucleoid-structure is not straightforward and also involves other NAPs and the major topoisomerases (Ohniwa *et al.*, 2006; Skoko *et al.*, 2006).

Fis binds as a dimer to DNA, using helix-turn-helix motifs to do so, and it bends its target sequence (Kostrewa *et al.*, 1991; Pan *et al.*, 1996). Data from compilations of Fis binding sites identified by ChIP-on-chip indicate that the typical Fis binding motif in DNA is 17 bp long, A + T-rich and has G/C residues at positions 2 and 16 (Cho *et al.*, 2008). Related A + T-rich DNA sequences are found throughout the *E. coli* genome and are frequently associated with promoters (Pedersen *et al.*, 2000).

III. THE Fis PROTEIN AND BACTERIAL PHYSIOLOGY

Fis regulates genes that contribute to fundamental cellular processes, such as the organization of the genetic material and the production of ribosomes and other key components of the translational machinery (Bosch *et al.*, 1990; Nilsson *et al.*, 1990; Ohniwa *et al.*, 2006; Ross *et al.*, 1990;

Schneider *et al.*, 2001; Skoko *et al.*, 2006). It also influences the expression of virulence genes in a variety of pathogens (Falconi *et al.*, 2001; Goldberg *et al.*, 2001; Kelly *et al.*, 2004; Lautier and Nasser, 2007; Lenz and Bassler, 2007). Despite its central role in such processes, mutants deficient in Fis protein expression are viable, although they are at a disadvantage when competing with otherwise isogenic wild type bacteria (Schneider *et al.*, 1997). Mutants lacking *fis* also have altered morphology when they grow at elevated temperature and they may display an extended lag phase (Filutowicz *et al.*, 1992; Nilsson *et al.*, 1990; Osuna *et al.*, 1995). High-level over-expression of the Fis protein results in changes in cell growth that are accompanied by an elevation in rRNA production but a decline in ribosome content, indicating that the Fis protein concentration must be kept within certain limits at each stage of growth if cellular physiology is not to be affected adversely (Richins and Chen, 2001). While it is not essential for growth, Fis certainly has wide-reaching effects on cellular functioning. Consistent with this is the finding that mutants with increased competitive fitness that have been isolated in long-term evolution experiments where *E. coli* cells are subcultured indefinitely in batch culture possess gain-of-fitness mutations that map to the *fis* gene (Crozat *et al.*, 2005).

The viability of *fis* knockout mutants hints at some redundancy in the systems to which the Fis protein contributes. It is possible that other members of the NAP group may substitute functionally for Fis in the mutant. On the other hand, none of the other proteins has obvious amino acid sequence similarity to Fis. Perhaps the molecular contributions made by Fis are not decisive but merely enhance processes that can continue, albeit less efficiently, in its absence. This certainly appears to be so in those cases where the matter has been investigated in detail (Lazarus and Travers, 1993; Merickel *et al.*, 1998; Ohniwa *et al.*, 2006; Schneider *et al.*, 1999; Zacharias *et al.*, 1992).

IV. THE CHARACTERISTIC EXPRESSION PATTERN OF Fis

Most studies of Fis protein expression have been carried out with bacteria growing in aerated batch culture. Here, the protein shows a characteristic growth-phase-dependent expression pattern in which Fis levels peak early and rapidly as the culture enters exponential growth and then decline thereafter. By the onset of stationary phase little or no Fis can be detected (Ball *et al.*, 1992; Keane and Dorman, 2003; Nilsson *et al.*, 1990). A great deal of effort has been expended to elucidate the molecular basis of this expression pattern. It seems that Fis is not subject to rapid turnover by proteolysis; its decline after the initial burst of expression seems to be due chiefly to dilution as the cells divide without synthesizing more

Fis protein. The key control point in Fis expression is transcriptional, and the *fis* mRNA expression pattern mirrors that of the protein product quite faithfully (Ball *et al.*, 1992; Keane and Dorman, 2003; Nilsson *et al.*, 1990). The *fis* gene has a single promoter and this is subject to negative autoregulation by the Fis protein, providing a simple regulatory loop in which high protein levels feed back onto transcription (Mallik *et al.*, 2004). However, there are several additional levels of control. The *fis* promoter is sensitive to DNA supercoiling and operates optimally as average negative supercoiling levels move further into the negative range (Schneider *et al.*, 2000). Negative DNA supercoiling is a feature of energized cells growing rapidly in the exponential phase of batch culture (Bordes *et al.*, 2003), a period when Fis levels are high. The *fis* promoter is also sensitive to the stringent response (Mallik *et al.*, 2004), as are many of the promoters that are targeted by Fis, such as those of stable RNA genes. This means that *fis* transcription is much less active when the bacterium experiences a shortage of charged tRNA, a signal that the protein synthetic machinery of the cell should be down regulated. Stringently regulated promoters usually contain a GC-rich discriminator sequence between the Pribnow box and the transcription start site at position +1; the *fis* promoter shares this feature (Schneider *et al.*, 2000). This sequence represents an energetic barrier to transcription initiation because it is hard to melt in relaxed DNA, the form of DNA that predominates in bacteria with a low energy charge. The presence of the discriminator sequence links *fis* transcription to the superhelical state of the chromosome and hence to the physiological state of the cell (Fig. 2.1). This linkage is particularly interesting in the light of evidence that the Fis protein regulates the transcription of the genes that encode the proteins responsible for introducing negative supercoiling (DNA gyrase) and removing it (DNA topoisomerase I) (Keane and Dorman, 2003; Schneider *et al.*, 1999; Weinstein-Fischer and Altuvia, 2007). In addition, Fis can directly influence cellular supercoiling levels by its ability to bind to intermediately supercoiled DNA molecules and to protect them from further changes to their linking number (Schneider *et al.*, 1997). Thus, it can be seen that Fis lies at the heart of the control network that governs the superhelicity of the genetic material in the cell (Fig. 2.1).

Recently, it was found that changes in growth conditions could modify the classical expression pattern of the *fis* gene. The intracellular pathogen *Salmonella enterica* serovar Typhimurium has a *fis* gene that shares the regulatory characteristics of its *E. coli* ortholog (Keane and Dorman, 2003). *S.* typhimurium expresses its invasive pathogenic properties best under laboratory conditions when it is grown in a poorly aerated high-osmolarity environment (Lee and Falkow, 1990), presumably because these conditions mimic certain features of the mammalian gut where invasion takes place in nature. Bacteria grown in this way sustain high levels of Fis

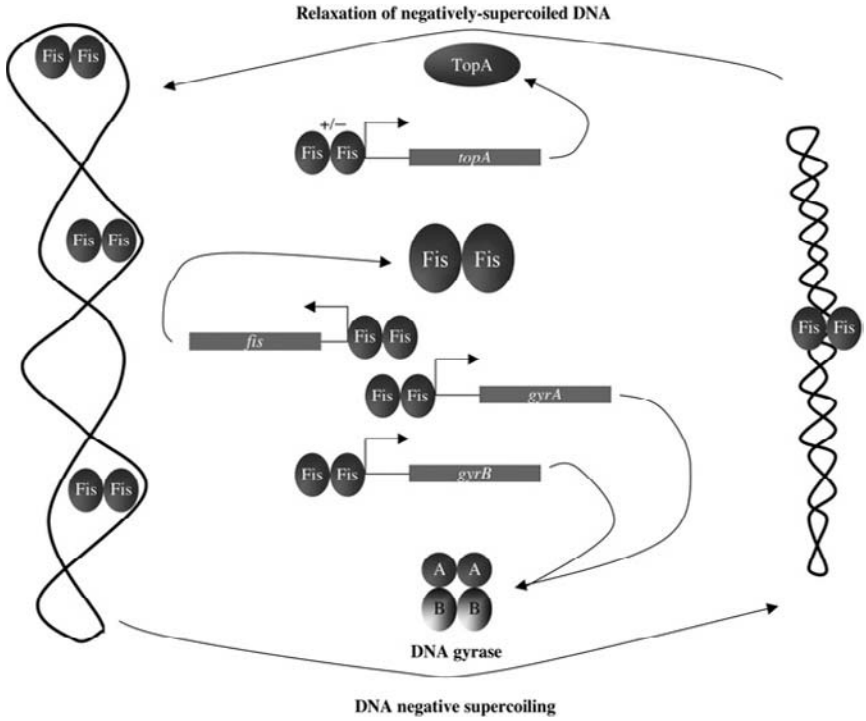

FIGURE 2.1 The Fis protein influences DNA supercoiling at a global level. The dimeric Fis protein represses the promoter of its own gene, *fis,* and those of the genes coding for the GyrA (*gyrA*) and GyrB (*gyrB*) subunits of the heterotetrameric DNA gyrase. Fis has a more complicated relationship with the gene coding for the monomeric DNA topoisomerase I (*topA*); here Fis acts positively when present at low concentrations but is a repressor at high concentrations. This dual input is indicated by the +/− symbol at the *topA* promoter. (The angled arrows upstream of each gene represent transcription start sites.) The Fis protein also binds to the chromosomal DNA where it can influence its availability as a substrate for DNA gyrase and DNA topoisomerase I. Thus Fis can influence the homeostatic control of global DNA supercoiling by modulating the expression of the genes coding for the main topoisomerases and by modulating their activities on the DNA substrate.

protein expression into stationary phase, especially if they are defective in expression of the RpoS stress and stationary phase sigma factor (Ó Cróinín and Dorman, 2007). This finding shows that Fis and RpoS have reciprocal expression states and is consistent with the fact that Fis regulates RpoS expression negatively at the level of transcription (Hirsch and Elliott, 2005). It is not clear how RpoS represses Fis expression; given that RpoS is a sigma factor it seems likely that the relationship is indirect.

V. Fis AND THE GLOBAL TRANSCRIPTION PATTERN

Our knowledge of Fis as a transcription regulator has benefited from both single-gene studies performed over almost two decades and more recent transcriptomic analyses. Much effort has been invested in understanding the mechanisms by which Fis influences the activation of promoters involved in the expression of stable RNA, both rRNA and tRNA in *E. coli*, molecules that lie at the heart of the translation process and bacterial physiology. The P_1 promoter of the *rrnB* operon in *E. coli* has three Fis binding sites centered at positions −71 (site I), −102 (site II), and −143 (site III) with respect to the transcription start site, +1 (Appleman *et al.*, 1998) (Fig. 2.2). Occupation of these sites correlates with the levels of Fis protein in the cell, which in turn is influenced by the phase of growth of the culture. The Fis dimer bound at site I is thought to influence transcription initiation through protein–protein interaction with the α subunit of RNA polymerase bound to the P_1 promoter. While Fis binding enhances the extent of transcriptional up regulation of *rrnB* following

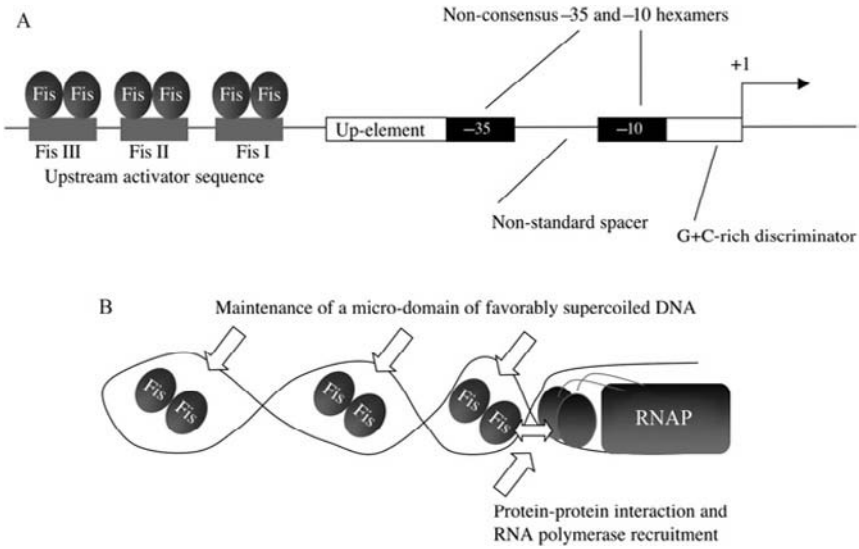

FIGURE 2.2 Regulation by the Fis protein of the transcription of a gene coding for stable RNA. (A) Summary of the main features of a Fis-dependent stable RNA operon promoter. The three binding sites for Fis are labeled with Roman numerals. The Up-element is a *cis*-acting DNA element that contacts the carboxyl-terminal domain of the α subunit of RNA polymerase. The angled arrow labeled +1 shows the location of the transcription start site. (B) The Fis protein can influence transcription by protein–protein contact with RNA polymerase (RNAP) and by the maintenance through protein–DNA contact of a microdomain of appropriately supercoiled DNA at the target promoter.

subculturing of the bacteria into fresh medium, the increase in promoter activity begins in advance of the build up of Fis in the cell (Appleman *et al.*, 1998). This points to an underlying Fis-independent mechanism of up regulation that is then boosted by the presence of the Fis protein. A strong candidate for this underlying mechanism is the degree of negative supercoiling of the *rrnB* promoter, with its known sensitivity to DNA supercoiling (Ohlsen and Gralla, 1992). The stringently-regulated *rrnB* P_1 promoter possesses a G + C-rich discriminator sequence (Appleman *et al.*, 1998), a feature that is difficult to melt in relaxed DNA (Schneider *et al.*, 2000). Since DNA relaxation accompanies the reduction in energy charge that is associated with starvation or entry into stationary phase (Bordes *et al.*, 2003), the G + C-rich discriminator imposes a barrier to transcription initiation. Elimination of the discriminator has been shown to abrogate this barrier in the case of the stringently-regulated *fis* promoter (Schneider *et al.*, 2000). The increased energy charge generated at the onset of exponential growth is manifested partly in an increase in the ratio of the concentration of ATP to ADP. This enhances the activity of DNA gyrase and results in the observed increase in the negative supercoiling of DNA (van Workum *et al.*, 1996). This DNA structural transition then enhances the ability of RNA polymerase to transcribe the *rrnB* promoter. Fis can modulate this process in two ways. One involves the creation of a microdomain of supercoiled DNA at the promoter (Fig. 2.2) and the other arises from the ability of Fis (and other DNA binding proteins such as IHF) to displace DNA twist from their binding sites to other regions in the DNA that may be recalcitrant to DNA melting, such as discriminator elements (Opel *et al.*, 2004; Sheridan *et al.*, 1998). Fis is also known to stabilize open transcription complexes once these have formed at the *rrnB* P_1 promoter (Zhi *et al.*, 2003). All of these observations are consistent with a physiological role for Fis is sustaining and enhancing molecular processes that are already underway, rather than being a primary initiator of those processes.

VI. THE H-NS PROTEIN, A UNIVERSAL REPRESSOR

The H-NS nucleoid-associated protein has been studied for over 35 years in the context of its DNA binding activity and influence on transcription *in vitro* (Cukier-Kahn *et al.*, 1972; Jacquet *et al.*, 1971). Around 20 years ago it became appreciated that mutations mapping to the gene coding for H-NS affect the expression of hundreds of genes, many of which are central to the physiological processes of the cell including those by which it adapts to changes in temperature, osmolarity, desiccation, and pH (Deighan *et al.*, 2000; Dorman *et al.*, 1990; Higgins *et al.*, 1988; May *et al.*, 1990). The application of transcriptomic and ChIP-on-chip methods has

confirmed this, and has also shown that H-NS binds to laterally acquired genes with a high A + T content (Dorman, 2007a; Grainger *et al.*, 2006; Lucchini *et al.*, 2006; Navarre *et al.*, 2006; Oshima *et al.*, 2006). This targeted repression is thought to be an important checkpoint in the acquisition of new genetic information because the new genes can be stored in an inert state while the bacterium develops a suitable regulatory regime for them (Lucchini *et al.*, 2006; Navarre *et al.*, 2006).

The H-NS protein is now recognized as a global repressor of transcription and as a DNA binding protein with an ability to form DNA-H-NS-DNA bridges both within and between DNA molecules (Dame *et al.*, 2006; Dorman, 2007b). An important corollary to the identification of the transcription repressor function of H-NS has been the discovery that many positive regulators of gene expression act as H-NS antagonists, i.e., as anti-repressors, an activity that they may possess in addition to an ability to activate transcription through protein–protein interactions with RNA polymerase (Stoebel *et al.*, 2008).

ChIP-on-chip data for H-NS and Fis binding in the chromosome of *E. coli* have revealed a remarkable degree of correspondence between the binding sites occupied by these two proteins (Grainger *et al.*, 2006). This is perhaps unsurprising when one considers their shared preference for binding to DNA sequences that have a high A + T content. Single-gene studies have shown that Fis can act antagonistically to certain H-NS-repressed promoters, including those of the ribosomal RNA operons (Afflerbach *et al.*, 1999; Dame *et al.*, 2002; Falconi *et al.*, 1999). Perhaps Fis plays a more general role in opposing the repressive activity of H-NS, at least at those phases of growth when Fis is abundant. Similarly, the Lrp NAP seems to target and upregulate or corepress genes that are prone to H-NS-mediated repression (McFarland *et al.*, 2008; Pul *et al.*, 2007). Interestingly, Fis regulates the transcription of the *hns* gene while Lrp contributes to the control of expression of *stpA,* the gene encoding StpA, an RNA-binding paralog of H-NS (Falconi *et al.*, 1996; Free and Dorman, 1997) that forms heterodimers with H-NS. These connections between the NAPs illustrate the tightly integrated nature of the global regulatory circuits of the cell.

VII. PROTEINS HU AND IHF

The HU protein represents an interesting case because it is a heterodimeric DNA binding protein whose α and β subunits can also form homodimers. In addition, the relative abundances of the α and β subunits vary as a function of growth, leading to a dynamic population of HU homodimers and its heterodimer in the cell (Claret and Rouvière-Yaniv, 1997). The three forms of the protein are not functionally equivalent; unlike the

other two, the b_2 form cannot promote the formation of negatively-supercoiled DNA (Claret and Rouvière-Yaniv, 1997) perhaps because it binds duplex DNA poorly (Pinson *et al.*, 1999).

The IHF protein also has an $\alpha\beta$ structure, although a_2 and b_2 homodimers can form. Transcriptomic studies in *Salmonella* indicate that the three forms of IHF ($\alpha\beta$, α_2, and β_2) control distinct but overlapping regulons of genes (Mangan *et al.*, 2006) IHF differs from HU in binding to a well-defined consensus DNA sequence (Ussery *et al.*, 2001). It frequently acts as a regulatory cofactor and its ability to bend DNA through 180° represents a key feature of its biological activity, not just in transcriptional control but also in other DNA-based transaction such as recombination and transposition (Haniford, 2006; Thompson *et al.*, 1987). Both HU and IHF have emerged as important influences on the expression (HU) and activity (IHF) of the stress and stationary phase sigma factor, RpoS, a pivotal player in the physiology of the cell.

VIII. RpoS AS A REGULATORY TARGET OF NAPs

The RpoS sigma factor is one of seven expressed by *E. coli* and its close relatives and it controls the expression of genes required in stationary phase and/or to adapt to stresses such as osmotic upshock (Hengge-Aronis, 2002). Several of the NAPs discussed in this review contribute to the control of RpoS expression. For example, the role of the Fis protein as a repressor of *rpoS* transcription during exponential growth (Hirsch and Elliott, 2005) has been mentioned earlier. The HU protein binds to *rpoS* mRNA to regulate RpoS expression posttranscriptionally (Balandina *et al.*, 2001). Proteolysis of RpoS is regulated indirectly by H-NS (Zhou and Gottesman, 2006); H-NS also has the ability to bind to and modulate the stability of *rpoS* mRNA (Brescia *et al.*, 2004). H-NS and RpoS also share a regulatory connection: each is controlled posttranscriptionally by the DsrA small regulatory RNA through a mechanism that depends on the Hfq RNA chaperone (Sledjeski *et al.*, 2001).

RpoS activity is also influenced by NAPs. Mutants that lack IHF are defective in expression of RpoS regulon genes in stationary phase, even though the level of RpoS is unaffected (Mangan *et al.*, 2006). This indicates that IHF plays an auxiliary role in the expression of the stationary phase regulon, an observation that is consistent with the finding that IHF levels peak as bacteria enter stationary phase (Thompson *et al.*, 1987). The Fis protein has also been found to act as a cofactor for RpoS at the osmotic-stress-and-stationary-phase inducible *proP* promoter (Xu and Johnson, 1995). At first glance, a role for Fis in stationary phase may seem counter-intuitive; however, it is in keeping with the finding that bacteria grown with low-aeration sustain Fis expression into the late phases of

batch growth (Ó Cróinín and Dorman, 2007). Both Fis and H-NS modulate the activities of RpoD- and RpoS-containing RNA polymerase at the *dps* promoter. Fis prevents promoter utilization by the polymerase containing RpoD but its absence in stationary phase means that RpoS-containing polymerase is able to initiate transcription. The H-NS protein blockades the promoter effectively when RNA polymerase contains RpoD but cannot do so when it contains RpoS (Grainger *et al.*, 2008). This finding has important implications for the differential effects of H-NS on promoter utilization depending on whether RpoD or RpoS is present in RNA polymerase.

IX. PERSPECTIVE

This short article has considered information drawn from both single-promoter and whole-genome studies. The amount of data coming from the latter is indeed daunting. Nevertheless, we must meet the intellectual and technical challenges posed by this explosion in information if the complexities of bacterial physiology and the regulatory processes that govern it are to be mastered. This is made all the more compelling by the promise that a genuinely thorough understanding of microbial systems may help to better human health and wellbeing.

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CHAPTER 3

Biodegradation of Pharmaceutical and Personal Care Products

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Abstract Medical treatments and personal hygiene lead to the steady release of pharmaceutical and personal care products (PPCPs) into the environment. Some of these PPCPs have been shown to have detrimental environmental effects and could potentially impact human health. Understanding the biological transformation of PPCPs is essential for accurately determining their ultimate environmental fate, conducting accurate risk assessments, and improving PPCP removal. We summarize the current literature concerning the biological transformation of PPCPs in wastewater treatment plants, the environment, and by pure cultures of bacterial isolates. Although some PPCPs, such as ibuprofen, are readily degraded under most studied conditions, others, such as carbamazepine, tend to be recalcitrant. This variation in the biodegradability of PPCPs can be attributed to structural differences, because PPCPs are classified by application, not chemical structure. The degradation pathways of octylphenol by *Sphingomonas* sp. strain PWE1, ibuprofen by *Sphingomonas* sp. strain Ibu-2, and DEET by *Pseudomonas putida* DTB are discussed in more detail.

I. INTRODUCTION

In recent decades, the use of pharmaceutical and personal care products (PPCPs) ranging from over-the-counter pain relievers to antibacterial soap has increased dramatically, with quantities now rivaling those of agrochemicals (Daughton and Ternes, 1999). Indeed, the five most commonly detected classes of organic wastewater contaminants detected in streams in the United States in a 1999–2000 study were PPCPs (Kolpin *et al.*, 2002). The occurrence of PPCPs in wastewater treatment plants (WWTPs) and in the environment has been extensively reviewed later in this chapter (Daughton and Ternes, 1999; Halling-Sorensen *et al.*, 1998; Heberer, 2002; Ternes *et al.*, 2004); however, less is known about the ability of microorganisms to degrade these bioactive compounds.

Although WWTPs are effective at removing some PPCPs from wastewaters (Clara *et al.*, 2005b; Joss *et al.*, 2005, 2006; Ternes *et al.*, 2004; Voets *et al.*, 1976; Watkinson *et al.*, 2007), many PPCPs and their metabolites have been detected in both the surface waters (Ahel *et al.*, 1994; Bendz *et al.*, 2005; Daughton and Ternes, 1999; Kolpin *et al.*, 2002; Lindstrom *et al.*, 2002; Singer *et al.*, 2002; Soares *et al.*, 2008) and aquatic sediments (Miller *et al.*, 2008; Singer *et al.*, 2002; Ying *et al.*, 2002; Yuan *et al.*, 2004). Wastewater treatment, however, is not the only source of PPCPs as some PPCPs have also been detected in groundwater impacted by landfill leachates (Holm *et al.*, 1995).

With the increased use of PPCPs and their consequent detection in the environment, the need for understanding how these compounds are

biotransformed has become apparent: we cannot accurately predict the risk these compounds pose until we have a better understanding of the nature and persistence of the metabolites generated during transformation. Several PPCPs and their metabolites have been shown to have toxicological effects. For example, octyl- and nonylphenols which are metabolites of nonionic surfactants (and collectively known as alkyphenols) are estrogenic (Isidori *et al.*, 2006; Jobling *et al.*, 1996; Nakamura *et al.*, 2002; White *et al.*, 1994). Commonly used fragrance components such as tonalide (AHTN) and galaxolide (HHCB) are not only estrogenic (Schreurs *et al.*, 2002; Seinen *et al.*, 1999), but also elicit acute toxicity in some aquatic organisms at environmentally relevant concentrations (Breitholtz *et al.*, 2003; Gooding *et al.*, 2006). Enhanced knowledge of PPCP biodegradation could lead to both improved removal of these compounds from wastewater as well as a more accurate estimate of the overall effects of PPCPs on human and environmental health (Daughton and Ternes, 1999; Kolpin *et al.*, 2002). In addition, determination of degradation pathways is necessary for tracking the ultimate fate of PPCP metabolites in the environment and for developing rational strategies for the design of less recalcitrant alternatives.

In light of the fact that PPCPs are grouped not on the basis of their chemical similarity, but rather by the applications for which they are used, the biological degradation pathways whereby PPCPs are metabolized are expected to be diverse. In this review, we attempt to summarize what is known in general concerning the role of biological degradation of PPCPs in wastewater as well as in natural environments. We also describe several instances in which the use of PPCPs as bacterial growth substrates has been demonstrated. Although less information is available regarding specific metabolic pathways of individual PPCPs we provide several examples detailing what is known about the metabolism of alkylphenols (APs) (specifically octylphenol), DEET (*N,N*-diethyl-*m*-toluamide), and ibuprofen.

II. WHAT ARE PPCPs?

The group of compounds collectively referred to as PPCPs consists of any substances used for medicinal or personal care purposes. The metabolites and transformation products of these substances can also be considered PPCPs (Daughton and Ternes, 1999). Within the medicinal PPCPs, substances used for any stage of medical care are included and can be either prescription or nonprescription. Diagnostic chemicals (i.e., the X-ray contrast medium iopromide), drugs for symptom mitigation (i.e., ibuprofen), curative agents (i.e., antibiotics or antineoplastics), and preventative treatments (i.e., 17α-ethinylestradiol (EE2) from birth control pills) are all

considered PPCPs (Daughton and Ternes, 1999). These substances which are designed to produce specific biological responses are also sometimes referred to as pharmaceutically active compounds (PhACs) (Heberer, 2002; Suárez *et al.*, 2005). Veterinary and illicit drugs can also be included in the PPCPs (Daughton and Ternes, 1999), but are not considered in this review.

Personal care products are substances used to maintain hygiene and general well-being. Some examples include the ingredients of cleansing products (i.e., nonionic surfactants), fragrances (i.e., synthetic musks), and protective products such as insect repellents (DEET) and antimicrobials (i.e., triclosan (TCS)). Several PPCPs may be found in a single product. For instance, ''antibacterial'' hand soap typically contains synthetic fragrance and TCS in addition to surfactants.

Though there is no chemical characteristic common to all PPCPs, most have at least one aromatic group (see Table 3.1 in (Daughton and Ternes, 1999)). They range from simpler compounds such as acetylsalicylic acid to more structurally complex ones such as iopromide. They may have natural origins, like many antibiotics or be completely synthetic, like TCS.

III. HUMAN INTERACTIONS WITH PPCPs

The fate of PPCPs depends in part on the application of the product, the chemical properties of the product, and to some extent the physiology of the user. Products which are applied externally, such as hygiene products and fragrances, typically enter the wastewater stream directly as components of grey water in the form of the unaltered parent compound. Internally administered PPCPs (or those which unintentionally enter the body) are subjected to the biochemical detoxification mechanisms of the human body that alter biological activity and facilitate excretion (Cunningham, 2004; Daughton and Ternes, 1999; Suárez *et al.*, 2008). These biochemical transformations are divided into two phases. In phase I, the compounds can be acted upon by monooxygenases (typically cytochrome P450s or flavin monooxygenases), reductases, or hydrolases to increase reactivity of the compound. Phase II typically involves conjugation of the compound with either sugars (glucuronidation) or peptides to improve solubility and facilitate excretion (Cunningham, 2004; Daughton and Ternes, 1999; Suárez *et al.*, 2008). The extent to which a PPCP becomes conjugated depends mainly on its chemical properties and varies greatly across PPCPs (Ternes *et al.*, 1999a). For example, ibuprofen can be hydroxylated or carboxylated, and either the parent compound or one of its metabolites can then be glucuronidated. All of these metabolites and the parent compound are excreted (Ternes *et al.*, 2004). Although up to 90% of some PPCPs are excreted as conjugates (Daughton and Ternes, 1999;

TABLE 3.1 Estimated biological transformation of selected PPCPs in WWTPs

Compound	PPCP Category	Predicted biodegradability[1]	Estimated biological transformation (%)	
			CAS	MBR
Ibuprofen	Analgesic	High	>90[2–7] 80[8,9] 70[10]	
Diclofenac		Low	15–40[2,5,6,8]	Not significant[3]
Naproxen		Moderate	50–70[2,6,8,10] Increased to>80[9] 93[5]	84[3]
Ketoprofen		NA	10–70[9] 65[5]	62[6]
Bezafibrate	Lipid regulator	NA	40–100[7]	>75[6,7]
Clofibric acid		Moderate	75[5]	
Gemfibrozil		Moderate	96[5]	
β-Lactam antibiotics	Antibiotic	NA	99[11]	
Quinolone antibiotics		NA	83[11]	
Lincosamide antibiotics		NA	11[11]	
Roxithromycin		Moderate	0–60[2] 20[12]	77[3]
Azithromycin		Low	0–20[12]	0–25[12]
Clarithromycin		Low	0–15[12]	50 (SRT ≤ 33 d)[12]; 90 (SRT 60–80 d)[12]
Erythromycin		Low	Not significant[12]	25 (SRT ≤ 33 d)[12]; 90 (SRT 60–80 d)[12]

(*continued*)

TABLE 3.1 (*continued*)

Compound	PPCP Category	Predicted biodegradability[1]	Estimated biological transformation (%)	
			CAS	MBR
Trimethoprim		NA	0–10[12]	36[3] 30(SRT ≤ 33 d)[12]; 90 (SRT 60–80 d)[12]
Sulfamethoxazole		Low	0–90[2]	
			Increased[5] 25[11] 50–70[10,12]	40–50[3,12]
Triclosan	Antimicrobial	NA	>75[13–15] 40 to>90[9] 50[16] 34[13] (one sample)	
Iopromide	X-ray contrast medium	Moderate	30–90[2] Not significant[10]	
Carbamazepine	Antiepileptic	Low	0–15[2,3] Increased to 80[8] <10[8] 30[5]	
Diazepam	Tranquilizer	Low	<10[8]	26[3]
Tonalide (AHTN)	Synthetic musk	Low	0–20[2]	
			10 to>80[7] 50[10]	50[3] >80[7]
Galaxolide (HHCB)		Low	0–40[2]	
			Increased[5] 20 to>80[7] 40[10]	50[3] >80[7]
Celestolide		NA		50[3]
Stearic (octadecanoic) acid	Surfactant	NA	98[5]	
Palmitic (hexadecanoic) acid		NA	98[5]	

Nonylphenol polyethoxylates (NPEO)		NA	0 to>90[7] 90[17] >92[18,19]	60–80[7]
Octylphenol polyethoxylates (OPEO)		NA	50 to>90[7]	60 to>90[7]
Nonylphenol	Degradation product of NPEO	NA	71[5] >99[18,20]	
Estrone (E1) (metabolite of E2)	Hormone	NA	Not significant[21 (Germany)] 40[10] 61[22] >80[9,21(Brazil)]	
17β-Estradiol (E2)		NA	45[10] 64[21 (Germany)] >80[9,21 (Brazil), 22]	
17α-Ethinylestradiol (EE2)		NA	Increased[21 (Germany)] 78[9,21 (Brazil)] 85[22]	
Estriol (E3)		NA	>80[8,22]	
EDTA	Preservative	NA	Not significant[23–25]	
N,*N*-diethyl-*m*-toluamide (DEET)	Insect repellent	NA	37–90[26]	

[1](Joss *et al.*, 2006); [2](Joss *et al.*, 2005); [3](Reif *et al.*, 2008); [4](Buser *et al.*, 1999); [5](Bendz *et al.*, 2005); [6](Quintana *et al.*, 2005); [7] (Clara *et al.*, 2005b); [8](Suárez *et al.*, 2005); [9](Nakada *et al.*, 2006); [10](Carballa *et al.*, 2004); [11](Watkinson *et al.*, 2007); [12](Göbel *et al.*, 2007); [13](Lindstrom *et al.*, 2002); [14](Singer *et al.*, 2002); [15](Federle *et al.*, 2002); [16](Voets *et al.*, 1976); [17](Lu *et al.*, 2008); [18](Planas *et al.*, 2002); [19](Zhang *et al.*, 2008); [20] (Chang *et al.*, 2005); [21](Ternes *et al.*, 1999b); [22](Baronti *et al.*, 2000); [23](Sýkora *et al.*, 2001); [24](Kari and Giger, 1996); [25](Madsen and Alexander, 1985); [26](Knepper, 2004)

Ternes, 1998), the chemical properties of others, such as iopromide which requires stability for its application, result in excretion of almost exclusively the parent compound (Bourin *et al.*, 1997; Suárez *et al.*, 2008). Taken as a whole, the parent compound and its metabolites are referred to by the FDA as structurally related substances (SRSs) (Daughton and Ternes, 1999).

IV. BIOLOGICAL TRANSFORMATION OF PPCPs DURING WASTEWATER TREATMENT

Most PPCPs enter WWTPs, also referred to as sewage treatment plants (STPs), as components of domestic, hospital, and industrial waste or via runoff (Daughton and Ternes, 1999; Suárez *et al.*, 2008). The number of PPCPs being detected in WWTPs is increasing, due not only to growth in the pharmaceutical and personal care industries (Daughton and Ternes, 1999), but also to improved methods of detection (Suárez *et al.*, 2008). Most PPCPs are discharged at a fairly constant rate (Daughton and Ternes, 1999; Joss *et al.*, 2005) which has led to them being called ''pseudo'' persistent pollutants (Barceló, 2007; Kosjek *et al.*, 2007b). The levels of PPCPs in WWTPs are typically in the part-per-trillion (ppt; ng l^{-1}) to part-per-billion (ppb; μg l^{-1}) (Daughton and Ternes, 1999; Suárez *et al.*, 2008). However, these concentrations remain a cause for concern since most of these compounds have been expressly employed because of their biological activity at low concentrations (Daughton and Ternes, 1999; Suárez *et al.*, 2008). In addition, drugs with similar biological targets can work synergistically or in unexpected ways (Daughton and Ternes, 1999), such as the estrogenic effects of APs (Isidori *et al.*, 2006; Jobling *et al.*, 1996; Nakamura *et al.*, 2002) and toxicity of some synthetic musks (Breitholtz *et al.*, 2003; Daughton and Ternes, 1999; Gooding *et al.*, 2006). Removal of PPCPs in WWTPs, which curtails their release into the aquatic environment, is necessary to minimize impacts in downstream aquatic environments, but also presents challenges for sewage sludge disposal since many PPCPs sorb to sludge without being degraded (Harrison *et al.*, 2006).

Most studies which examine the fate of PPCPs in WWTPs measure only the influent and effluent concentrations of the parent compound, not the wide array of known or possible SRSs. This omission can confound results because conjugates can act as a reservoir of the parent PPCP (Heberer, 2002; Ternes *et al.*, 2004, 1999a,b). Although the conjugates are often not biologically active, they can be enzymatically cleaved to release the parent PPCP (Daughton and Ternes, 1999; Joss *et al.*, 2005; Ternes *et al.*, 2004). Some studies (i.e., Göbel *et al.*, 2007; Joss *et al.*, 2005) have reported discrepancies in the mass balance of certain PPCPs (e.g., the antibiotic sulfamethoxazole), with more PPCPs being found in the effluent of the biological treatment step of conventional activated sludge (CAS) and

membrane bioreactor (MBR) WWTPs than were found in the influent. One explanation proposed for this discrepancy is the cleavage of unmeasured influent PPCP conjugates which were then cleaved and measured as the parent compound in the effluent (Göbel *et al.*, 2007; Joss *et al.*, 2005). For some compounds, such as macrolide antibiotics, an alternative explanation may be the gradual release of the compounds from feces particles (Göbel *et al.*, 2007).

Once they have entered the WWTP, PPCPs can be removed by several routes. Depending on the sorption coefficient (K_d), sorption to solids (sludge) can play a significant role in the removal of the parent compound (Daughton and Ternes, 1999; Joss *et al.*, 2005; Suárez *et al.*, 2008). In one study, nearly all removal of the synthetic musks galaxolide and tonalide was attributed to sorption to sludge (Joss *et al.*, 2005). It has been determined that for compounds whose K_d is less than 300 L kg^{-1}; however, sludge sorption is not a relevant removal mechanism and therefore decreases in PPCP concentration are usually attributed to biological transformation (Joss *et al.*, 2005).

Microbiological transformation of PPCPs could be the result of either catabolism (use of the compound as a carbon and energy source) or cometabolism (coincidental transformation of the compound without use as a carbon or energy source (Alexander, 1999b)). In either case, the transformation can result in complete mineralization (e.g., to CO_2 and water), degradation to lower molecular weight products, or minor chemical modification. Measurement of only the parent compound, however, makes it impossible to determine which of these fates a PPCP encounters during ''biological removal'' processes (Daughton and Ternes, 1999; Joss *et al.*, 2005). The metabolic fate of PPCPs can impact the aquatic environments receiving WWTP effluent and will therefore be referred to collectively as biological transformation rather than ''removal.'' For example, if the PPCP is completely mineralized, it has been removed from the system and can no longer have any impact. Conjugation, however, as mentioned earlier, acts as a reservoir for the parent PPCP which can be released by cleavage in the environment and exert the same biological effects as the untransformed parent compound.

In some cases, transformation of one PPCP results in the formation of another. This is the case for the hormone 17β-estradiol (E2) which is initially transformed into estrone (E1) (Carballa *et al.*, 2004; de Mes *et al.*, 2005; Suárez *et al.*, 2008; Ternes *et al.*, 1999a), often resulting in no apparent removal of E1 or an increase in its concentration during biological wastewater treatment (Carballa *et al.*, 2004; Ternes *et al.*, 1999a). The transformation of estrogens in WWTPs, as well as in the environment, has been further reviewed by de Mes *et al.* (2005).

Several studies have reported on the presence of PPCP metabolites produced during biological treatment of wastewater (Eichhorn *et al.*, 2005;

Kosjek *et al.*, 2007a,b; Miao *et al.*, 2005; Pérez *et al.*, 2006; Quintana *et al.*, 2005; Ternes *et al.*, 1999b; Zhong *et al.*, 2003; Zwiener *et al.*, 2002), which have been reviewed further by Kosjek *et al.* (2007b). The PPCP metabolites investigated include those of ketoprofen (Quintana *et al.*, 2005), diclofenac (Kosjek *et al.*, 2007a), ibuprofen (Quintana *et al.*, 2005; Zwiener *et al.*, 2002), naproxen (Kosjek *et al.*, 2007a; Zhong *et al.*, 2003), benzafibrate (Quintana *et al.*, 2005), trimethoprim (Eichhorn *et al.*, 2005), and the estrogens E2 and mestranol (Ternes *et al.*, 1999b). Metabolites have even been detected from the more recalcitrant PPCPs like iopromide (Pérez *et al.*, 2006) and carbamazepine (Miao *et al.*, 2005). When five acidic pharmaceuticals (ibuprofen, ketoprofen, naproxen, diclofenac, and bezafibrate) were incubated with activated sludge from a MBR, four of the five pharmaceuticals were transformed, but to varying extents (Quintana *et al.*, 2005). No transformation of diclofenac was observed (Quintana *et al.*, 2005). Ibuprofen and naproxen were completely mineralized, but the metabolism of both ketoprofen and bezafibrate resulted in the accumulation of stable metabolites that resisted further degradation (Quintana *et al.*, 2005). Although these metabolites were not detected in the effluent of the actual WWTP (Quintana *et al.*, 2005), these results further emphasize that the disappearance of the parent compound is not equivalent to removal of the PPCP and that studies examining the metabolic pathways by which PPCPs are degraded are critical to understanding their fate in WWTPs and the environment.

The extent of biological transformation of PPCPs in WWTPs varies greatly between compounds, but reported values for most compounds remain fairly constant (Table 3.2). For example, several studies have found that at least 90% of the analgesic ibuprofen undergoes biotransformation (Bendz *et al.*, 2005; Buser *et al.*, 1999; Joss *et al.*, 2005; Paxeus, 2004; Quintana *et al.*, 2005; Reif *et al.*, 2008), although exceptions have been reported (Carballa *et al.*, 2004; Suárez *et al.*, 2005). This contrasts with reports regarding the biotransformation of the antiepileptic drug carbamazepine, where only ~15% is reported to be lost due to biological transformation (Clara *et al.*, 2004; Joss *et al.*, 2005; Reif *et al.*, 2008; Suárez *et al.*, 2005). Joss *et al.* (2006) used activated sludge from both CAS reactors and MBRs in stirred batch reactors to determine the biodegradation rates of 35 PPCPs. This information was used to classify the biodegradability of PPCPs (Joss *et al.*, 2006). For all 35 PPCPs, pseudo first-order kinetics were observed and degradation constants (k_{biol}) were calculated, taking volatilization and sorption into account (Joss *et al.*, 2006). It was generally found that the k_{biol} using the MBR activated sludge was slightly lower than that for CAS (Joss *et al.*, 2006). Based on the k_{biol}, three biodegradation classes were defined: low removal ($k_{biol} < 0.1$ L g ss^{-1} d^{-1}; < 20% predicted removal), moderate removal (0.1 L g ss^{-1} $d^{-1} < k_{biol} < 10$ L g ss^{-1} d^{-1}; 20–90% predicted removal), and high removal ($k_{biol} > 10$ L g ss^{-1} d^{-1}; > 90%

TABLE 3.2 Bacterial strains and consortia which grow on PPCPs

Compound	Strains/consortia	Closest taxonomic identification	Source	Study
Estrone (E1)	BP1	*Phyllobacterium myrsinacearum*	Compost	(Pauwels *et al.*, in press)
	BP2	*Ralstonia pickettii*		
	BP3	*Pseudomonas aeruginosa*		
	BP7	*Pseudomonas*		
	BP8, BP10	*Acinetobacter*		
	CYH	*Sphingomonas*	Artificial sandy aquifer	Ke *et al.* (2007)
	Y 50158	*Rhodococcus zopfii*	Activated sludge	Yoshimoto *et al.* (2004)
	Y 50156, Y 50157, Y 50155	*R. equi*		
	Mixed culture R1	*Achromobacter xylosoxidans* and *Ralstonia*	Activated sludge	Weber *et al.* (2005)
17β-Estradiol (E2)	BP1	*P. myrsinacearum*	Compost	Pauwels *et al.* (in press)
	BP2	*R. pickettii*		
	BP3	*P. aeruginosa*		
	BP7	*Pseudomonas*		
	BP8, BP10	*Acinetobacter*		
	CYH	*Sphingomonas*	Artificial sandy aquifer	Ke *et al.* (2007)
	LHJ3	*Agromyces*		

(*continued*)

TABLE 3.2 (*continued*)

Compound	Strains/consortia	Closest taxonomic identification	Source	Study
	KC8	*Sphingomonas*	Activated sludge	Yu *et al.* (2007)
	AcBE2–1^T	*Denitratisoma oestradiolicum* gen. nov., sp. nov.	Activated sludge	Fahrbach *et al.* (2006)
	Y 50158	*R. zopfii*	Activated	Yoshimoto
	Y 50156, Y 50157, Y 50155	*R. equi*	sludge	*et al.* (2004)
	ARI-1^T	*Novosphingobium tardaugens* sp. nov.	Activated sludge	Fujii *et al.* (2003)
	Mixed culture R1	*A. xylosoxidans* and *Ralstonia*	Activated sludge	Weber *et al.* (2005)
Estriol (E3)	BP1	*P. myrsinacearum*	Compost	Pauwels *et al.*
	BP2	*R. pickettii*		(in press)
	BP3	*P. aeruginosa*		
	BP7	*Pseudomonas*		
	BP8, BP10	*Acinetobacter*		
	LHJ3	*Agromyces*	Artificial sandy aquifer	Ke *et al.* (2007)
	Y 50158	*R. zopfii*	Activated	Yoshimoto
	Y 50156, Y 50157, Y 50155	*R. equi*	sludge	*et al.* (2004)

17α-Ethinylestradiol	Y 50158	*R. zopfii*	Activated sludge	Yoshimoto *et al.* (2004)
	Y 50156, Y 50157, Y 50155	*R. equi*		
	Sphingobacterium sp. JCR5	*Sphingobacterium*	Activated sludge (of oral contraceptive factory)	Haiyan *et al.* (2007)
EDTA	DSM 9103	*Phyllobacteriaceae/ Mesorhizobia* family	Mixture of activated sludge and soil	Weilenmann *et al.* (2004)
	BNC1	α-Proteobacteria	EDTA-containing industrial sewage	Nortemann (1992)
	Unnamed	*Agrobacterium*	Activated sludge	Lauff *et al.* (1990)
	Mixed culture	*Methylobacterium, Variovorax, Enterobacter, Aureobacterium, Bacillus, Pseudomonas,* unidentified gram-positive	River water	Thomas *et al.* (1998)

(*continued*)

TABLE 3.2 (*continued*)

Compound	Strains/consortia	Closest taxonomic identification	Source	Study
	Mixed culture	Not determined	EDTA-containing industrial sewage	Nortemann (1992)
	Mixed culture	Not determined (source of DSM 9103)	Mixture of activated sludge and soil	Gschwind (1992)
Iminodisuccinate (EDTA substitute)	BY6	*Agrobacterium tumefaciens*	Activated sludge	Cokesa *et al.* (2004)
Polyethylene glycol	Gra PEG 1, Gra PEG 2	*Bacteroidaceae* (*Pelobacter venetianus* sp. nov.)	Mud	Schink and Stieb (1983)
	Ko PEG 2	*Bacteroidaceae* (*Pelobacter venetianus* sp. nov.)	Activated sludge	
	Unnamed	Unidentified	Soil	Fincher and Payne (1962)
Ibuprofen	Ibu-2	*Sphingomonas*	Activated sludge	Murdoch and Hay (2005)
4-Acetamidophenol	ST1	*Pseudomonas*	Pharmaceutical-contaminated soil	Ahmed *et al.* (2001)

Acetylsalicylic acid	Unnamed	*Acinetobacter lwofii*	Distilled water	Grant *et al.* (1970)
NPEOs	BCaL1	*Acinetobacter*	Activated sludge	Di Gioia *et al.* (2004)
	BCaL2	*Stenothrophomonas*		
	Unnamed	*P. putida*	Activated sludge	John and White (1998)
	Multiple unnamed	*Acinetobacter, Shewanella, Proteus, Aeromonas,* undetermined	Activated sludge	Barberio *et al.* (2001)
Nonylphenols	TTNP3	*Sphingomonas*	Activated sludge	Tanghe *et al.* (1999)
	JC1	*Pseudomonas*	River sediment	Yuan *et al.* (2004)
	AN3	Gram-negative (grows anaerobically)	River sediment	Chang *et al.* (2004)
	S. xenophaga Bayram	*S. xenophaga* Bayram	Activated sludge	Gabriel *et al.* (2004)
	S-3^{T}	*S. cloacae* sp. nov.	Activated sludge	Fujii *et al.* (2001)
OPEOs	10 Unnamed	*P. putida*	Rice paddies	Nishio *et al.* (2002)
	Unnamed	*Burkholderia cepacia*		
	TX1	*P. nitroreducens*	Rice paddies	Chen *et al.* (2006)
	G-3	*Sphingomonas*	Topsoil	Nishio *et al.* (2005)

(*continued*)

TABLE 3.2 (*continued*)

Compound	Strains/consortia	Closest taxonomic identification	Source	Study
Octylphenols	TTNP3	*Sphingomonas*	Activated sludge	Tanghe *et al.* (1999)
	PWE1	*Sphingomonas*	Activated sludge	Porter and Hay (2007)
Tetradecyltrimethylammonium (TDTMA) environmental source unknown	*P. putida* A ATCC 12633 Liffourrena *et al.* (2008)	*P. putida* A ATCC 12633	ATCC; original	
n-dodecyltrimethylammonium chloride (DTAC)	7–6	*Pseudomonas*	Activated sludge	Takenaka *et al.* (2007)
Hexadecyltrimethylammonium chloride	B1	*Pseudomonas*	Activated sludge	van Ginkel *et al.* (1992)
Alkylbenzene sulfonates	Unnamed	*Bacillus*	Activated sludge	Willets and Cain (1972)
N,N-diethyl-*m*-toluamide (DEET)	DTB	*P. putida*	Activated sludge	Rivera-Cancel *et al.* (2007)
Antibiotics (18 from different groups)	75 Unnamed	*Burkholderiales, Xanthomonadales, Pasteurellales, Enterobacteriales, Pseudomonadales,*	Soil	Dantas *et al.* (2008)

		Sphingomonadales, *Rhodospirillales*, *Rhizobiales*, *Sphingobacteriales*, *Flavobacteriales*, *Actinomycetales*		
Benzylpenicillin (Penicillin G)	Unnamed	*P. fluorescens*	Lake Sediment	Johnsen (1977)
	KT 512, KT 513, KT 514, KT 515	*Leptospira*	Soil	Kameda *et al.* (1961)
Chloramphenicol	Unnamed	*Streptomyces*	Soil	Abd-el-malek *et al.* (1961)
Triclosan	TriRY	*P. putida*	Compost	Meade *et al.* (2001)
	TR1	*Alcaligenes xylosoxidans* subsp. *denitrificans*		
	Consortium	Multiple unidentified taxa (contains TCS-mineralizing *Sphingomonas* sp. strain RD1)	Activated sludge	Hay *et al.* (2001)

predicted removal) (Joss *et al.*, 2006). In that study, only four of the studied PPCPs fell into the high removal classification: ibuprofen, paracetamol, 17β-estradiol, and estrone (Joss *et al.*, 2006), whereas 17 (including carbamazepine) fell into the low removal class (Joss *et al.*, 2006). With few exceptions, the extent of degradation predicted by Joss (Joss *et al.*, 2006) agreed with other values reported in the literature.

Although the most reliable predictor of the biological transformation of individual PPCPs is the inherent degradability of the compound, variations in the operating conditions of WWTPs can also impact the biological transformation of PPCPs. Specific conditions which may effect removal of PPCPs in WWTPs are temperature, retention time, and the reactor type.

Several studies have noted improved biological transformation of certain PPCPs with increased temperature (Suárez *et al.*, 2008; Ternes *et al.*, 1999b). For example, Ternes *et al.* (1999b) found that even though up to 83% of the synthetic estrogen EE2 was removed by the aerated tank of a WWTP in Brazil (>20 °C), no significant removal was achieved by similar treatment in Germany (average 2 °C). Although sorption was not accounted for in this study, sorption does not completely explain the removal of EE2 (de Mes *et al.*, 2005; Suárez *et al.*, 2008). No effect of temperature, however, has been reported in other studies (Göbel *et al.*, 2007; Joss *et al.*, 2005). One possible explanation for the disparate observations of temperature effect could be the different temperature ranges studied (2 °C and over 20 °C (Ternes *et al.*, 1999b) versus 12–21 °C (Joss *et al.*, 2005)) or the compounds examined (hormones (Ternes *et al.*, 1999b) versus nine nonhormonal PPCPs (Joss *et al.*, 2005)).

Another factor which can influence the biological transformation of PPCPs in WWTPs is retention time, both hydraulic retention time (HRT) and solids retention time (SRT). It might be expected that a shorter HRT would reduce the amount of biological transformation of PPCPs due to less time spent in the reactor. Several studies have found that, specifically in the case of ibuprofen and ketoprofen, a decreased HRT reduces biological transformation (Suárez *et al.*, 2008; Tauxe-Wuersch *et al.*, 2005; Ternes, 1998). In one study of a constructed wetland designed to further treat the secondary effluent of a WWTP, an extremely long HRT of 1 month appeared to improve the removal efficiencies of most PPCPs examined, including the more recalcitrant diclofenac and carbamazepine (Matamoros *et al.*, 2008). A study by Reif *et al.* (2008), however, found that variations in HRT between 12 and 24 h had no effect on the biological transformation of twelve PPCPs including ibuprofen. Similarly, there was no difference in the removal of select antibiotics in a fixed bed reactor (FBR) with an HRT of 1 h compared to CAS treatment with an HRT of 33 h (Göbel *et al.*, 2007). While this was attributed to a higher active biomass in the FBR (Göbel *et al.*, 2007) direct comparisons between different reactor types is problematic.

Perhaps more influential on biological transformation of PPCPs than HRT is SRT. Several studies have reported that increased SRT improves biological transformation of PPCPs (Clara *et al.*, 2005a; Göbel *et al.*, 2007; Reif *et al.*, 2008; Suárez *et al.*, 2005, 2008). Clara *et al.* (2005a) determined that, for most of the PPCPs they investigated, an $SRT_{10\ ^\circ C}$ (SRT at 10 °C) of at least 10 days is adequate for high removal rates, as measured by difference between influent and effluent concentration. Exceptions included inconsistent results for EE2 and diclofenac and no improvement with increased $SRT_{10\ ^\circ C}$ for carbamazepine (Clara *et al.*, 2004, 2005a). Coincidentally, an $SRT_{10\ ^\circ C}$ of at least 10 days corresponds to the design parameters for nitrogen removal and therefore WWTPs designed for nitrogen removal should be reasonably effective in removal of PPCPs (Clara *et al.*, 2005a). For some PPCPs, however, a longer SRT may substantially improve removal. For example, a two- to threefold increase in removal of trimethoprim and several macrolide antibiotics was observed in an MBR with an SRT of 60–80 days (running between 12 and 19 °C) compared to an SRT of 16–33 days (Göbel *et al.*, 2007).

One way to increase and more accurately control SRT as compared to CAS treatment plants is the use of MBRs (Artiga *et al.*, 2005; Reif *et al.*, 2008). In MBRs, a microfiltration or ultrafiltration membrane is used to retain suspended solids, increasing the concentration of suspended solids and biomass in the bioreactor as compared to CAS. Due to the increased SRT and biomass in MBRs, it has been hypothesized that MBRs would have improved biological transformation of PPCPs as compared to CAS. Most studies that have compared MBR and CAS treatment, however, have found that SRT itself is actually more important than reactor type when it comes to biological transformation of PPCPs (Clara *et al.*, 2005a; Göbel *et al.*, 2007; Joss *et al.*, 2005; Suárez *et al.*, 2008).

Increased SRT is thought to improve biological transformation of PPCPs in WWTPs for several related reasons. The slower SRT allows more time for the growth of bacteria and other microbes with longer generation times (Reif *et al.*, 2008), some of which may be critical for the biological transformation of certain PPCPs. For example, *Sphingomonas* sp. RD1, which has been shown to mineralize the antibacterial compound TCS (Hay *et al.*, 2001), has a doubling time of approximately 11 h at 18 °C (Kagle, unpublished data) compared to a doubling time of about 3 h for *Escherichia coli* under similar conditions (Cooper *et al.*, 2001). Allowing more time for growth may also improve the biodiversity within the reactor (Göbel *et al.*, 2007), increasing the chances of obtaining organisms in the reactor with the ability to degrade PPCPs. Finally, WWTPs have been traditionally designed for removal of compounds of natural origin (Daughton and Ternes, 1999), but often microbial populations need time to adapt, or acclimate, to anthropogenic

PPCPs before they can be degraded (Clara *et al.*, 2005a; Daughton and Ternes, 1999; Reif *et al.*, 2008; Ternes *et al.*, 2004).

Acclimation can consist of a variety of processes, from the population to genetic level (Alexander, 1999a). First, as mentioned earlier, the population of bacteria capable of metabolizing the compound can increase. In WWTPs, however, it is not just the microbes responsible for the actual metabolism that are important; other microbes may indirectly improve the degradation of pollutants without actually transforming it. For example, during the degradation of nonylphenol polyethoxylate surfactants (NPEOs) it was found that a *Bacillus* involved in floc-forming enhanced the NPEO degradation of two other strains capable of growing on NPEOs as their sole carbon and energy source (Di Gioia *et al.*, 2004). Second, even if the appropriate organisms are present in high enough numbers, in many instances the genes necessary for PPCP degradation are not constitutive and therefore need to be induced (Alexander, 1999a). Absence of gene induction may also be responsible for the observed delay in degradation of ibuprofen in sewage sludge prior to its rapid transformation (Buser *et al.*, 1999). Another example is that of *Sphingomonas* sp. strain RD1, a WWTP isolate which requires at least 2 mg of TCS per liter before the genes which encode the degradation of TCS are transcribed (Junker and Hay, 2004). Thus, although the biological potential for TCS biotransformation in WWTPs exists, the low concentration typically found in WWTPs ($\mu g\ l^{-1}$) may limit biotransformation (Federle *et al.*, 2002; Lindstrom *et al.*, 2002; Singer *et al.*, 2002; Stasinakis *et al.*, 2007). This so-called ''threshold'' effect has been reported to be a significant factor in the persistence of low concentrations of agricultural and industrial pollutants in the environment (Alexander, 1999a,c). Finally, many recalcitrant compounds are not utilized until other more easily degraded carbon sources have been depleted (Alexander, 1999a). The best known example of this is the diauxic growth of *E. coli* on a mixture of glucose and lactose or sorbitol. Although *E. coli* is capable of using either as a growth substrate, the catabolism of lactose is only initiated after glucose has been consumed (Monod, 1949). This phenomenon has also been observed in environmental samples for the organic pollutants 4-chlorophenol and 2,4-dichlorophenol (Kuiper and Hanstveit, 1984). That carbon source preference occurs with PPCPs is supported by the fact that in some studies it has been observed that providing additional carbon sources inhibits PPCP degradation (Chang *et al.*, 2004; Yuan *et al.*, 2004).

The successful removal of PPCPs by a WWTP can also be impaired by uncontrollable acts of nature. The importance of WWTP homeostasis on the extent of PPCP transformation was reported by Ternes (1998) who noted that excessive rain runoff from a storm event decreased the removal of nonsteroidal anti-inflammatory drugs (NSAIDs) and lipid regulators from 60% to 5% (Ternes, 1998).

V. BIOLOGICAL TRANSFORMATION OF PPCPs IN THE ENVIRONMENT

The incomplete removal of PPCPs by WWTPs results in the release of these micropollutants into receiving waters (Heberer, 2002; Jones *et al.*, 2005; Singer *et al.*, 2002; Ternes *et al.*, 2004). Although improvements to WWTPs can decrease the levels of PPCPs released (Miller *et al.*, 2008), many studies, (Daughton and Ternes, 1999; Halling-Sorensen *et al.*, 1998; Heberer, 2002), have detected PPCPs in rivers, streams, lakes, and other bodies of water as well as in their sediments. We know much less about the biological fate of these compounds in the environment than we do in WWTPs.

Most studies which investigate the biological transformation of PPCPs in the environment do so in collected environmental samples incubated in the laboratory rather than measuring the transformation *in situ*. For example, in lab experiments, the biological transformation of APs and alkylphenol ethoxylates (APEOs) have been examined in aerobic and anaerobic sediments from lakes (Chang *et al.*, 2004; Yuan *et al.*, 2004), rice paddies(Shibata *et al.*, 2006), and estuaries (Lu *et al.*, in press) as well as in river (Manzano *et al.*, 1999) and lake (Mann and Boddy, 2000) water. In aerobically incubated sediments, the reported half lives of APs range from 9 days for octyphenol (OP) in rice paddies (Shibata *et al.*, 2006) to 99 days for nonylphenol (NP) in some lake sediments (Yuan *et al.*, 2004). The degradation of the alkylphenol ethoxylates takes slightly longer, due to the removal of the ethylene oxide monomers which typically precedes degradation of the alkylphenol itself (Giger *et al.*, 1984; Swisher, 1987). Under anaerobic conditions, Chang and Yuan report similar values for NP and nonylphenol monoethoxylate (NP1EO) half lives in lake sediments (Chang *et al.*, 2004; Yuan *et al.*, 2004), but in rice paddy sediment no anaerobic degradation of OP was observed (Shibata *et al.*, 2006).

In lake water, it was found that although the primary degradation of nonylphenol polyethoxylates (NPEOs) proceeded quickly, short-chain ethoxylates (NP1–3EO) accumulated and did not fully degrade within 33 days (Mann and Boddy, 2000). This degradation was more complete in the dark compared to 12 h light–dark cycles, possibly due to less competition for resources between heterotrophic bacteria and algae (Mann and Boddy, 2000). Similar results were found in river water, with about 65% mineralization (32–70% depending on temperature) of NPEOs over 30 days due mainly to the removal of ethylene oxide monomers (Manzano *et al.*, 1999). Total mineralization of NP was not observed (Manzano *et al.*, 1999).

In addition to surfactants such as APs in the environment (further reviewed by Ying (2006)), the potential biological degradation of other PPCPs in environmental matrices has been investigated in the laboratory. In microcosms prepared with sediment from the Santa Ana River,

biological transformation of ibuprofen and gemfibrozil was observed, whereas naproxen was not transformed (Lin *et al.*, 2006). The biological transformation of ibuprofen was also observed in fortified lake water with a half life of approximately 20 days, with a preference for the S enantiomer (Buser *et al.*, 1999). Using cultivated river water biofilms from the South Saskatchewan and Elbe Rivers, Winkler *et al.* (2001) showed that ibuprofen is rapidly degraded and they also detected the ibuprofen metabolites carboxyibuprofen and hydroxyibuprofen. They observed the transient accumulation of carboxyibuprofen whereas the metabolite hydroxyibuprofen persisted (Winkler *et al.*, 2001). These metabolites, however, only accounted for 10% of the added ibuprofen and provided little insight into the main biodegradation mechanism (Winkler *et al.*, 2001). In the same study, no biological transformation of clofibric acid (a pharmacologically active degradation product of many blood lipid regulators) was observed in the same river biofilms (Winkler *et al.*, 2001).

Several studies have attempted to document the environmental fate of PPCPs *in situ*, but determining that biological mechanisms are responsible for removal is challenging. For example, Bendz *et al.* (2005) measured the concentrations of 27 PPCPs at several sites downstream of a WWTP for 7 km along the Höje River. They showed a decrease in the concentrations of several PPCPs including ibuprofen, naproxen, and diclofenac, but how much removal was due to abiotic factors such as exposure to UV light or sequestration versus biological transformation was not determined (Bendz *et al.*, 2005). The concentrations of other PPCPs, however, remained stable (i.e., trimethoprim) or fluctuated (i.e., sulfamethoxazole, carbamazepine, and gemfibrozil) (Bendz *et al.*, 2005) suggesting that biological transformation of these compounds was slow or nonexistent. To overcome the challenges associated with determining if *in situ* biological degradation is occurring some investigators have examined changes in the isotopic ratios of racemic PPCPs. For example, Buser *et al.* (1999) found changes in the enantiomeric ratios of *S* and *R* ibuprofen found in Lake Greifensee (Buser *et al.*, 1999), which suggested biodegradation was occurring. The usefulness of this methodology, however, is limited to racemic mixtures.

Studies of the fate of the biocides TCS and triclocarban (TCC) in river water as wells as freshwater and estuarine sediments provide some insight into the *in situ* biological transformation of these PPCPs (Lindstrom *et al.*, 2002; Miller *et al.*, 2008; Singer *et al.*, 2002). In the case of TCS, an increase in the concentration of methyl-TCS in surface waters was suggestive of biological transformation (Lindstrom *et al.*, 2002). In both freshwater and estuarine sediments, however, TCS has been found to persist for at least 30 years (Miller *et al.*, 2008; Singer *et al.*, 2002). Biological transformation of TCS, however, does proceed under certain environmental conditions (Miller *et al.*, 2008). For example, TCS was

recently found to be more labile in the oxic sediments of the Chesapeake Bay than in the anoxic sediments of Jamaica Bay (Miller *et al.*, 2008). This influence of oxygen availability agrees with the observed aerobic degradation of TCS (Hay *et al.*, 2001; Kagle, 2004; Meade *et al.*, 2001). Conversely, TCC is more persistent than TCS in oxic estuarine sediments, but under anoxic conditions, TCC appears to be reductively dechlorinated (Miller *et al.*, 2008), being used as a terminal electron acceptor during anaerobic respiration.

As suggested earlier, a variety of environmental factors can influence the biological transformation of PPCPs in environmental samples. In general, an increase in temperature increases biological transformation (Chang *et al.*, 2004; Manzano *et al.*, 1999; Yuan *et al.*, 2004). For example, at 7 °C 68% of NPEO in river water was degraded in 30 days whereas 96% was degraded at 25 °C (Manzano *et al.*, 1999). Depending on the metabolic mechanism, oxygen availability can impact the rate and extent of the biological transformation of PPCPs (Chang *et al.*, 2004; Yuan *et al.*, 2004). For example, Shibata *et al.* (2006) showed that although the half life of OP was only 9–19 days under aerobic conditions in rice paddies, OP was not degraded at all under flooded, anaerobic conditions.

The available alternative electron acceptors under anaerobic conditions can also be a factor. In anaerobic river sediments, NP was degraded most quickly under sulfate-reducing conditions, followed by methanogenic conditions, and most slowly under nitrate-reducing conditions (Chang *et al.*, 2004; Yuan *et al.*, 2004). This may be significant considering sulfate reducers are a major component of river sediment (Chang *et al.*, 2004; Yuan *et al.*, 2004). Also, the addition of alternate electron acceptors (manganese oxide and ferric chloride) can inhibit the anaerobic degradation of some compounds (Chang *et al.*, 2004; Yuan *et al.*, 2004).

As discussed before in reference to PPCP degradation in WWTPs, the availability of alternative carbon sources in addition to the PPCPs, specifically in the case of NP and NP1EO, can also inhibit their degradation (Chang *et al.*, 2004; Yuan *et al.*, 2004). As with any biological activity, other stressors such as heavy metals or increased salt concentration can also inhibit PPCP biodegradation (Chang *et al.*, 2004; Yuan *et al.*, 2004).

In surface waters, an important environmental factor is UV light. Although light exposure may increase competition for resources between algae and heterotrophic bacteria (Mann and Boddy, 2000), more typically it results in abiotic transformation of pollutants to intermediates that can then be further degraded. Many PPCPs, including TCS and sulfonamides, can undergo photolysis (Baran *et al.*, 2006; Lindstrom *et al.*, 2002). At pH $>$ 8, TCS is rapidly photolysed (Lindstrom *et al.*, 2002). This is the main degradation mechanism for TCS even in neutral and slightly acidic surface waters (Singer *et al.*, 2002; Tixier *et al.*, 2002). This photolysis, however, can result in the production of transformation products that

are more toxic than the parent compound. For TCS this has been evidenced by the formation of chlorinated dioxins as a result of UV exposure (Latch *et al.*, 2003; Lores *et al.*, 2005; Mezcua *et al.*, 2004). Photolysis of sulfonamides, on the other hand, results in phototransformation products which are typically more susceptible to biological transformation than the parent compounds (Baran *et al.*, 2006).

Biological transformation of PPCPs in environmental samples often requires adaptation or acclimation of the microbial community. For example, the degradation of NPEOs by the microflora of lakes (Mann and Boddy, 2000) and rivers (Manzano *et al.*, 1999) generally exhibits a lag time of several days, although the amount of lag time is highly influenced by temperature (Manzano *et al.*, 1999). Adaptation of the microbial community to NP degradation was also shown under both aerobic (Chang *et al.*, 2004; Yuan *et al.*, 2004) and anaerobic conditions (Chang *et al.*, 2004). Lin *et al.* (2006) found that with subsequent additions of ibuprofen and gemfibrozil to river microcosms, degradation of these compounds proceeded more rapidly, indicative of adaptation of the microbial community. For ibuprofen, this observation is supported by that fact that river biofilms adapted to ibuprofen degradation quickly degraded its metabolite hydroxyibuprofen, whereas hydroxyibuprofen was found to accumulate in unadapted biofilms (Winkler *et al.*, 2001). These studies, however, do not address the mechanism of adaptation or acclimation responsible for PPCP degradation. Unfortunately, very little is known about this process in natural systems, however, it is likely to involve an increase in the population of microbes capable of degrading the PPCP of interest, induction of necessary degradation genes, or some combination thereof.

VI. GROWTH ON PPCPs

The use of several PPCPs as growth substrates has been demonstrated for microbes from both WWTPs and natural environments. Examples of bacterial isolates, mixed cultures, and consortia capable of growth on select PPCPs are summarized in Table3.2. One of the most extensively studied groups of PPCPs in this respect is the nonionic surfactants, APEOs. Bacterial strains capable of growth on APEOs and their octyl- and nonylphenol metabolites have been isolated from activated sludge (i.e., Ajithkumar *et al.*, 2003; Barberio *et al.*, 2001; John and White, 1998; Porter and Hay, 2007; Tanghe *et al.*, 1999), rice paddies (Chang *et al.*, 2004; Chen *et al.*, 2006; Nishio *et al.*, 2002; Yuan *et al.*, 2004), and topsoil (Nishio *et al.*, 2005). Although most of these are aerobic bacteria, there is at least one report of bacterial isolates growing on NP and NP1EO anaerobically (Chang *et al.*, 2004). One such anaerobic isolate, AN3, was shown to degrade 94.9% of the supplied NP and 90.4% of the NP1EO in 63 days

(Chang *et al.*, 2004). Most of the APEO-degrading isolates are gram-negative (Ajithkumar *et al.*, 2003; Barberio *et al.*, 2001; Chang *et al.*, 2004; Chen *et al.*, 2006; John and White, 1998; Nishio *et al.*, 2002, 2005; Porter and Hay, 2007; Tanghe *et al.*, 1999; Yuan *et al.*, 2004). The aerobic degradation of APs by *Sphingomonas* sp. strain PWE1 (Porter and Hay, 2007), is described in further detail later.

Other surfactants have also been shown to support the growth of bacteria. More than 30 years ago, a *Bacillus* species capable of using anionic alkylbezne sulfonates as the sole carbon and sulfur source was isolated from sewage sludge and the metabolic pathway it employed was characterized (Willets and Cain, 1972). It was proposed that the sulfate and alkyl groups were oxidatively cleaved from the aromatic core (Willets and Cain, 1972). The resulting sulfate was then assimilated via adenosine sulfatophosphate, the alkyl side chain via β-oxidation, and the *p*-hydroxybezoate via an *ortho*-cleavage pathway (Willets and Cain, 1972).

Quaternary ammonium compounds (QACs) are cationic surfactants with a variety of uses including antistatic agents, emulsifiers, cosmetics, and hair rinses as well as antimicrobial products (Ahlstroem *et al.*, 1997; Korai and Takeichi, 1970; Kouma *et al.*, 2003; Liffourrena *et al.*, 2008; Takenaka *et al.*, 2007). Although it has been proposed that the antimicrobial properties of QACs might hinder their biodegradation (Liffourrena *et al.*, 2008; Nishiyama *et al.*, 1995; van Ginkel, 1991), QACs have also been shown to support the growth of bacterial isolates. Based on previous studies which found that *Pseudomonas* strains which could degrade QACs in coculture with another bacterium (Dean-Raymond and Alexander, 1977) or could use a portion of the QAC as its carbon source (van Ginkel *et al.*, 1992), Liffourrena *et al.* (2008) screened 14 *Pseudomonas* strains for their ability to grow using tetradecyltrimethylammonium (TDTMA) as a sole carbon and nitrogen source. Only one strain, *P. putida* A ATCC 12633 was found to have this ability (Liffourrena *et al.*, 2008). In another study, *Pseudomonas* sp. Strain 7–6 was isolated using a low concentration (0.05%) of the QAC *n*-dodecyltrimethylammonium chloride (DTAC) as its sole carbon source (Takenaka *et al.*, 2007). By gradually increasing the DTAC concentration on each transfer, however, an adapted variant of strain 7–6 was obtained which could grow using up to 0.4% DTAC as its sole carbon and nitrogen source (Takenaka *et al.*, 2007).

In addition to surfactants, other common ingredients of PPCPs can support the growth of bacteria. The chelating agent ethylenediaminetetraacetic acid (EDTA) is often used as a preservative for pharmaceutical and hygiene products and also has a variety of medicinal uses. Both mixed cultures (Gschwind, 1992; Nortemann, 1992) and isolates (Bohuslavek *et al.*, 2001; Lauff *et al.*, 1990; Nortemann, 1992; Payne *et al.*, 1998; Satroutdinov *et al.*, 2003; Weilenmann *et al.*, 2004; Witschel *et al.*, 1997) have been shown to aerobically mineralize EDTA, using it as the

sole carbon and nitrogen source. The metabolic pathway utilized by the isolates DSM 9103 (Gschwind, 1992; Weilenmann *et al.*, 2004) and BNC1 (Nortemann, 1992) have been further characterized (Kluner *et al.*, 1998; Nortemann, 1999; Payne *et al.*, 1998; Satroutdinov *et al.*, 2000; Witschel and Egli, 1997–1998; Witschel *et al.*, 1997, 1999). The EDTA substitute iminodisuccinate can also support the growth of bacteria (Cokesa *et al.*, 2004) and critical enzymes involved in its catabolism have been identified (Cokesa *et al.*, 2004). Polyethylene glycol (PEG), a synthetic polymer of various molecular weights used in the manufacture of pharmaceuticals and cosmetics, has been reported to be degraded both aerobically (Fincher and Payne, 1962; Haines and Alexander, 1975; Hosoya *et al.*, 1978; Kawai *et al.*, 1977, 1978; Ogata *et al.*, 1975; Patterson *et al.*, 1970) and anaerobically (Schink and Stieb, 1983) by mixed and pure cultures of bacteria.

The active ingredients of personal care products can also support the growth of bacterial isolates. For example, *P. putida* strain DTB which is capable of utilizing DEET as a sole carbon and nitrogen source, was isolated from sewage sludge (Rivera-Cancel *et al.*, 2007) and is discussed further below. Numerous other active ingredients can also support the growth of bacteria, even compounds as toxic as antibiotics. Although one might expect that antibiotics and other antimicrobials would present unique challenges to bacterial growth, the use of a number of such compounds as growth substrates has recently been reported (Dantas *et al.*, 2008). Dantas *et al.* (2008) isolated bacteria capable of growing on at least 13 different antibiotics from the soil of each of 11 different habitats. Interestingly, the isolates were phylogenetically diverse across gram-negative and gram-positive phyla and also tended to be highly cross-resistant to other antibiotics at therapeutically relevant levels (Dantas *et al.*, 2008). Bacteria had previously been isolated from natural environments which could grow using chloramphenicol (Abd-el-malek *et al.*, 1961) and penicillin-G (benzylpenicillin) (Johnsen, 1977; Kameda *et al.*, 1961). A consortium of bacteria isolated from activated sludge able to grow using the antimicrobial compound TCS has also been reported (Hay *et al.*, 2001). One organism which can mineralize TCS as measured by release of ^{14}C-labeled CO_2 from ^{14}C-TCS, *Sphingomonas* sp. RD1, was isolated from that consortium (Hay *et al.*, 2001). Another study also reported the isolation of two strains from compost, *P. putida* TriRY and *Alcaligenes xylosoxidans* subsp. *denitrificans* TR1, with the ability to use TCS as their sole carbon and energy source (Meade *et al.*, 2001).

Both natural and synthetic estrogens such as those found in birth control pills have been shown to support the growth of bacterial isolates. Isolates that can use the natural estrogens E1 (Ke *et al.*, 2007; Pauwels *et al.*, in press; Yoshimoto *et al.*, 2004), E2 (Fahrbach *et al.*, 2006; Fujii *et al.*, 2003; Ke *et al.*, 2007; Pauwels *et al.*, in press; Yoshimoto *et al.*, 2004; Yu *et al.*, 2007), and estriol (E3) (Ke *et al.*, 2007; Pauwels *et al.*, in press; Yoshimoto

et al., 2004) have been reported. Weber *et al.* (Weber *et al.*, 2005) reported on a defined mixed culture of *A. xylosoxidans* and *Ralstonia* sp. capable of growing on either E1 or E2. Although most studies have looked exclusively at aerobic growth on estrogens, Ke *et al.* (Ke *et al.*, 2007) examined their anoxic catabolism. Two isolates, LHJ3 (*Agromyces*) and CYH (*Sphingomonas*), were identified which could grow using E2 as a sole carbon source (Ke *et al.*, 2007). Although more recalcitrant in some studies (Joss *et al.*, 2006; Ke *et al.*, 2007), the synthetic estrogen EE2 has been reported to support the growth of bacteria (Haiyan *et al.*, 2007; Yoshimoto *et al.*, 2004) and the cometabolism of EE2 by bacteria growing on E1, E2, or E3 has also been observed (Pauwels *et al.*, in press).

Relatively few bacteria capable of growing on pharmaceuticals other than antibiotics, estrogens, and analgesics have been isolated. Of the common pain relievers, acetylsalicylic acid and 4-acetamidophenol have been reported to support the growth of bacterial isolates (Ahmed *et al.*, 2001; Grant *et al.*, 1970). More recently, we have reported on the ability of a *Sphingomonas* strain (Ibu-2) to grow on Ibuprofen as a sole carbon and energy source (Murdoch and Hay, 2005). The pathway by which Ibu-2 catabolizes ibuprofen is discussed in the following section.

VII. PATHWAYS FOR THE DEGRADATION OF SELECTED PPCPs

Although the isolation of pure cultures of microorganisms typically requires concentrations of PPCPs that are not environmentally relevant, lessons learned from the study of individual isolates capable of degrading industrial pollutants (Jeon *et al.*, 2003; Madsen and Alexander, 1985) suggest that this approach is likely to yield valuable insights regarding the degradation of PPCPs. Compared to agrochemicals and industrial pollutants, the degradative pathways of relatively few PPCPs have been elucidated. Below are the three examples of catabolic pathways of selected PPCPs (octylphenol, ibuprofen, and DEET) by bacterial isolates.

A. Octylphenol degradation by *Sphingomonas* sp. strain PWE1

APEOs are common nonionic surfactants with both industrial and household applications such as wetting agents, plasticizers, dispersants, degreasers, institutional cleaners, and in personal care products (Naylor *et al.*, 1992). NPEOs comprise the majority of the APEOs produced (80%) with octylphenol polyethoxylate (OPEOs) making up the remainder (Staples *et al.*, 1999). Whereas NPEOs are produced from a mixture of at least 22 different *para*-substituted isomers of nonylphenol (Wheeler *et al.*, 1997), only one OP isomer is utilized in OPEO production. In the

environment, primary aerobic microbial degradation of APEOs sequentially removes the ethoxy units (Swisher, 1987) until one or two remain. Under aerobic conditions, the remaining ethoxy units can be removed (Giger *et al.*, 1984), releasing the estrogenic nonylphenol (NP) and octylphenol (OP) (Isidori *et al.*, 2006; Jobling *et al.*, 1996; Nakamura *et al.*, 2002; White *et al.*, 1994). Although NPEOs are used in higher quantities than OPEOs, the degradation product OP has been shown to be 10 times more estrogenic than any isomer of NP (White *et al.*, 1994).

Most studies of bacterial biodegradation of long-chain APs (i.e., OP and NP) thus far suggest catabolism via ring hydroxylation and cleavage, without apparent side-chain modification, although side-chain modification has been suggested to occur in some cases (Yuan *et al.*, 2004). Analyses of the metabolism of NP and OP typically reveal the disappearance of the phenol moiety and the appearance of alcohols corresponding in length and branching pattern to the alkyl side chain of the original alkylphenol (Corvini *et al.*, 2004; Fujii *et al.*, 2000; Gabriel *et al.*, 2004; Tanghe *et al.*, 2000). These observations have been made with only a few exceptions (Telscher *et al.*, 2005). Taken together, these observations suggest ring cleavage is the main mechanism for biodegradation.

Metabolites (i.e., substituted hydroquinones and nonanol) detected by Corvini *et al.* suggest that *Sphingomonas* sp. strain TTNP3 catabolizes NP isomers via a type II *ipso* substitution mechanism (Corvini *et al.*, 2004, 2005, 2006, 2007) followed by catabolism of the resulting hydroquinone and alkyl metabolites (Corvini *et al.*, 2006). *Ipso* substitution is a mechanism whereby a group is added during electrophilic substitution of an aromatic ring and temporarily shares the same position as the leaving group (not hydrogen) (Abbie's dissertation). With respect to NP metabolism, oxygen is added to the same carbon that harbors the alkyl substituent on the *para*-substituted phenol and typicallys results in replacement of the alkyl substituent by the oxygen atom to produce hydroquinone and an alkyl ion, although it may results in rearrangements producing either an alkoxyphenol or an alkyl substituted hydroquinone (Corvini *et al.*, 2006). In type II *ipso* substitution the resulting alkyl ion is a carbocation whereas for type I it is a carboanion. A similar degradation mechanism has been suggested for *S. xenophaga* Bayram (Gabriel *et al.*, 2007; Kohler *et al.*, 2008).

Sphingomonas sp. strain PWE1 was isolated from activated sludge and can grow on OP as its sole carbon and energy source (Porter and Hay, 2007). Based on the previous studies suggesting *ipso*-substitution as the initial step in alkylphenol catabolism by *Sphingomonads*, molecular and metabolic analyses were used to further characterize the catabolic pathway for OP in PWE1. Several OP metabolites of PWE1 were identified that were consistent with the type II *ipso* substitution mechanism discussed above including hydroquinone (Porter and Hay, 2007). Porter therefore cloned and expressed a gene from PWE1 in *E. coli* that conferred the

ability to produce hydroquinone from OP. Expression of that gene, labeled o*pdA* (for OP-degradation), in *E. coli* also lead to the production of 2,4,4-trimethyl-1-pentene and 2,4,4-trimethyl-1-pentanol (Porter and Hay, 2007). The presence of these metabolites strongly suggests OP degradation by PWE1 is initiated through type II *ipso* substitution and is encoded by a single gene (Fig. 3.1).

In silico analyses of *opdA* revealed a conserved monooxygenase domain and a flavin adenine dinucleotide (FAD)-binding domain (Porter and Hay, 2007) and a weak similarity to a putative polyketide hydroxlase (Porter and Hay, 2007). A genetic investigation of two other *Sphingomonads* suggested to degrade APs via type II *ipso* substitution, *Sphingomonas* sp. strain TTNP3 and *S. xenophaga* Bayram, revealed genes homologous to o*pdA* (Wise, 2008). As with *opdA,* when heterologously expressed, the cloned homologous genes from TTNP3 and Bayram were both able to produce 2,4,4-trimethyl-1-pentene from OP (Wise, 2008). In further support of the necessity of *opdA* for alkylphenol degradation in these strains, neither its gene nor its protein could be detected in a

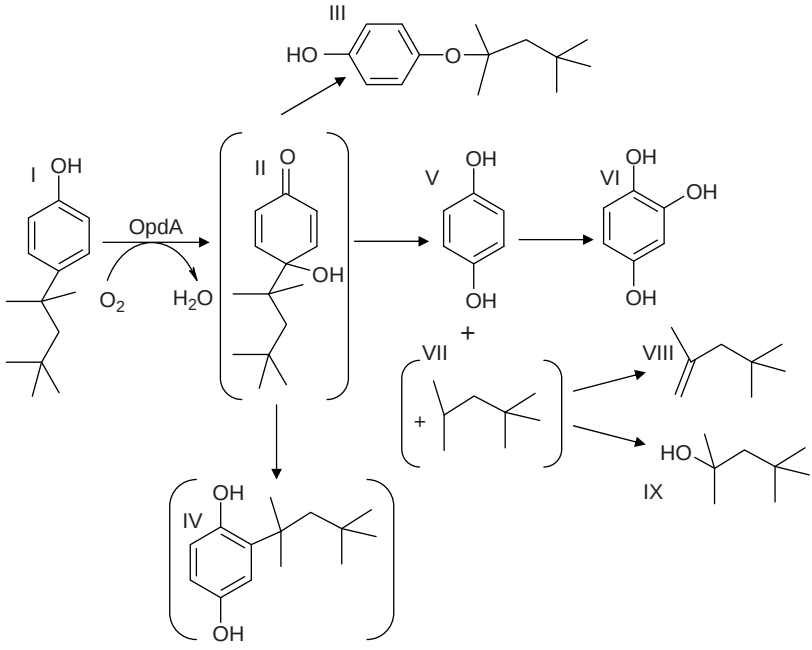

FIGURE 3.1 Proposed pathway for OP degradation by *Sphingomonas* sp. strain PWE1. I: OP; II: *ipso* hydroxylation intermediate; III: octyloxyphenol; IV: 2-octylbenzenediol; V: HQ; VI: 1,2,4-trihydroxybenzene; VII: alkyl side chain carbocation; VIII: 2,4,4-trimethyl-1-pentene; IX: 2,4,4-trimethyl-1-pentanol. Putative intermediates are in parentheses.

TTNP3 mutant unable to degrade nonylphenol (Porter *et al.*, submitted for publication).

PWE1 appears to use the putative flavin monooxygenase OpdA to initiate degradation of OP by adding a hydroxyl group to the alkyl-substituted carbon of OP (Fig. 3.1) (Porter and Hay, 2007). This intermediate, through type II *ipso* substitution, then converts to either octyloxyphenol or hydroquinone and a carbobcation corresponding to the octyl sidechain (Porter and Hay, 2007). This genetic and metabolic evidence suggest this mechanism for alkylphenol degradation may be conserved, at least within *Sphingomonads* (Porter and Hay, 2007; Wise, 2008), several of which have been reported to grow on hydroquinone (Corvini *et al.*, 2006).

B. Ibuprofen degradation by *Sphingomonas* sp. strain Ibu-2

Ibuprofen (2-(4-isobutylphenyl)-propionic acid) is the third most consumed drug in the world (Buser *et al.*, 1999), being used for its analgesic, antipyretic, and anti-inflammatory properties. Although, as discussed before, ibuprofen is readily biologically transformed under most wastewater treatment and environmental conditions, little is known concerning the routes by which environmental bacteria degrade ibuprofen. Some studies have detected ibuprofen metabolites in WWTPs or the environment (Buser *et al.*, 1999; Chen and Rosazza, 1994; Kolpin *et al.*, 2002; Zwiener *et al.*, 2002) and others have investigated the degradation of similar compounds (Andreoni *et al.*, 1992; Defrank and Ribbons, 1976, 1977; Eaton, 1996, 1997; Ismail *et al.*, 2003; Kuge *et al.*, 1991; Mohamed *et al.*, 2002; Sparnins and Chapman, 1976; Van Den Tweel *et al.*, 1988), but the most extensively characterized metabolism of ibuprofen is that of the ibuprofen-utilizing *Sphingomonas* sp. strain Ibu-2 (Murdoch and Hay, 2005).

Studies of the catabolism of compounds structurally similar to ibuprofen have revealed several different metabolic strategies. In some cases, transformation begins with the aromatic ring. *P. putida* F1 carrying the *cmt* operon initiates degradation of cumate (4-isopropylbenzoate) with dioxygenation at the 2,3 position of the aromatic ring followed by *meta* cleavage of the ring (Defrank and Ribbons, 1976, 1977; Eaton, 1996, 1997). *Streptomyces rimosus* has been shown to hydroxylate 2-phenylpropionic acid (2PPA) to 4-hydroxy-2PPA (Kuge *et al.*, 1991). The well-characterized homoprotocatechuate (Sparnins and Chapman, 1976) and homogentisate (Van Den Tweel *et al.*, 1988) pathways, wherein the ring is initially monooxygenated have also been implicated in the degradation of aromatic acids. In other instances, the initial site of modification is the acid substituent. In *E. coli* K12, phenylacetic acid is first converted to phenylacetyl-CoA and undergoes further metabolism that is reminiscent of β-oxidation (Ismail *et al.*, 2003). On the other hand, 2PPA (Andreoni *et al.*, 1992) and

tropic acid (2-phenyl-3-hydroxypropionic acid) (Mohamed *et al.*, 2002) catabolism by *P. cepacia* begins with their decarboxylation.

Sphingomonas sp. strain Ibu-2 was isolated from activated sewage sludge for its ability to use ibuprofen as a sole carbon and energy source (Murdoch and Hay, 2005). In addition to ibuprofen, resting cells of Ibu-2 can transform phenylacetic acid, 3- and 4-tolylacetic acid, 2PPA, and 2-(4-tolyl)-propionic acid (Murdoch and Hay, 2005). Chiral capillary electrophoresis (CE) analysis of the supernatant of Ibu-2 grown on racemic ibuprofen revealed a slight preference for *R*-ibuprofen, but both isomers were ultimately metabolized to the same extent (Murdoch and Hay, 2005).

The transient appearance of a yellow color in the supernatant ibuprofen-grown Ibu-2 cultures suggested *meta* cleavage of the aromatic ring during catabolism (Murdoch and Hay, 2005). The presence of *meta* cleavage in Ibu-2 was confirmed by poisoning of ibuprofen-grown Ibu-2 cultures with the *meta* cleavage inhibitor 3-fluorocatechol, examining enzyme activity of cell-free extracts, and performing GC–MS analysis of the supernatant of ibuprofen-grown cultures (Murdoch and Hay, 2005). Cell-free extracts of Ibu-2 produced yellow *meta* cleavage product from catechol, 3-methylcatechol, and 4-methylcatechol, but not from ibuprofen directly (Murdoch and Hay, 2005).

GC–MS analyses revealed that the downstream products of *meta* cleavage by ibuprofen grown cells, interestingly were not of dihydroxylated ibuprofen as might have been expected, but were of isobutylcatechol (Murdoch and Hay, 2005). The production of 4-isobutylcatechol during ibuprofen catabolism by Ibu-2 was confirmed through GC–MS analysis of the supernatant of 3-fluorocatechol-poisoned ibuprofen-grown cultures of Ibu-2 (Murdoch and Hay, 2005). Resting cells of Ibu-2 treated with 3-fluorocatechol also produced the corresponding catechols from phenylacetic acid, 3- and 4-tolylacetic acid (Murdoch and Hay, 2005). These analyses suggested that the removal of the propionic acid side chain of ibuprofen and dioxygenation to 4-isobutylcatechol precedes *meta* cleavage in the catabolism of ibuprofen by Ibu-2.

Genetic analysis of Ibu-2 was done by screening a fosmid library of Ibu-2 DNA expressed in *E. coli* for ability to produce hydroxylated intermediates of ibuprofen. This revealed several genes proposed to be involved in ibuprofen catabolism (Murdoch and Hay, 2006). A cluster of five putative genes was identified, named *ipfABDEF*, which were most similar to genes for large and small subunits of aromatic dioxygenases (*ipfA* and *ipfB*), enzymes for the addition or removal of acyl groups (*ipfD*), and coenzyme A ligases (*ipfF*) (Murdoch and Hay, 2006). No putative function could be assigned to *ipfE*, although it did group with a domain of unknown function (Murdoch and Hay, submitted for publication). Further analysis of *ipfF* revealed that it conferred coenzyme A ligase activity toward both phenylacetic acid and ibuprofen (Murdoch and

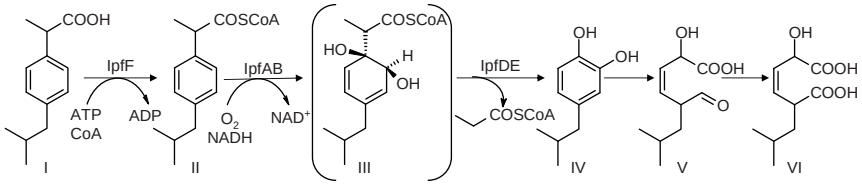

FIGURE 3.2 Proposed pathway for ibuprofen degradation by *Sphingomonas* sp. strain Ibu-2. I: ibuprofen; II: ibuprofen-CoA; III: 1,2-*cis*-diol-2-hydroibuprofen-CoA; IV: 4-isobutylcatechol; V: 5-formyl-2-hydroxy-7-methylocta-2,4-dienoic acid; VI: 2-hydroxy-5-isobutylhexa-2,4-dienedioic acid. Putative intermediates are in parentheses.

Hay, 2006). No transformation of ibuprofen was detected in an *ipfF* knockout, suggesting it is required for the first step in the metabolic pathway (Murdoch and Hay, 2006).

Through a combination of metabolite and genetic analyses, a pathway for the degradation of ibuprofen has been proposed (Fig. 3.2) (Murdoch and Hay, 2005, 2006). Like phenylacetic acid metabolism in *E. coli* K12 (Ismail *et al.*, 2003), ibuprofen degradation by Ibu-2 appears to be initiated by the formation of ibuprofen-CoA, most likely catalyzed by the coenzyme A ligase encoded by *ipfF* (Murdoch and Hay, 2006). This is followed by dioxygenase attack (encoded by *ipfAB*) to form a putative 1,2-*cis*-diol-2-hydroibuprofen-CoA (Murdoch and Hay, submitted for publication). Based on sequence similarity to enzymes of known function, the putative acyl transferase *ipfD* is likely to be involved in the subsequent removal of the propionic acid side chain to form 4-isobutylcatechol which is then further metabolized via *meta* cleavage (Murdoch and Hay, submitted for publication). Although this metabolic pathway shares some aspects of both phenylacteic acid metabolism by *E. coli* K12 (i.e., ligation to coenzyme A) and cumate metabolism by *P. putida* F1 (i.e., *meta* cleavage), it represents a unique hydrid of these two pathways. The metabolites produced during Ibuprofen metabolism by Ibu-2 are short-lived and would not have been detected using the techniques previously employed to monitor ibuprofen degradation in environmental samples (Zwiener *et al.*, 2002). It remains to be seen, however, if ibuprofen is metabolized in the environment via an Ibu-2-like pathway or by some as yet unknown mechanism.

C. Deet degradation by *P. putida* DTB

DEET is a widely used insect repellent that acts by inhibiting the ability of biting insects to sense the attractants lactic acid (Dogan *et al.*, 1999) and 1-octen-3-ol (Ditzen *et al.*, 2008) which are present in human sweat and breath. Annual domestic usage of DEET is estimated to be 1.8 million kg (U.S. Environmental Protection Agency, 1998). It was one of the

organic wastewater contaminants most frequently detected in U.S. streams in a study conducted by the U.S. Geological Survey in 1999 and 2000, being detected in 74% of the streams surveyed at concentrations up to 1.1 μg l^{-1} (Kolpin *et al.*, 2002).

The fate of DEET in the environment is not well-known. A study by Knepper (2004) revealed that DEET was degraded to some extent in WWTPs in Germany, but only after a period of adaptation and with influent concentrations over 1 μg l^{-1}. The frequent detection of DEET in aquatic environments in the U.S. reported by Kolpin *et al.* (2002) and Sandstrom *et al.* (2005) might be a consequence of limited degradation by microbes in WWTPs.

There are very few published examples of microbial metabolism of DEET. One of these reported the partial degradation of DEET by the fungi *Cunninghamella elegans* and *Mucor ramannianus* R-56 (Seo *et al.*, 2005). These fungi metabolize DEET by *N*-oxidation and *N*-deethylation to produce *N,N*-diethyl-*m*-toluamide-*N*-oxide and *N*-ethyl-*m*-toluamide. *C. elegans* also produced *N*-ethyl-*m*-toluamide-*N*-oxide. A second example is the aerobic degradation of DEET by *P. putida* DTB (Rivera-Cancel *et al.*, 2007). *P. putida* DTB was isolated from activated sludge based on its ability to utilize DEET as a sole carbon an energy source.

High performance liquid chromatographic (HPLC) analysis of the supernatant of DTB grown on DEET revealed the disappearance of DEET and transient appearance of two metabolites: 3-methylbenzoate and 3-methylcatechol (Rivera-Cancel *et al.*, 2007). When cultures of DTB were incubated with either DEET or its metabolite 3-methylcatechol they accumulated a yellow intermediate indicative of *meta* cleavage of 3-methylcatechol (Rivera-Cancel *et al.*, 2007) (Fig. 3.3).

The transient accumulation of 3-methylbenzoate suggests that the hydrolysis of diethylamine from the amide bond of DEET is the first

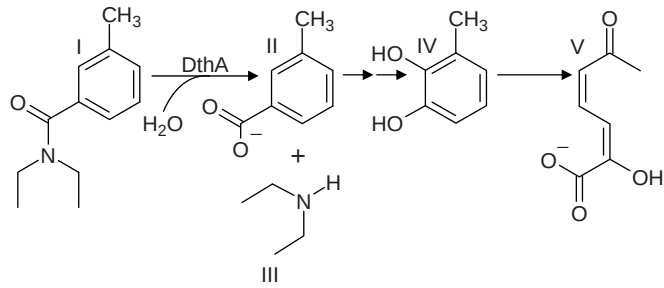

FIGURE 3.3 Proposed pathway for DEET degradation by *Pseudomonas putida* DTB. I: DEET; II: 3-methylbenzoate; III: diethylamine; IV: 3-methylcatechol; V: 2-hydroxy-6-oxo-hepta-2,4-dienoate.

step in degradation. The stoichiometric accumulation of diethylamine in DTB cultures grown on DEET was confirmed using GC–MS (Rivera-Cancel *et al.*, 2007). Although these results indicate that the strain DTB is not capable of using diethylamine (and consequently DEET) as a nitrogen source, two organisms from the genuses *Arthrobacter* and *Pseudomonas* that form part of the consortium from which DTB was isolated are capable of utilizing diethylamine as a sole carbon and nitrogen source (Rivera-Cancel, unpublished data).

Rivera-Cancel *et al.* (2007) screened a fosmid library of DTB DNA in *E. coli* for the ability to produce diethylamine from DEET and susequently identified one gene, *dthA*, which was responsible for hydrolysis of DEET's amide bond (Rivera-Cancel *et al.*, 2007). Subcloning and heterologous expression of *dthA* in *E. coli* confirmed that this gene encoded the ability to hydrolyze DEET to 3-methylbenzoate and diethylamine (Rivera-Cancel *et al.*, 2007).

Sequence analysis suggested that DthA was related to the α/β hydrolase fold family of enzymes. This is a diverse family of enzymes that includes lipases, proteases, and esterases, among others (Nardini and Dijkstra, 1999). *In silico* analyses further revealed similarity to a few proteins of known function including the cocaine esterase of *Rhodococcus* sp. MB1 (Bresler *et al.*, 2000) (24% identity) and the glutaryl-7-aminocephalosporanic acid acylase from *Brevibacillus laterosporus* J1 (Aramori *et al.*, 1991) (22% identity). These results were consistent with the observation that DthA-mediated DEET hydrolysis was completely inhibited by the serine protease inhibitor AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride).

Although these results suggest that the metabolic potential for DEET degradation exists, at least in engineered environments such as WWTPs, it remains unclear how DEET is actually degraded in natural environments and why it persists at low $\mu g\ l^{-1}$ levels.

VIII. CONCLUSIONS

The biodegradation of PPCPs faces several challenges given their diverse chemical structures, steady release into the environment, and low environmental concentrations. Significant progress has been made, however, in understanding the role of microbial metabolism in the transformation and removal of PPCPs in WWTPs and natural aquatic systems. There are consistent patterns, such as the observation of periods of acclimation, in both WWTPs and environmental samples. Although the biodegradability of PPCPs appears to be mainly determined by their chemical structure, manipulation of WWTP variables such as temperature, SRT, and possibly even composition of the microbial community could be investigated as methods to improve the removal of PPCPs from wastewater. Further

studies of the metabolites formed during the biodegradation of more PPCPs are needed in order to perform accurate mass balances of the environmental fate of PPCPs and to begin to understand what risks these degradation products might pose. Given what the scientific community has learned regarding the biotransformation of industrial and agricultural pollutants in the recent past, it is likely that additional effort regarding the isolation and characterization of PPCP-degrading microorganisms could lead to an increased understanding of the degradative pathways by which PPCPs are transformed and provided us with the insights necessary to reduce the environmental impact of these compounds.

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CHAPTER 4

Bioremediation of Cyanotoxins

Christine Edwards and **Linda A. Lawton**

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Abstract

Cyanobacteria are a diverse group of mainly aquatic microorganisms which occur globally. Eutrophication (nutrient enrichment) of water bodies, often as a result of human activities, results in prolific grow of cyanobacteria that develop into a thick scum or bloom. Many of these blooms are toxic due to the production of hepatotoxins (microcystins and cylindrospermopsin) and/or neurotoxins (saxitoxins and anatoxins) posing a serious health hazard to humans and animals. The presence of these cyanotoxins is of particular concern in drinking water supplies where conventional water treatment often fails to eliminate them. Hence, there is significant interest in water treatment strategies that ensure the removal of cyanotoxins, with the exploitation of microbes being on such possible approach.

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As naturally occurring compounds it is assumed that these toxins are readily biodegraded. Furthermore, there is no significant evidence of their accumulation in the environment and their relative stable under a wide range of physico-chemical conditions, suggests biodegradation is the main route for their natural removal from the environment. Microcystins, as the most commonly occurring toxins, have been the most widely studied and hence form the main focus here. The review provides an overview of research into the biodegradation of cyanotoxin, including evidence for natural bioremediation, screening and isolation of toxin biodegrading bacteria, genetic and biochemical elucidation of a degradation pathway along with attempts to harness them for bioremediation through bioactive water treatment processes.

I. INTRODUCTION

Cyanobacteria (blue-green algae) are a diverse group of ancient (3.5 billion year old) autotrophs found in many habitats but especially associated with water bodies such as lakes and ponds. Nutrient enrichment of water bodies (eutrophication), particularly elevated levels of nitrate and phosphate, often occurs due to agricultural practices, sewage disposal, and industrial activities. Following eutrophication, cyanobacteria are typically found to dominate the phytoplankton community, growing to high density and forming a thick scum of biomass, commonly referred to as a bloom. Blooms cause many problems for both the natural ecosystem and water users. In a natural ecosystem they can result in significant fish-kills usually thought to be due to the depletion of oxygen that is rapidly consumed by heterotrophic bacteria which proliferate as the bloom dies and decays. However, some fish mortalities have been traced to the presence of toxins. Where water is being used for drinking water or industrial processes, the presence of bloom material can result in blocked filters and drinking water can also become tainted with odorous compounds (especially geosmin and methylisoborneol) released by certain cyanobacteria. Of most concern, however, are the highly toxic metabolites known collectively as cyanotoxins which are typically observed in at least half of the blooms analyzed. Cyanotoxins are now known to be ubiquitous, reported around the globe in association with cyanobacterial blooms particularly in lakes, reservoirs, ponds, and rivers with low flow rates as may be observed in times of drought or excessive abstraction.

Cyanotoxins are a group of structurally and biochemically diverse toxic secondary metabolites produced by cyanobacteria which have been responsible for human and animal poisonings. The most commonly observed and studied group of cyanotoxins are the microcystins, a group of chemically related cyclic heptapeptides observed to be potent

hepatotoxins and class 2B carcinogens (Grosse *et al.*, 2006). Nodularins are similar in both their chemistry and biological activity although they differ structurally in that they are composed of only five amino acids. The other main group of cyanotoxins is the neurotoxins, fast acting toxins namely the saxitoxins, anatoxins-a and the rarely observed anatoxin-a(s). Finally, the most recently discovered cyanotoxin, cylindrospermopsin, a potent protein synthesis inhibitor, is of growing concern due its apparent increasing prevalence.

It is not surprising that increasing efforts are now being made to monitor and minimize the human health risks associated with cyanotoxins. An important aspect of this is a robust knowledge of the persistence of the toxins in aquatic systems, including an understanding of their biodegradation and assessment of the toxicity of degradation product. Furthermore, there is growing interest in bioremediation for the efficient removal of these hazards from potable water supplies.

II. HEPATOXIC PEPTIDES—MICROCYSTINS AND NODULARINS

Microcystins are the most commonly occurring cyanotoxins, produced by several genera of cyanobacteria including benthic and planktonic species, hence they are the most widely studied. The microcystins are a group of >70 variants where the core cyclic structure is comprised of seven amino acids, cyclo-(D-Ala^1-X^2-D-MeAsp^3-Z^4-Adda^5-D-Glu^6-Mdha^7), where X and Z represent variable L-amino acids and Adda is the unusual amino acid (2S,3S,8S,9S,)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Fig. 4.1). As well as variation in amino acids at positions 2 and 4, there are many additional variants with minor modifications such as methylation and demethylation. Nomenclature is based on the variable amino acids present in position 2 and 4, for example, MC-LR consists of leucine (L) at position 2 and arginine (R) at position 4. Nodularins are a small group of related pentapeptides where the structure of nodularin is cyclo-(D-MeAsp^1-L-Arg^2-Adda^3-D-Glu^4-Mdhb^5) where Mdhb represents *N*-methyldehydrobutyrine (Mazur-Marzec *et al.*, 2006). Toxicity, LD_{50} based on i.p. mouse bioassay, ranges from 50 to >1,000 μg kg^{-1}, depending on the variant, with the most toxic being MC-LR and MC-LA.

The toxicity of microcystins and nodularins is attributed to their potent inhibition of essential protein phosphatase enzymes (PP1 and PP2A) which results in cellular chaos, most noticeable in liver where there is a high degree of necrosis and hemorrhage. Since toxicity has been correlated with inhibition of either PP1 or PP2A, the inhibition of these enzymes has been exploited as a useful tool for detection of these compounds and their total toxicity.

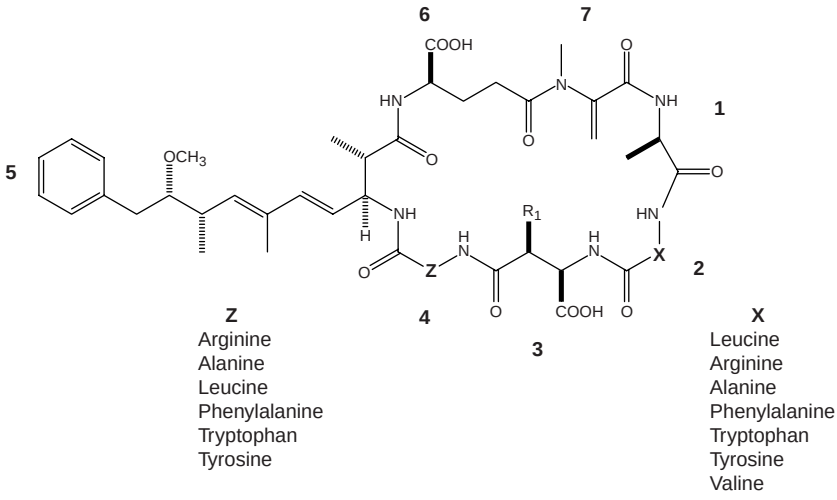


FIGURE 4.1 Generic structure of microcystins; cyclo-(D-Ala[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-Glu[6]-Mdha[7]) where X and Z represent variable L-amino acids. Additional variants occur as a result of minor modifications, such as methylation, demethylation and modification to Adda (isomerization to 6(Z)-Adda, hydroxylation and acetoxylation giving DMAdda and ADMAdda, respectively).

Microcystins have been responsible for many animal and human toxicoses and are reviewed elsewhere (Van Apeldoom *et al.*, 2007; Zurawell *et al.*, 2005) with the most notable incident being the death of 76 dialysis patients in 1996, who suffered from acute liver failure at a clinic in Caruaru, Brazil, after water contaminated with microcystins was used in their renal dialysis treatment (Carmichael *et al.*, 2001). As well as acute toxicity, exposure to low concentrations over a long period has been linked in the cause of primary liver cancer in studies in China (Ueno *et al.*, 1996). Another study implicated the chronic exposure to microcystins as the cause of colorectal cancer (Zhou *et al.*, 2002). The potential chronic toxicity suggested by these studies, combined with toxicology data led the WHO to establish a guideline of 1 μg L^{-1} as a maximum concentration of MC-LR in drinking water (WHO, 1998). In addition, the International Agency for Research on Cancer (IARC) classified MC-LR as a potential carcinogen (Group 2B) in June 2006 (Grosse *et al.*, 2006). Insufficient data were available to classify other microcystins or nodularin but it would be pertinent to assume they are potential carcinogens.

A. Persistence and biodegradation

Microcystins remain within the cells of actively growing cyanobacteria and are only released into the water upon senescence or induced lysis, by use of algicides. The microcystins may then be removed by adsorption

onto sediments, physicochemically degraded and biodegraded. However, the cyclic nature of microcystins renders them highly stable and potentially recalcitrant, presenting a hazard to the aquatic environment and its users. Biodegradation would appear to be the main fate for most microcystins in aquatic systems although it is dependent on environmental conditions, and high levels may persist for several weeks, posing a serious health hazard and a challenge to water utility management. In one study MC-LR persisted in the surface water for 2 weeks and was still detectable after a month (Jones and Orr, 1994).

The process of natural bioremediation for removal of microcystins from water bodies has been the subject of many studies. All of these studies have adopted a similar protocol, essentially the collection of water or sediment samples as a source of natural microbial biota followed by addition of microcystins in the laboratory then their disappearance monitored over a period of time, typically by HPLC. The experimental design includes sterile controls, to confirm that removal is due to biological activity as opposed to adsorption or physicochemical degradation.

In one of the first detailed studies crude extracts of *Microcystis*, containing MC-LR at a concentration of 1 mg L^{-1} was degraded by natural bacterial flora (indigenous microflora) in water from the Murrumbidgee River in 5 d after an initial lag phase of 3 d (Jones *et al.*, 1994). Similar lag phases and half-lives have been reported from numerous studies (Cousins *et al.*, 1996; Edwards *et al.*, 2008) furthermore we observed similar half-lives for MC-LR degradation regardless of the time of year water samples were collected (Table 4.1). Jones *et al.* (1994) observed no lag phase on re-addition of MC-LR to water samples, suggesting either an increase in the number of microcystin-degrading bacteria or induction of enzymes for rapid removal of MC-LR. In the same study, river water supplemented with acetate or glucose exhibited no lag phase but the rate of degradation was reduced, suggesting that the degradation process was based on constitutive enzymes rather than a specialized inducible system. It was suggested by Saito and co-workers (2003a) that long lag phases

TABLE 4.1 Half-lives of MC-LR biodegradation in Scottish waters

	Half-life (d)			
Water body	March 2005	August 2005	November 2005	November 2006
Forfar Loch	9.0	10.5	8.0	nt
Loch Rescobie	4.0	4.5	6.5	5.0
River Carron	13	10.5	16.5	nt

nt = not tested.

observed in water from Japanese lakes were due to specific induction mechanisms. This hypothesis, where the lag period represents the induction of specific microcystin-degrading enzymes, was shown to be unlikely in work by Lam *et al.* (1995) where differences in the lag phase were associated with the numbers of bacteria present. In addition they demonstrated that loss of the MC-LR peak by HPLC was paralleled with the loss of toxicity, as observed by utilization of the protein phosphatase inhibition assay indicating that degradation products were no longer toxic.

Another study, demonstrated that the most rapid loss of microcystins occurred when crude extracts of microcystin-containing cyanobacteria were incubated with water or sediment samples taken from a eutrophic lake during or after a hepatotoxic bloom (Rapala *et al.*, 1994). In this study removal of MC-LR (approximately 120 μL^{-1}) and [D-Asp^3]MC-LR (approximately 90 μL^{-1}) occurred within 4 d. Degradation was shown to take twice as long when water was taken from an area of the same lake where no bloom had occurred suggesting the presence of microcystin-containing blooms encourages the establishment of microbial populations capable of degrading microcystins and other peptides. They also demonstrated that water from an oligotrophic and a humic lake had indigenous, microcystin-degrading populations, with complete removal of microcystins by 8 and 14 d, respectively. Most likely the longer degradation observed in the humic lake was due to high concentration of organic carbon as an additional source of nutrients. Similar rates of degradation were obtained by Christoffersen *et al.* (2002) who found rapid degradation of both MC-LR and microcystins in crude cell lysates added to water from several lakes frequently exposed to microcystin-containing blooms, although no lag phases were observed, probably due to high numbers of bacteria.

Most studies have focused on aerobic biodegradation in samples of surface waters from selected water bodies, however, processes occurring in the sediment play a vital role in the aquatic habitat. Holst *et al.* (2003) examined removal of ^{14}C-labeled MC-LR from sediments which allowed determination of degradation with the evolution of $^{14}CO_2$ as mineralization was achieved. This approach, combined with the protein phosphatase inhibition assay for quantification, demonstrated microbes in the sediment reduced the concentration of MC-LR from 70 to 20 $\mu g\ L^{-1}$ in 1–2 weeks. In contrast, the same sample was able to reduce MC-LR from 100 to 20 $\mu g\ L^{-1}$ within 1 d under anoxic ($<0.3\%\ O_2$) conditions when nitrate was added. The simultaneous production of N_2O suggested that the degradation of MC-LR in the sediments may be coupled to denitrification thus providing a further mechanism for microcystin removal. This importance of sediments in elimination of microcystins from the aquatic environment was supported in a recent study in Lake Taihu, China which demonstrated rapid biodegradation of microcystins in sediments, which

also contained a naturally occurring pool of microcystins (Chen *et al.*, 2008). This detailed study also evaluated the degradation capability of natural microflora in surface water, water overlaying sediment and the sediment at several sites. Most rapid degradation was observed in the sediment, followed by the overlaying water, with slowest biodegradation observed in the surface water. Toruńska and co-worker (2008) investigated the biodegradation of nodularin under a range of conditions, finding that seawater plus sediment resulted in the most rapid degradation, again illustrating the importance of benthic bacteria.

While biodegradation of microcystins has been clearly demonstrated in the laboratory, only a few studies have extended this to the field. Christoffersen *et al.* (2002) used transparent microcosms in one eutrophic lake to monitor fate of microcystins and changes in bacterial community to follow up laboratory experiments. Lysate of *Microcystis* sp. containing total microcystin of 54 μg L^{-1} and bloom extract (2.3 μg L^{-1}) was degraded rapidly in the fixed volume microcosms with only trace amounts detectable at 8 d. Based on this study it would take 3.8 and 1.3 d to reduce the concentration of dissolved microcystin in the *Microcystis* and bloom lysate, respectively, to below the WHO guideline level of to 1 μg L^{-1} (although this is generally viewed as a guideline for concentration in drinking as opposed to raw water). Bacterial abundance and production rates increased immediately after addition of the lysates and correlated with the reduction of dissolved organic carbon (DOC), peaking after 1–2 d. There was only a temporary increase in species diversity on analysis by density gradient gel electrophoresis (DGGE) in the laboratory experiments, and no significant changes in diversity/abundance in the field experiment.

The prediction that degradation of MC-LR was a result of the activity of aquatic bacteria was supported by the successful culturing of bacterial isolates capable of utilizing MC-LR as a sole carbon and energy source.

B. Biodegrading bacteria

To date only a small number of bacteria with the capability of degrading microcystins have been isolated and they all belong to the family Proteobacteria (Table 4.2). Until recently the only bacteria characterized which were capable of degrading these cyclic peptides were of the genus *Sphingomonas*. However, in the last few years, Rapala *et al.* (2005) described a novel bacterium, *Paucibacter toxinivorans* capable of degrading microcystins and nodularin, and a strain isolated in Japan, Y2, was classified as *Sphingosinicella microcystinivorans* (Maruyama *et al.*, 2006). All of these bacteria have been isolated and screened using similar traditional microbiological approaches, for example, one Finnish study where 100 bacterial strains were isolated from water and sediment in freshwater

TABLE 4.2 Isolated microcystin/nodularin degrading bacteria

Bacteria	MC degraded	MC not degraded	Reference
Sphingomonas ACM-3962	LR, RR	NOD	Bourne *et al.* (1996)
Sphingomonas MD-1	LR, YR, RR	NOD	Saito *et al.* (2003b)
Sphingomonas Y2[a]	LR, RR, YR, 6(Z)-Adda		Park *et al.* (2001)
Sphingomonas B9	LR, RR, 3-DMMLR, -DHLR, LR-Cys, NOD	LF, 6(Z)-AddaLR, 6(Z)-AddaRR	Harada *et al.* (2004)
Sphingomonas 7CY	LR, RR, LY, LW, LF	NOD-har	Ishii *et al.* (2004)
Paucibacter toxinvorans	LR, YR, NOD		Rapala *et al.* (2005)
Sphingopyxis witflariensis LH21	LA. LR		Ho *et al.* (2007)
Burkholderia	LR, [D-leu^1]LR		Lemes *et al.* (2008)

[a] Reclassified as *Sphingosinicella microcystinivorans* (Maruyama *et al.*, 2006).

lakes, found 17 were capable of degrading microcystins, three of which were also capable of degrading nodularin (Lahti *et al.*, 1998). It may be this classic approach of targeting microcystin-exposed population which could explain the small number and/or diversity of isolates so far found to be associated with the degradation of microcystins.

Although still relying on initial bacterial isolation, a recent study (Manage *et al.*, 2007) utilized the Biolog MT2 plate as a tool to screen cultures for their ability to metabolize microcystins as a sole carbon and energy source. This rapid assay has the advantage of only requiring small volume cultures and low amounts of toxins (which can be of limited availability and high cost) in a practical 96-well microplate format. The plates contain a tetrazolium redox dye which forms a purple product if bacteria are able to oxidize the carbon source, microcystin in this case. *P. toxinivorans*, the known microcystin degrader, clearly demonstrates the potential of this assay (Fig. 4.2) to rapidly evaluate bacterial isolates to determine their suitability for further study with potential exploitation in

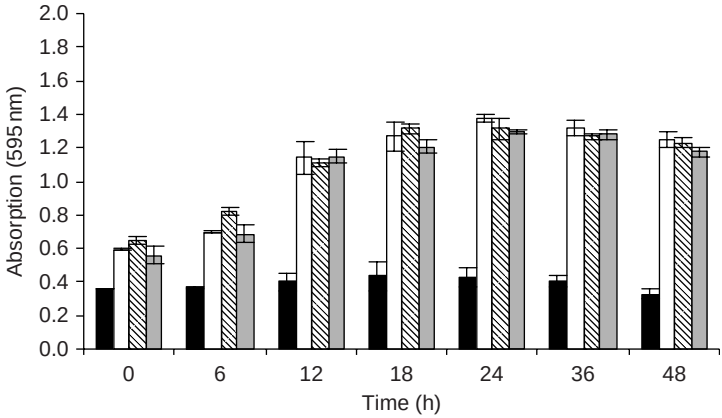


FIGURE 4.2 Metabolism of MC-LR by *Paucibacter toxinovorans* (DSMZ-16998) in Biolog MT2 plate with control (■), 0.1 (□), 1.0 (▨) and 10 μg mL^{-1} (▩) of MC-LR.

bioremediation. It allows a number of variables to be readily assessed, such as, different microcystin variants, concentration of microcystin, and other cyanotoxins (Fig. 4.2).

C. Characterization of MC-LR degradation pathway

While there have been many studies investigating the biodegradation of microcystins, only a single pathway has been elucidated to date and is summarized in Fig. 4.3. Early work by Bourne *et al.* (1996) characterized the enzymatic pathway in a *Sphingomonas* sp., ACM-3962, responsible for the degradation of MC-LR. Experiments with protease inhibitors on cell extracts coupled with structural elucidation of the degradation products indicated that MC-LR was hydrolyzed by a metalloprotease, referred to as microcystinase (MlrA), at the Adda–Arg bond to give the linear peptide, acyclo MC-LR (NH_2-Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg-OH). The conversion of MC-LR to its linear form resulted in a 160-fold reduction in toxicity protein phosphatase inhibition. This enzyme was shown to be produced constitutively when the *Sphingomonas* was grown on peptone media thus not specifically induced to hydrolyze microcystins. This was shown to be the case in a study where a cytoplasmic cell extract of *Sphingomonas* B9 was shown to be able to hydrolyze a range of nontoxic cyanobacterial peptides (nostophycin, microcyclamide, aeruginopeptin 95-A, and microviridin I) in addition to the microcystins (Kato *et al.*, 2007). A serine protease (MlrB) facilitated hydrolysis of the Ala–Leu bond of the linear MC-LR to give rise to a tetrapeptide, NH-Adda-Glu-Mdha-Ala-OH which was only 20 times less toxic than the parent MC-LR.

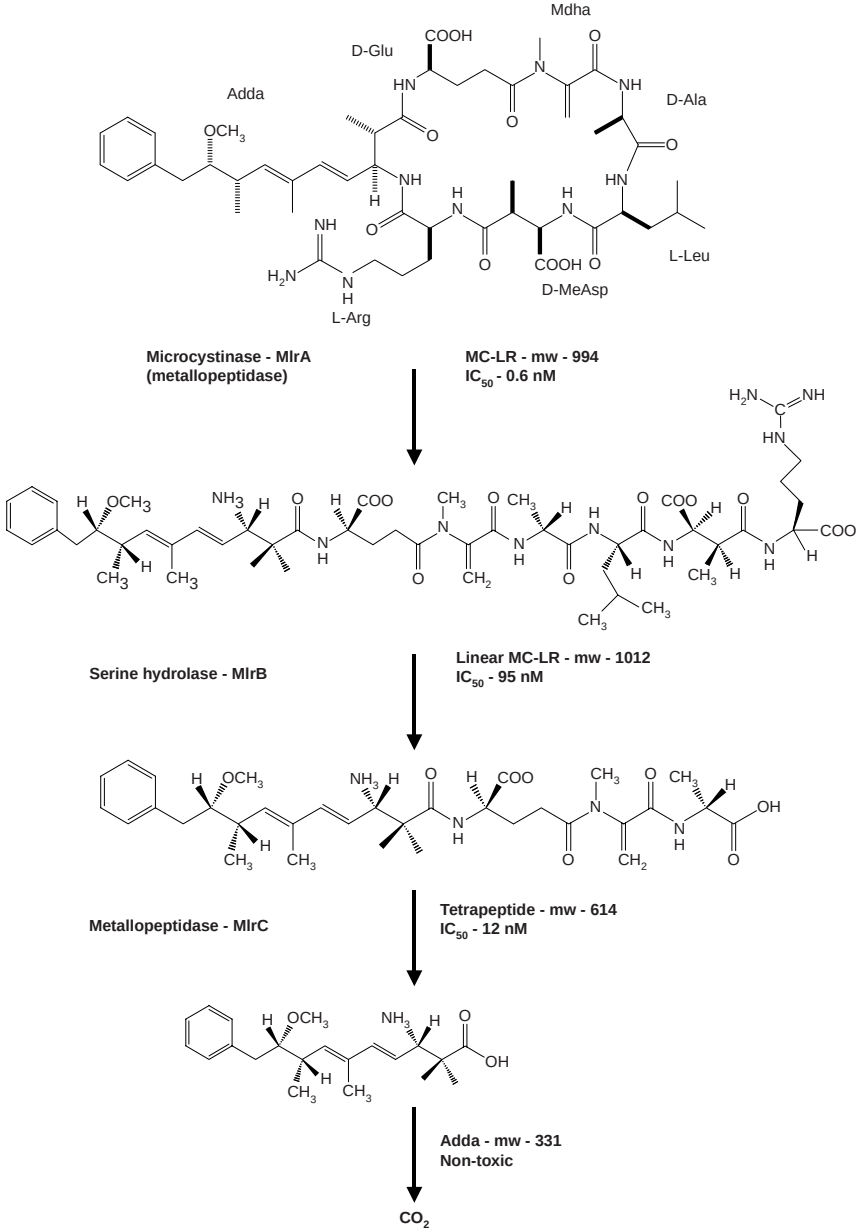


FIGURE 4.3 The only characterized biodegradation pathway where the MC-LR is hydrolyzed at the Adda–Arg bond to give the linear peptide. Further hydrolysis results in the formation of a tetrapeptide , followed by amino acids, Adda, the only one to be identified in degradation experiments (Harada *et al.*, 2004). Studies with ^{14}C MC-LR have resulted in the evolution of $^{14}CO_2$, indicating some mineralization had occurred (Holst *et al.*, 2003, Hyenstrand *et al.*, 2003). Toxicity data are based on inhibition of protein phosphatase.

A third enzyme (MlrC), responsible for hydrolysis of the tetrapeptide was identified, although the degradation intermediate, Adda was not identified until later degradation studies using a Japanese *Sphingomonas* isolate (B-9) where Adda was purified and identified (Harada *et al.*, 2004). A few studies have also investigated whether mineralization is achieved, that is, where the terminal product is CO_2. Using ^{14}C-labeled MC-LR, Holst *et al.* (2003) and Hyenstrand *et al.* (2003), demonstrated that a proportion on the ^{14}C MC-LR was converted to $^{14}CO_2$, although complete mineralization was not achieved.

Cloning and molecular characterization resulted in the identification of four genes which encoded the three hydrolytic enzymes along with a putative oligopeptide transporter (Bourne *et al.*, 2001). The genes termed *mlrA, mlrB*, and *mlrC*, encode the enzymes MlrA, MlrB, and MlrC, respectively, with *mlrD* representing the oligopeptide transporter. All the clone gene sequences along with the 16S rDNA sequence of the *Sphingomonas* sp. have been placed in the GenBank database.

Several other bacterial isolates have been shown to degrade MC-LR via this pathway, indicated by analysis of the degradation intermediates, these include *Sphingomonas* B-9. The ability of this isolate to degrade other microcystins and nodularin was investigated and revealed that peptides with the Adda–Arg bond were successfully degraded while MC-LF, with Adda–Phe bond and 6(z)-Adda MC-LR and 6(z)-Adda MCRR were not significantly degraded (Imanishi *et al.*, 2005). Another Japanese *Sphingomonas* isolate, 7CY, was shown to degrade a wider range of microcystins, including MC-LR, -RR, -LY, -LW, and -LF but it was unable to degrade NOD-Har (nodularin variant where arginine is replaced by homoarginine) unless MC-RR was present (Ishii *et al.*, 2004). Experiments with additional carbon sources were not able to mimic the result obtained when MC-RR, suggesting that either MC-RR itself or a degradation product induced enzymes capable of metabolizing the NOD-Har. This data suggested that alternative degradation pathways exist.

Screening for the *mlr*A gene has been used as another approach to detect potential microcystin degraders. In a study by Ho *et al.* (2007), 32 bacteria were isolated from a sand filter on R2A media. Only one bacterium had the four *mlr* genes and was able to efficiently degrade MC-LR and MC-LA. It was identified as a novel bacterium *Sphingopyxis witflariensis* LH21. This bacterium was used to demonstrate rapid degradation of MC-LR and MC-LA at high concentrations, 2 and 3 mg L^{-1}, respectively, in bioreactor studies, where removal of toxins and toxicity were determined by HPLC, protein phosphatase inhibition and cell-based cytotoxicity assay (Ho *et al.*, 2007)

To date, the degradation pathway described (Fig. 4.3), which proceeds via initial hydrolysis of the Adda–Arg bond is the only one characterized. Homology of *mlr*A gene sequences between strains of *Sphingomonas* Sp.

isolated from diverse global locations has led to the development of molecular probes based on 16S rDNA and the *mlr*A gene, to advance the understanding of the broader ecology (Bourne *et al.*, 2006). These techniques will facilitate further investigations into the occurrence and exploitation of these bacteria.

However, despite these advances, there have been very few detailed studies on the biodegradation of multiple microcystins and the number of reported isolates capable of microcystin degradation is still extremely low.

It is highly unusual for such a small number of bacteria to be capable of degrading an organic compound especially via a single pathway. However, in the last two years there has been evidence that other degradation pathways do indeed exist based on the identification of novel intermediates. Amé *et al.* (2006) reported on a strain *Sphingomonas* (CB4) that degraded MC-RR initially by demethylation prior to complete removal by 72 h. In studies on bacterial degradation in Scottish freshwater, we tentatively identified a number of novel degradation products of MC-LF and nodularin (Edwards *et al.*, 2008), supporting the hypothesis for the existence of greater diversity of organisms and pathways.

D. Exploitation of microcystin-degrading bacteria

The removal of organic pollutants by biologically active filters has been recognized for many years, and over the last 10 years there has been a focus in some countries, particularly Australia, on the exploitation of microcystin-degrading bacteria in water treatment processes. Removal of microcystins was shown to be more efficient once natural microbial populations had developed compared to direct removal by adsorption to the granular activated carbon (GAC) filter in a water treatment plant (Newcombe *et al.*, 2003). In this study, feed water containing MC-LR and MC-LA at concentrations between 4 and 20 μg L^{-1} was passed through a GAC filter at Myponga Water Treatment Plant. A high concentration of natural organic material (NOM; represented by a DOC of 6 mg L^{-1}) reduced the capacity of the column resulting in some microcystin breakthrough, although breakthrough was higher for MC-LA (approximately 40%) compared to MC-LR (<10%). Material from this filter was used for laboratory removal studies and after 16 d removal of both toxins to below detectable levels was achieved. Experiments with mixed populations isolated from the GAC material also degraded the microcystins, providing good evidence that they were responsible for the removal. Interestingly only populations isolated from the inlet had this capability, those isolated from the center of the filter were unable to degrade the microcystins. In another study on GAC filters, Wang *et al.* (2007) provided

further evidence that microbial biodegradation provided an additional mechanism of microcystin removal.

Dissolved microcystins (MC-LR and MC-LA at 20 μg L^{-1}) have also been removed successfully, by biodegradation, from laboratory columns packed with sand from a filter bed at a water treatment plant under both slow and rapid filter conditions (Ho *et al.*, 2006). As with the GAC filter the microbes involved in biodegradation of the microcystins were located in the upper region of the column, this was supported by determination of the biomass and biomass activity using the leucine aminopeptidase assay (LAP), an assay based on enzyme hydrolysis of large numbers of peptides, and amino acids. No breakthrough was observed from the column which had been pre-exposed to microcystins, albeit 6 months prior to the experiment, compared to detection of microcystins for 3 d in effluent from a ''virgin'' column and a column containing autoclaved sand. However, after this lag period, no microcystins were detectable, suggesting that a suitable number and or species of microbes had been established and were affecting breakdown of the microcystins. In contrast, in another study, it took 7 months for a sand to develop a microbial population capable of degrading microcystins (Wang *et al.*, 2007). This suggests that the microbial flora of the source water and possible other physical parameters are important in the development of a viable filtration system.

Improved removal of microcystins was achieved in a full scale slow sand filter with an intact and active *schmutzdecke* (upper biofilm layer on sand filter) compared to removal by adsorption alone (Grützmacher *et al.*, 2002). While dissolved microcystins were efficiently removed (>95%) the physical removal of cells was initially >85% but this dropped to 43% as the temperature dropped to 4 °C in autumn. The physical removal of cyanobacterial cells may appear effective but they may remain viable and continue to produce toxin, or lyse releasing toxin which may not be degraded if conditions are not suitable. These aspects must be included in future studies. However, consideration of biological removal of cyanobacterial cells was the main focus of a study on a biofilm where the indigenous nanoflagellate *Monas* sp. was shown to prey on cells of *Microcystis viridis*, removing both cells and toxins (Saito *et al.*, 2003c). Removal of cells correlated with removal of toxins suggesting that they were removed by direct ingestion, rather than a process of release followed by microbial degradation. Small scale studies using photoautotrophic biofilms, comprising largely of green algae, were shown to contribute to the removal of dissolved microcystins, supporting their potential incorporation into drinking water treatment processes (Babica *et al.*, 2005). Indeed rapid elimination ($t_{½}$ = 20 h) was achieved using a biofilm that had been pre-cultivated in the presence of toxic cyanobacteria.

Lower cost approaches for water treatment include bank filtration whereby water travels though the soil down a hydraulic gradient, as

with carbon and sand filters some toxin removal is achieved by adsorption. Increased efficiency is dependant on the microbial populations present that will bring about biodegradation. Miller and Fallowfield (2001) demonstrated that the type of soil was important for removal of MC-LR and nodularin where soils with high organic or clay content resulted in complete toxin removal within 10–16 d compared to only minor removal by the soil with high sand content.

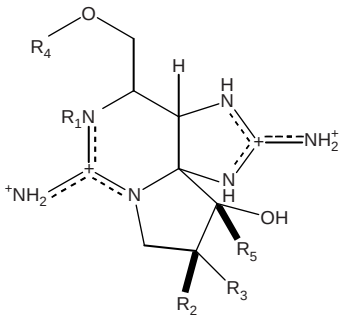
An approach that has potential for remediation of cyanotoxins in aquatic ecosystems and water treatment plants is the use of immobilized biodegrading bacteria. A study in Japan isolated several bacteria from a eutrophic lake and selected the organism that most readily degraded MC-LR and MC-RR at concentrations of 2 mg L^{-1}. Isolate B9 was capable of completely degrading these high concentrations within a day (Tsuji *et al.*, 2006). The resultant strain was immobilized on several substrates; cellulose, polyester (Marimo; spherical) and polyester (Fabios; pillar shaped) and evaluated efficiency of dissolved toxin removal was assessed in a bioreactor. Excellent removal (100%) was achieved using the B9 immobilized on polyester (Fabios) and very low OD at 660 nm indicated very low leakage of bacteria from the substrate. This removal efficiency was proved highly reproducible during a 72 d experiment where MC-RR was added on a regular basis. In addition to the efficient removal of dissolved toxin, particulate toxins from a healthy culture of *M. viridis* NIES 102 were also removed within 24 h using the B9/polyester complex, a process clearly involving cell lysis followed by biodegradation. This work has obvious potential and full scale-up assessment, stability of filters and an evaluation of all factors influencing the process (e.g., temperature, water quality, nutrients) is now required.

III. OTHER TOXINS

A. Saxitoxins

This group of toxins, produced by marine dinoflagellates and freshwater cyanobacteria, are potent neurotoxins which exert their effect by blocking sodium channels. Saxitoxins are carbamate alkaloids, which may be doubly sulfated as in the C-toxins, singly sulfated as in the gonyautoxins (GTX) or non-sulfated as in the saxitoxins. In addition there are decarbomyl variants (Fig. 4.4). As with the microcystins, there are differences in toxicity between the chemical variants.

While the occurrence of these toxins in freshwater is globally less significant than that of microcystins, it has localized importance in Australia where *Anabaena circinalis* is common and produces a range of saxitoxins. In 1992 (Negri *et al.*, 1995), a 1,000 km bloom of *A. circinalis*



Toxin variant	R_1	R_2	R_3	R_4	R_5
STX	H	H	H	$CONH_2$	OH
NEOSTX	OH	H	H	$CONH_2$	OH
GTX1	OH	H	OSO_3^-	$CONH_2$	OH
GTX2	H	H	OSO_3^-	$CONH_2$	OH
GTX3	H	OSO_3^-	H	$CONH_2$	OH
GTX4	OH	OSO_3^-	H	$CONH_2$	OH
GTX5	H	H	H	$CONHSO_3^-$	OH
GTX6	OH	H	H	$CONHSO_3^-$	OH
C1	H	H	OSO_3^-	$CONHSO_3^-$	OH
C2	H	OSO_3^-	H	$CONHSO_3^-$	OH
dcSTX	H	H	H	H	OH
dcGTX2	H	H	OSO_3^-	H	OH
dcGTX3	H	OSO_3^-	H	H	OH

FIGURE 4.4 Generic structure of neurotoxic saxitoxins, which may be doubly sulfated as in the C-toxins, singly sulfated as in the gonyautoxins (GTX) or non-sulfated as in the saxitoxins (STX and NEOSTX). In addition there are decarbomyl variants.

developed along the Murray–Darling River, causing many animal deaths and a state of emergency. More recently the genera capable of producing saxitoxins has extended to include *Aphanizomenon, Lyngbya*, and *Cylindrospermopsis*, indicating a more widespread occurrence of these toxins. Little work has been done on the biodegradation of saxitoxins, although the increasing occurrence of these toxins warrants a better understanding.

A recent study indicated that saxitoxins are susceptible to bacterial degradation during passage through bioactive treatment plant, however, while the predominant C-toxins did decrease there was in increase in GTX2 and GTX3, a result of structural modification during the biological treatment (Kayal *et al.*, 2008). The GTX-toxins are more toxic than the C-toxins which is a cause for concern and warrants further detailed investigation.

B. Cylindrospermopsin

Cylindrospermopsin (CYN) is an unusual sulfated alkaloid (Fig. 4.5), produced by several genera of cyanobacteria and was implicated in the poisoning of 120 people in Palm Island, Queensland, Australia in 1979 (Saker and Griffiths, 2003). People were hospitalized with severe gastroenteritis after drinking water from a reservoir where a bloom of *Cylindrospermopsis raciborskii* was treated with copper sulfate, which resulted in cell lysis and release of toxin, now known to be cylindrospermopsin. This toxin causes severe necrosis of the liver, hence it is often referred to as a hepatotoxin.

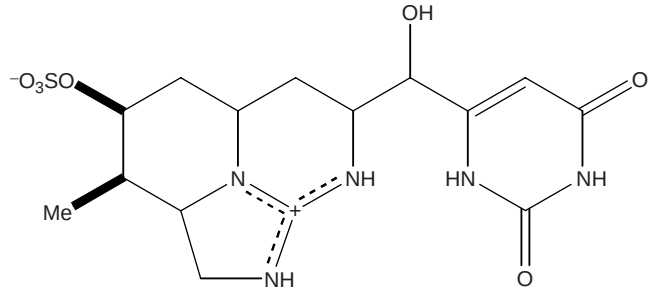


FIGURE 4.5 Hepatotoxic alkaloid cylindrospermopsin.

Other organs may also be affected and its ability to inhibit protein synthesis suggests it may be carcinogenic and potentially genotoxic. Relatively little work has been done on the toxicology of cylindrospermopsin, but the evidence to date suggests that there could be serious long term effects from chronic exposure in addition to the reported acute effects.

Once considered restricted to tropical regions, this sulfated alkaloid, has now been detected in many temperate locations. To date there have been few studies on the biodegradation of CYN. Biodegradation was suggested as an important process for the removal of CYN from contaminated water by Chiswell (1999), where a natural population of bacteria was shown to rapidly degrade CYN in dam water after a lag period, and no degradation was observed in autoclaved (i.e., microbe-free) dam water. However, use of copper sulfate to lyse the cyanobacteria, also affected the natural bacterial population resulting in dissolved CYN remaining in the water column for 3 months.

Degradation of CYN was observed in water taken from a water body in Queensland, Australia, with complete removal of CYN and DEOXY-CYN within 8–15 d. A degradation product was partially characterized by LC-MS and tentatively identified as a sulfated intermediate (Chiswell *et al.*, 2004). In contrast, a study investigating biodegradation of CYN with bacterial communities from two water bodies in Spain, one having frequent exposure to CYN, the other rarely, observed no degradation over 40 d experiment (Wormer *et al.*, 2008).

A recent study by Smith *et al.*, (2008) demonstrated that CYN was degraded by indigenous microbial flora in waters with a history of *Cylindrospermopsis* blooms, although this was shown to be concentration dependent, suggesting that above a certain concentration CYN may be needed to induce bacterial degradation. Despite isolation of many bacteria from CYN enriched cultures, only a single isolate (*Delftia* sp.) capable of degrading CYN has been obtained (Smith, 2005). Biologically active filters have been shown to remove both cells of *Cylindrospermopsis* and dissolved toxin once they have been ripened or conditioned (Garnett *et al.*, 2003).

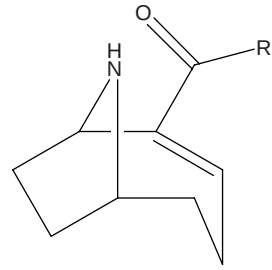


FIGURE 4.6 Structure of alkaloid neurotoxins, anatoxin-a (R = CH_3) and homoanatoxin-a (R = CH_2CH_3).

C. Anatoxin-a

Anatoxin-a and its analog homoanatoxin-a are neurotoxic alkaloids produced by a few genera of cyanobacteria, *Anabaena, Oscillatoria, Aphanizomenon*, and *Raphidiopsis*. These secondary amines (Fig. 4.6) are potent toxins, binding to nicotinic acetylcholine receptors, causing persistent stimulation which can result in death (Van Appeldoom *et al.*, 2007). There are very few reports on the persistence and biodegradation of this neurotoxic alkaloid, reflecting its limited occurrence and availability compared to other cyanobacterial toxins. Early work by Kiviranta *et al.* (1991) resulted in the isolation of a *Pseudomonas* sp. capable of rapid degradation of anatoxin-a, with a reported rate of 6–30 μg mL^{-1} per 3 d. Further work on natural bioremediation of anatoxin-a by Rapala *et al.* (1994) demonstrated the removal of anatoxin-a by microbial populations from water and sediments from eutrophic, oligotrophic, and humic lake. In all sediment and water samples, regardless of bloom history and previous exposure, anatoxin-a had been completely removed by day 8 despite use of high concentrations of anatoxin-a (approximately 2,500 μg L^{-1}).

There have been no studies on biodegradation pathways/intermediates, although nontoxic variants have been identified in cultures of the cyanobacterium *Raphidiopsis mediterranea* and it has been indicated that this is the result of *in vivo* oxidation (Namikoshi *et al.*, 2004).

IV. CONCLUSIONS

It is evident from the numerous studies that natural bioremediation plays an important role in the elimination of cyanotoxins from aquatic ecosystems, and a reasonable understanding of this process combined with regular monitoring has enabled practical management strategies in many countries, thus ensuring the safety of the drinking water supply.

While it is clear that there is potential in harnessing microbes for bioremediation of cyanotoxins, there remain many challenges, in particular the chemical diversity and stability of the toxins themselves. Most of the studies to date have focused on one or two microcystin variants, clearly this must be expanded with additional toxicity analysis. As many cyanobacteria produce multiple classes of toxins, greater effort is needed to ensure these organisms and their toxins can be successfully eliminated by an efficient microbial population.

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CHAPTER 5

Virulence in *Cryptococcus* Species

Hansong Ma and **Robin C. May**[1]

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Abstract

Cryptococcus neoformans and *Cryptococcus gattii* are the cause of life-threatening meningoencephalitis in immunocompromised and immunocompetent individuals respectively. The increasing incidence of cryptococcal infection as a result of the AIDS epidemic, the recent emergence of a hypervirulent cryptococcal strain in Canada and the fact that mortality from cryptococcal disease remains high have stimulated intensive research into this organism. Here we outline recent advances in our understanding of *C. neoformans* and *C. gattii*, including intraspecific complexity, virulence factors, and key signaling pathways. We discuss the molecular basis of cryptococcal virulence and the interaction between these pathogens and the host immune system. Finally, we discuss future challenges in the study and treatment of cryptococcosis.

I. *CRYPTOCOCCUS* AND CRYPTOCOCCOSIS

The genus *Cryptococcus* contains 39 heterobasidiomycetous fungal species characterized as variously encapsulated budding yeasts, of which only *Cryptococcus neoformans* and *Cryptococcus gattii* are commonly considered as the causative agents of cryptococcosis (Casadevall and Perfect, 1998). *C. neoformans* was first identified as a human pathogen in the 1890s (Buschke, 1895; Busse, 1894). It exists predominantly as a vegetative haploid form and is heterothallic with each cell existing as one of two distinct mating types: MATa or MATα. In response to nutrient limitation, cells of opposite mating type mate to form the filamentous teleomorph (Kwon-Chung, 1975, 1976). Under the microscope, most clinical isolates of *C. neoformans* appear as encapsulated spherical yeasts in both tissue and culture (Mitchell and Perfect, 1995). The capsule size varies according to the strain and culture conditions with most isolates having a medium-sized capsule resulting in a total diameter of 4–10 μm (Fig. 5.1A). Poorly encapsulated strains have diameters of only 2–5 μm whereas heavily encapsulated isolates can have a cell diameter of up to 80 μm (Casadevall and Perfect, 1998).

C. neoformans can cause human infections following inhalation of the small airborne propagule (believed to be either basidiospores or poorly encapsulated yeast cells) originating from certain environments such as soil and avian habitats. Therefore, the lung is invariably the portal of entry and initial site of infection (Casadevall and Perfect, 1998). However,

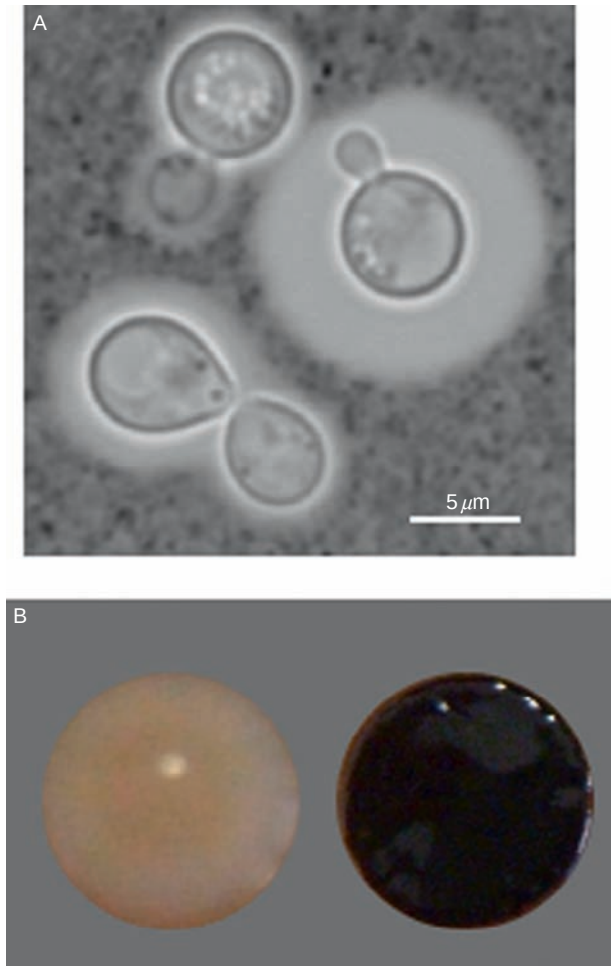

FIGURE 5.1 (A) India ink staining reveals the capsule (of various sizes) around budding *C. neoformans* cells; (B) Melanin and nonmelanin forming colonies of *C. gattii* serotype B on L-DOPA medium after 7 days at 25 °C.

C. neoformans not only has the ability to simply colonize the host's respiratory tract without causing disease (latency) in immunocompetent individuals (Garcia-Hermoso *et al.*, 1999), but is also capable of disseminating to any organ of the human body, with a predilection for the central nervous system (CNS). The resulting meningoencephalitis represents the most severe form of the disease and is uniformly fatal if untreated (Casadevall and Perfect, 1998).

Conventional nomenclature classified *C. neoformans* into five serotypes (A, B, C, D, and AD) and three varieties: *C. neoformans* var. *neoformans* (serotype D), *C. neoformans* var. *grubii* (serotype A), and *C. neoformans* var.

gattii (serotype B and C) (Franzot *et al.*, 1999; Kwon-Chung *et al.*, 1982). Each serotype is characterized by a specific structure of glucuronxylomannan (GXM), the main capsule component (Cherniak *et al.*, 1995). In the last decade, a number of DNA genetic typing techniques have been used to genotype and study the epidemiology of *C. neoformans* species. These techniques include electrophoretic karyotyping by pulsed field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), DNA hybridization studies, amplified fragment length polymorphism (AFLP), polymerase chain reaction (PCR) fingerprinting, and multi locus sequence typing (MLST) (Boekhout *et al.*, 1997, 2001; Brandt *et al.*, 1995; Currie *et al.*, 1994; Litvintseva *et al.*, 2006; Meyer *et al.*, 1999; Ruma *et al.*, 1996; Varma and Kwon-Chung, 1992). These techniques resulted in the elevation of *C. neoformans* var. *gattii* to the species level, based on genetic variability and lack of evidence for genetic recombination between *C. neoformans* and *C. gattii* (Kwon-Chung *et al.*, 2002). Moreover, *C. gattii* differs from *C. neoformans* in phenotypic characters, natural habitat, epidemiology, clinical manifestations of disease, and response to antifungal treatment (Casadevall and Perfect, 1998; Chen *et al.*, 2000; Sorrell, 2001; Speed and Dunt, 1995). The *C. neoformans–C. gattii* species complex is further divided into nine major molecular types or genotypes (Fig. 5.2).

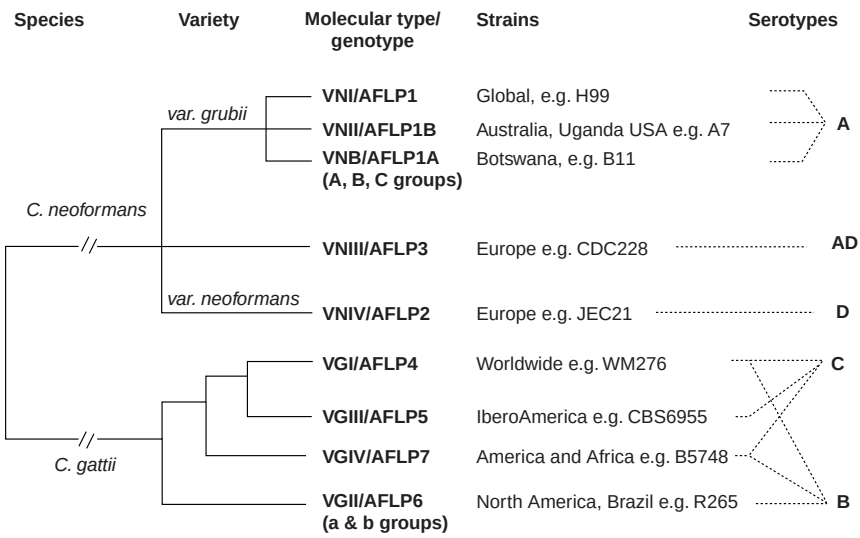

FIGURE 5.2 A schematic phylogeny of the *C. neoformans–C. gattii* species complex. For *C. neoformans*, two monophyletic lineages, corresponding to the varieties *grubii* and *neoformans*, are clearly present along with the hybrid population. Within *C. neoformans*, serotypes correspond to genotypes. For *C. gattii*, four monophyletic lineages corresponding to the previously described genotypic groups are consistently found but in this group serotypes and genotypes do not necessary correlate with each other.

C. neoformans var. *grubii* isolates correspond to molecular types VNI, VNII, and VNB; *C. neoformans* var. *neoformans* corresponds to VNIV; and serotype AD isolates correspond to molecular type VNIII. *C. gattii* corresponds to four molecular types: VGI, VGII, VGIII, and VGIV, and a recent study by Bovers *et al.* has proposed to treat these four molecular types as different taxa (varieties), just like var. *neoformans* and var. *grubii* (Bovers *et al.*, 2008).

A. *C. neoformans*

C. neoformans usually infects immunocompromised patients (although some exceptions have recently been reported, e.g., (Chen *et al.*, 2008b)). It can be found in the environment worldwide, and is commonly associated with pigeon guano or soil (Casadevall and Perfect, 1998). Most *C. neoformans* isolates are serotype A or serotype D. A and D serotypes diverged about 18 million years ago and have always been described as varieties, not as separate species (Fan *et al.*, 1994; Xu *et al.*, 2000). Nevertheless, a recent proposal is that these two varieties of *C. neoformans* should be described as different species (Bovers *et al.*, 2008), because they have diverged to such an extent that normal mating is no longer possible (Sun and Xu, 2007), and comparison of their genomes shows that there has not been any recent DNA exchange between these two varieties (Kavanaugh *et al.*, 2006). Further detailed studies are required to analyze the ongoing speciation events within this clade.

Serotype A is the predominant serotype of *C. neoformans* isolated from infected patients, responsible for 95% of all *C. neoformans* infections (Hull and Heitman, 2002). It is subdivided into three molecular types: VNI (AFLP1), VNII (AFLP1B), and VNB (AFLP1A) according to MLST and AFLP analysis (Boekhout *et al.*, 2001; Bovers *et al.*, 2008; Litvintseva *et al.*, 2006) (Fig. 5.2). Such sub-classification is confirmed by recent comparative genome hybridization (CGH) data (Hu *et al.*, 2008). VNI is the most common molecule type, contributing 78% of *C. neoformans* isolates (Meyer *et al.*, 1999). The VNB cluster can be further separated into three groups: VNB-A, VNB-B, and VNB-C (Litvintseva *et al.*, 2006). Initially, VNB strains were found only in Botswana (Litvintseva *et al.*, 2006), but more recently they have also been recovered from Brazilian pigeon droppings and patients in Rwanda, Portugal, and Brazil (Bovers *et al.*, 2008).

Serotype D strains are found globally, but they are more prevalent in areas with temperate climates, such as Europe, where 30% of isolates are serotype D (Dromer *et al.*, 1996). This restricted distribution may be due to the fact that serotype D strains are more susceptible to killing by high temperature than cells of serotype A (Martinez *et al.*, 2001). The clinical manifestations of human infections caused by serotype A or D are similar,

although differences in virulence potential in animal models have been reported (Barchiesi *et al.*, 2005; Lin *et al.*, 2008)

Serotype AD is the result of a fusion event between a serotype A strain and serotype D strain followed by impaired meiosis due to genomic incompatibilities (Boekhout *et al.*, 2001; Cogliati *et al.*, 2001; Lengeler *et al.*, 2001; Xu *et al.*, 2002). AD strains are therefore diploid (or aneuploid), containing two sets of chromosomes, and possessing two mating type alleles. Serotype AD strains are relatively common: a recent analysis of environmental and clinical populations of *C. neoformans* in North America revealed that ~7.5% of strains isolated from the environment are AD hybrids (Litvintseva *et al.*, 2005a). Thus far, the majority of the globally isolated serotype AD strains originate in Africa (Litvintseva *et al.*, 2007).

B. *C. gattii*

C. gattii was first described after being isolated from a leukemic patient in 1970 (Vanbreuseghem and Takashio, 1970). It mainly infects individuals with no immunological defects, although AIDS-associated *C. gattii* infections have also been reported (Chen *et al.*, 2000, 2008b; Litvintseva *et al.*, 2005b). It has been consistently isolated from decaying wood of several tree species, especially the red gum group of eucalyptus trees (*Eucalyptus* ser. *Exsertae Blakely*) (Ellis and Pfeiffer, 1990, 1992; Fortes *et al.*, 2001; Krockenberger *et al.*, 2002; Lazera *et al.*, 2000). The geographic distribution of *C. gattii* was originally thought to be limited to tropical and subtropical regions of the world (Kwon-Chung and Bennett, 1984). However, recent studies have revealed its worldwide distribution. For instance, VGI (AFLP4) strains were found to be the most widely distributed (Campbell *et al.*, 2005; Chen *et al.*, 2008b; Meyer *et al.*, 2003); Strains of the VGII (AFLP6) type are found in areas like Australia and America (Fraser *et al.*, 2005; Kidd *et al.*, 2004, 2005; Meyer *et al.*, 2003); The VGIII (AFLP5) type predominates IberoAmerican countries (Meyer *et al.*, 2003) and can also be found in India (Bartlett *et al.*, 2007), whilst the VGIV (AFLP7) type, which has been associated with infections in HIV-positive patients (Bovers *et al.*, 2006; Litvintseva *et al.*, 2005b), is found in South Africa (Meyer *et al.*, 2003) and Central America (Bartlett *et al.*, 2007) etc.

Until recently, *C. gattii* has been under-studied because *C. gattii* infections comprise only 1% of cryptococcosis cases worldwide. Even in areas like Australia, where *C. gattii* is endemic, the rate of infection is 0.94 cases per million residents per year (Chen *et al.*, 2000; Sorrell, 2001). However, a recent outbreak of cryptococcosis caused by *C. gattii* has stimulated detailed investigation of this organism. This ongoing outbreak was first noted in 1999 on Vancouver Island, British Columbia (BC), Canada. Between 2002 and 2006, the average annual cryptococcosis incidence rate was 6.5 cases/million in BC and 27.9 cases/million on Vancouver

Island (Control, 2007). In addition to human infections, cryptococcal disease has been diagnosed in animals such as dogs, cats, horses, and even porpoises. In fact, veterinary cases have been diagnosed two to three times more frequently than human cases (Lester *et al.*, 2004). So far, the fungus has infected more than 176 individuals and spread from Vancouver Island to other regions of Canada and the Pacific Northwest (MacDougall *et al.*, 2007).

Interestingly, the majority (>97%) of cryptococcal isolates from the island have been found to belong to the VGII molecular type, with the rest being VGI (Kidd *et al.*, 2004). These VGII isolates have been further separated into two discrete subtypes: a major form common in environmental and clinical isolates (VGIIa/AFLP6a, hypervirulent, e.g., CDCR265), and a rare minor form presented by one clinical and several environmental samples (VGIIb/AFLP6b, with attenuated virulence, e.g., CDCR272) (Fraser *et al.*, 2005; Kidd *et al.*, 2004, 2005). So far, the VGIIa genotype has accounted for 78% of the examined veterinary cases and 87% of the human cases on Vancouver Island (Bartlett *et al.*, 2007). Surprisingly, MLST (Kidd *et al.*, 2005) and gene genealogy analysis (Fraser *et al.*, 2005) revealed that VGIIa and VGIIb strains found on Vancouver Island share similar or identical genotypes with isolates from other parts of world. For example, the VGIIa genotype was also shared by the NIH444 strain (from a patient in Seattle, 1971, which is considered as the potential origin of the VGIIa subtype), CBS7750 (from a *Eucalyptus* tree in San Francisco, 1992), and isolates from other parts of the North America (e.g., KB10455). The VGIIb genotype was also observed among environmental and clinical isolates from Australia (e.g., Ram005, NT-13), as well as a clinical isolate from Thailand (MC-S-115) (Fraser *et al.*, 2005; Kidd *et al.*, 2005). A recent study confirmed the global distribution of the outbreak genotypes (Meyer *et al.*, 2007). The wide distribution of Vancouver genotypes in other geographical areas makes it difficult to accurately determine a specific origin. Current hypotheses are that the species is either a long-term resident of BC (ancient population), or represents a particularly virulent genotype that may be well adapted to the local conditions and has been recently introduced to BC. For instance, Fraser *et al.* reported that the VGIIa and VGIIb strains from Vancouver Island shared 14 identical loci after examining 30 alleles, and hypothesized that VGIIa isolates might be the result of same-sex mating (α/α) between a VGIIb isolate and a second unknown VGII isolate in Australia, in transit or in the Pacific Northwest (Fraser *et al.*, 2005). However, Meyer *et al.* revealed that there were VGIIa and VGIIb isolates recovered as early as in 1986 in South America, suggesting that these genotypes may have been present for a long time in the Americas rather than being a result of a recent recombination event as suggested by Fraser *et al.* (Meyer *et al.*, 2007).

C. Other species

Besides *C. neoformans* and *C. gattii*, there are at least 37 other cryptococcal species found in a wide variety of environmental locations, such as Antarctica, the Himalayas, and saline water (Casadevall and Perfect, 1998). However, since most of them are not able to survive in mammalian tissue due to the relatively high body temperature and host immune system, infection caused by these species is rare (Kordossis *et al.*, 1998; Krajden *et al.*, 1991; Kunova and Krcmery, 1999; Loison *et al.*, 1996). Among those causing non-*neoformans/gattii* cryptococcosis, *Cryptococcus laurentii* (20 cases) and *C. albidus* (18 cases) are responsible for most (80%) of such infections (Khawcharoenporn *et al.*, 2007). The transmission, virulence factors and host immune response to these species resembles that of *C. neoformans* (Ikeda *et al.*, 2000; McCurdy and Morrow, 2003), although the level of laccase activity is lower than that seen in *C. neoformans* (Ikeda *et al.*, 2002). A systematic review of non-*neoformans* infection can be found in Khawcharoenporn *et al.*, 2007.

D. Cryptococcosis

Following inhalation of the infectious particle, a primary pulmonary lymph-node complex is formed. In most cases, symptoms do not develop, indicating that most immunocompetent people either clear or control the infection before widespread symptomatic dissemination occurs (Casadevall and Perfect, 1998). Yet frequently the yeast will reside in a dormant state, probably within the lymph-node complex (Baker, 1976). Among patients with significant alterations of immunity, including patients with prolonged corticosteroid administration, hematological malignancies, or HIV infection, however, disseminated disease is often seen. *Cryptococcus* can cause localized infections in any organ involving the skin, eyes, myocardium, bones, joints, lungs, prostate gland, urinary tract, or CNS (Perfect, 1989). Dissemination may occur from a primary infection. For example, it was reported that acute infection could occur when immunocompromised individuals are exposed to large numbers of cryptococcal cells (Nosanchuk *et al.*, 2000b). However, there is increasing evidence indicating that dissemination is the result of reactivation of dormant disease (Perfect, 1989). For instance, it has been reported that patients coming from tropical areas can be diagnosed with *C. gattii* cryptococcosis long after they have left their countries of origin (Dromer *et al.*, 1992). Similarly, Garcia-Hermoso and colleagues analyzed cryptococcal clinical isolates recovered from patients diagnosed with cryptococcosis in France but born in Africa. The RAPD profiles of these isolates were significantly different from that of those from 17 European patients, suggesting that *Cryptococcus* can be acquired long before the infection

develops, as these patients had been living in France for approximately 10 years and had not been in contact with an African environment for as long as 13 years (Garcia-Hermoso *et al.*, 1999).

Cryptococcosis occurs in both animals and humans, but animal-to-human or human-to-human transmission has not been documented, other than rare examples of iatrogenic transmission (Lin and Heitman, 2006) and a mother-to-child transmission (Sirinavin *et al.*, 2004). The clinical presentation of cryptococcosis can be acute or chronic, and manifestation varies depending on stage of the disease. Typical symptoms associated with meningoencephalitis are significantly raised cerebrospinal fluid (CSF) opening pressure (>25 cm H_2O) (occurs in more than 50% of patients with HIV-associated cryptococcal meningitis) (Graybill *et al.*, 2000), resulting in headache, fever, altered mental status, visual loss, dementia, or even coma (Casadevall and Perfect, 1998). For pulmonary cryptococcosis, symptoms range from asymptomatic pulmonary nodules to acute respiratory distress syndrome (Casadevall and Perfect, 1998; Saag *et al.*, 2000). According to a recent study in 166 patients, symptoms including cough (58%), dyspnea (46%), and fever (38%) are the most frequent manifestations of infection (Baddley *et al.*, 2008). Both *C. neoformans* and *C. gattii* affect the lung and CNS. However, the infections caused by the two species have important differences in epidemiology, clinical presentation, and therapeutic outcome (Kwon-Chung and Bennett, 1984; Sorrell, 2001). For instance, *C. gattii* appears to invade the brain parenchyma more commonly than *C. neoformans*, and in *C. gattii* infected patients, pulmonary infections and pulmonary mass-like lesions are more common (Mitchell and Perfect, 1995; Speed and Dunt, 1995).

Since 1981, infections due to *Cryptococcus* have been a major cause of morbidity and mortality in individuals with depressed immune system as a consequence of the AIDS epidemic, as 5–10% of all individuals with CD4+ lymphopenia develop life-threatening cryptococcosis (Steenbergen and Casadevall, 2003). Nowadays, cryptococcosis ranks as one of the three common life-threatening opportunistic infections in people with AIDS worldwide (Levitz and Boekhout, 2006). Even though the prevalence of cryptococcosis in HIV-infected individuals has declined because of highly active antiretroviral therapy, it remains epidemic in Africa and Southeast Asia, where up to 30% of AIDS patients are affected (Bicanic and Harrison, 2004; Idnurm *et al.*, 2005). In fact, cryptococcosis has been recognized as an AIDS-defining illness in areas like Zimbabwe, where 91% of AIDS patients are infected (Mwaba *et al.*, 2001). Although less common, cryptococcosis in HIV-negative patients also has a high mortality rate (Kiertiburanakul *et al.*, 2006), particularly in areas such as northern Brazil, where *C. gattii* is endemic and accounts for 62.7% of all cryptococcosis cases (Nishikawa *et al.*, 2003).

1. Antifungal therapy

Untreated cryptococcal meningitis is uniformly fatal, although survival can range from years to only a few weeks (Mwaba *et al.*, 2001). There are several well-established antimicrobial reagents for treatment, and amphotericin B, a polyene introduced in the mid-1950s, was the first effective therapy developed. Amphotericin B binds to ergosterol in the fungal plasma membrane to cause increased permeability to protons and monovalent cations such as potassium (Brajtburg *et al.*, 1990). It was also found to stimulate inflammatory cytokine production from innate immune cells through CD14 and Toll-like receptors (TLRs) (Sau *et al.*, 2003). In many resource-poor areas where amphotericin B is not available, fluconazole, a triazole that inhibits fungal ergosterol synthesis is widely used (Jarvis and Harrison, 2007). It has excellent absorption and CSF penetration and is widely available at low cost in generic form. However, the slow response to therapy with fluconazole means that it is better suited to long-term maintenance therapy than initial therapy (Bozzette *et al.*, 1991; Powderly *et al.*, 1992). Flucytosine (5-Fc) is another commonly used anticryptococcal drug. It is a synthetic antimycotic compound and was initially developed as an anticancer drug in the 1970s. It has no intrinsic antifungal capacity, but after it has been taken up by *Cryptococcus*, it is converted into 5-fluorouracil (5-Fu), a pyrimidine analogue that inhibits fungal RNA and DNA synthesis (Vermes *et al.*, 2000). Flucytosine is commonly prescribed in combination with amphotericin B, because such combination has been shown to have higher efficiency compared to amphotericin B alone in both non-HIV-associated and HIV-associated infection (Bennett *et al.*, 1979; Brouwer *et al.*, 2004; van der Horst *et al.*, 1997). The optimal current therapy is with amphotericin B 0.7–1 mg/kg/day plus flucytosine 100 mg/kg/day for two weeks, followed by fluconazole 400 mg/day for 8 weeks and 200 mg/day thereafter (Bicanic and Harrison, 2004).

The emergence of antifungal drug resistance has not been a major problem to date in areas like Australia and New Zealand (Chen *et al.*, 2000). However, in sub-Saharan Africa, resistance can be very high. For instance, in Nairobi Kenya, flucytosine resistance was observed in 21% of cryptococcal strains and only 23.8% of these strains were susceptible to fluconazole (65% susceptible in a dose-dependent manner and 11.2% resistant) (Bii *et al.*, 2007). Differences in the antifungal susceptibilities of the two species of *Cryptococcus* have also been reported. A study conducted by Trilles *et al.* found that *in vitro*, *C. gattii* was less susceptible to seven antifungal compounds as compared with *C. neoformans*, although both showed equal susceptibility to amphotericin B and flucytosine (Trilles *et al.*, 2004).

2. Immunotherapy

Immunotherapeutic strategies, mainly based on introducing antibodies and cytokines, have been developed to restore and boost host defense mechanisms to *Cryptococcus*. Antibodies against capsular and cell wall have been demonstrated to provide protection in animal models of cryptococcal infection (Casadevall *et al.*, 1998; Dromer *et al.*, 1987; Mukherjee *et al.*, 1992; Rachini *et al.*, 2007; Sanford and Stollar, 1990). However, adjunctive use of antibody therapy in mice with established cryptococcal infection was also reported to cause cardiovascular collapse and death in some strains of mice due to the release of platelet-activating factor (Lendvai *et al.*, 2000; Savoy *et al.*, 1997). Nevertheless, a murine IgG1 (Mab 18B7) has reached phase I trial in patients recovering from HIV-associated cryptococcal meningitis (Larsen *et al.*, 2005) and radioimmunotherapy (radiation was delivered by specific radio-labeled antibodies leading to antibody-specific killing of *Cryptococcus*) is under evaluation in the murine model (Dadachova *et al.*, 2004).

Several cytokines (Th1 type) have been shown to augment the antifungal activity of effectors cells against cryptococcal infection. In a murine cryptococcal infection, administration of IL-12 resulted in up to 10-fold decreases in the cryptococcal burden in the CNS. Significantly, the combination of fluconazole with IL-12 showed synergistic effects on reducing organism burden (Clemons *et al.*, 1994). Similarly, the importance of interferon-γ (IFNγ) in the clearance of cryptococci, especially from the CSF, has been demonstrated by several groups (Kawakami *et al.*, 1996; Siddiqui *et al.*, 2005; Zhou *et al.*, 2007), and IFNγ can potentiate amphotericin B mediated reduction of infection in the brain (Lutz *et al.*, 2000). A recent phase II study to evaluate the safety and antifungal activity of adjuvant recombinant interferon (rIFN)-γ1b in HIV patients with acute cryptococcal meningitis showed a trend towards improved mycological and clinical success without adverse effects on CD4 count or HIV viral load (Pappas *et al.*, 2004).

With the use of molecular biology, several genes and their encoded proteins have now been identified which may help elicit a protective immune response. One such group is the mannoproteins. Mannoproteins are a group of glycoproteins present in the capsule (discussed in detail in Section II). They are recognized by the mannose receptor and presented to T cells by dendritic cells (Levitz and Specht, 2006; Mansour *et al.*, 2006). Recent *in vivo* and *in vitro* studies have reported that mannoproteins were the major T cell antigenic determinants from *C. neoformans* and both CBA/J and C57BL/6 mice benefited from immunization with mannoproteins (Mansour *et al.*, 2004; Specht *et al.*, 2007). Another molecule with therapeutic potential is a synthetic oligodeoxynucleotide containing an unmethylated CpG motif (CpG-ODN). CpG-ODN is a TLR ligand, which

was found to protect mice from infection with *C. neoformans* by altering the Th1-Th2 cytokine balance toward a Th1-biased immune response (Edwards *et al.*, 2005; Miyagi *et al.*, 2005). Combination of CpG-ODN with antifungal chemotherapy or with mannoproteins seems to provide a beneficial effect in a murine model of pulmonary and disseminated infection (Dan *et al.*, 2008; Kinjo *et al.*, 2007), suggesting a rationale for vaccination strategies that combine mannosylated antigens with TLR ligands to achieve synergistic promotion of host defense against *C. neoformans* infection.

3. Outcomes

The mortality from cryptococcosis remains unacceptably high. The last US Mycoses Study Group treatment trial of HIV-associated cryptococcal meningitis showed the lowest mortality to date, which is still 9.4% at 10 weeks (Bicanic and Harrison, 2004). In France, Dromer *et al.* observed an overall mortality rate of 6.5% in the first 2 weeks and 11.5% over the next 10 weeks (Dromer *et al.*, 2007). In Southeast Asia, even in the context of amphotericin B based therapy, acute mortality has ranged from 22% to more than 40% (Brouwer *et al.*, 2004; Imwidthaya and Poungvarin, 2000). For instance, with amphotericin B plus flucytosine, 34% of patients with *C. gattii* meningitis in Papua New Guinea died during their first admission, at a median of 8 days (Seaton *et al.*, 1996). In African areas where amphotericin B is not available, results with fluconazole monotherapy at 200 mg/day or fluconazole plus flucytosine in combination showed 44% mortality at 8 weeks (Mayanja-Kizza *et al.*, 1998; Mwaba *et al.*, 2001). The main reasons for the ongoing high mortality of cryptococcal disease include the inadequacy of current antifungal therapy, restricted access to some drugs in many areas and the problem of raised CSF pressure (Antinori, 2006; Bicanic and Harrison, 2004; Jarvis and Harrison, 2007; Perfect, 2007).

E. Genome sequencing project

The genome sequence of five cryptococcal strains (JEC21, B3501A, H99, WM276, and R265) has been completed (Hu *et al.*, 2008). The JEC21 genome (sequenced at TIGR) comprises a total of 20 Mb of DNA, containing approximately 6572 genes (Loftus *et al.*, 2005), 10% of which are unique to *C. neoformans* (Idnurm *et al.*, 2005). The intron-rich genome encodes a transcriptome abundant in alternatively spliced (4.2% of transcriptome) and antisense messages (53 genes). The genome is also rich in transposons (~5%), many of which cluster at centromeric regions. The presence of these transposons results in genetic plasticity and may be responsible for karyotype instability and phenotypic variation (Loftus *et al.*, 2005). The sequence difference between JEC21 and B3501A (another

serotype D isolate, sequenced at Stanford University) is restricted to 50% of their genomes, which overall are 99.5% identical at the sequence level (Loftus *et al.*, 2005). The genome sequencing project has been reviewed recently by Idnurm *et al.* (Idnurm *et al.*, 2005).

As a basidiomycete fungus, *C. neoformans* is evolutionarily distinct from ascomycete fungi such as *Saccharomyces cerevisiae*, the fission yeast *Schizosaccharomyces pombe*, and many common human fungal pathogens including *Candida albicans* and *Aspergillus fumigatus* (Hull and Heitman, 2002). The completed *C. neoformans* and *C. gattii* genome sequences permit comparative genomics with fungi from other phyla, although a detailed comparison based on all five cryptococcal genomes has not yet been undertaken. In addition, the availability of these sequences has made the construction of tiling microarrays and CGH studies feasible. CGH in combination with physical mapping and sequencing has already been used to study the genome variability within *C. neoformans* species and potentially allows for detailed characterization of the genome of emerging clinically significant strains (e.g., isolates from the Vancouver Outbreak) in the future (Hu *et al.*, 2008). These studies will provide important information on the mechanisms of genome microevolution in these pathogens.

II. VIRULENCE FACTORS

C. neoformans and *C. gattii* have a number of well-defined virulence factors, which strongly influence the degree of pathogenicity of individual isolates. A recent study by Rodrigues *et al.* demonstrated that *C. neoformans* was able to secrete vesicles containing many of its virulence factors, including GXM, laccase, urease, and phospholipase B (Rodrigues *et al.*, 2008). The extracellular vesicles manifested various sizes and morphologies, including electron-lucid membrane bodies and electron-dense vesicles. During disseminated cryptococcosis, measurable levels of cryptococcal products are detected in the body fluid of patients (Gordon and Vedder, 1966), suggesting that these ''virulence factor delivery bags'' may represent an efficient and general way of delivering pathogenesis-related molecules to the extracellular environment by *C. neoformans* (Rodrigues *et al.*, 2008). Below several well-characterized virulence factors are discussed in detail.

A. Capsule

The importance of capsule as a virulence factor was demonstrated by the observation that acapsular variants of *C. neoformans* very rarely cause human disease (Alspaugh *et al.*, 1998). The capsule is composed of 90–95%

GXM and 5% galactoxylomannan (GalXM) (Rakesh *et al.*, 2008). GXM is a large polymer with a repeating structure of α-1,3-mannose with β-D-xylopyranosyl, β-D-glucuronosyl and 6-*o*-acetyl branching. This structure determines the serotype of *C. neoformans* and *C. gattii*, because different capsule structures can be distinguished by antibodies. GalXM is an α-1,6 galactan that contains branches of β-1,3-galactose-α-1,4-mannose-α-1,3 mannose (Vaishnav *et al.*, 1998). It has a much smaller mass than GXM: $1.01 \times 10^5 \text{gmol}^{-1}$ versus $1.7–7.4 \times 10^6 \text{gmol}^{-1}$ (McFadden *et al.*, 2006). In addition to GXM and GalXM, several mannoproteins (<1%) such as MP-98 and MP-99 have been identified within the cryptococcal capsule (Huang *et al.*, 2002; Levitz *et al.*, 2001). So far, a total of 53 mannoproteins are predicted by genomic databases (Levitz and Specht, 2006). These mannoproteins share several structural features, including N-terminal signal sequences, serine/threonine (S/T)–rich C-terminal regions, and glycosylphosphatidylinositol (GPI) anchor motifs. When mannosylated and glycosylated, they act as critical cryptococcal antigens responsible for stimulating T-cell responses by promoting dendritic cell maturation and activation (Mansour *et al.*, 2004; Pietrella *et al.*, 2005; Specht *et al.*, 2007).

Many *C. neoformans* genes in capsular synthesis and formation have been identified. Chang *et al.* cloned and sequenced four genes (*CAP10, CAP59, CAP60*, and *CAP64* genes) responsible for capsule synthesis in serotype D isolates. Each of these capsule genes is required for virulence in a murine model (Chang and Kwon-Chung, 1994, 1998, 1999; Chang *et al.*, 1996). *CAP59*, the first capsule-associated gene isolated, encodes a transmembrane protein (Chang and Kwon-Chung, 1994; Chang *et al.*, 1995), which is involved in the process of GXM export (Garcia-Rivera *et al.*, 2004). *CAP64*, the second capsule-associated gene identified, was used to complement an acapsular strain (602), resulting in capsule production and a fatal infection in mice (Chang *et al.*, 1996, 1997). *CAP60* and *CAP10* are the other two characterized capsule genes, which encode proteins localized to the nuclear membrane and cytoplasm respectively (Chang and Kwon-Chung, 1998, 1999). All four *CAP* genes have been shown to be essential in capsule synthesis, but the biochemical function of their products is ill defined. There are many other genes involved in, but not essential to, capsule formation. For instance *CAS1* and *CAS3* are involved in the acetylation of GXM (Janbon *et al.*, 2001; Moyrand *et al.*, 2004), whilst *UXS1* and *UGD1* along with *CAS31*, *CAS32*, *CAS33*, *CAS34*, and *CAS35* are important for proper xylosylation of GXM (Bar-Peled *et al.*, 2001; Moyrand *et al.*, 2004). Genome analysis identified more than 30 new genes that are likely to be involved in capsule biosynthesis, including a family containing seven members of the capsule-associated (*CAP64*) gene and a second family of six capsule-associated (*CAP10*) genes (Loftus *et al.*, 2005).

The capsule is important for *C. neoformans* survival in its host, where it increases the fitness of *C. neoformans* by providing direct protection for the yeast. For instance, the capsule inhibits phagocytosis of *C. neoformans* by professional phagocytes in the absence of opsonins (Kozel and Gotschlich, 1982) and resists phagosome digestion (Tucker and Casadevall, 2002). Capsular material also acts directly against the host. In macrophages, *C. neoformans* releases polysaccharide from its capsule into vesicles around the phagosome and accumulation of these vesicles in the cytoplasm of the host cell results in macrophage dysfunction and lysis (Feldmesser *et al.*, 2000; Tucker and Casadevall, 2002). High levels of capsular polysaccharide antigens in the CSF can change the osmolarity of the CSF, thereby affecting its outflow and leading to increased intracranial pressure, headaches, and visual disturbance (Denning *et al.*, 1991). In addition, capsular material was reported to repress the migration of host phagocytes (e.g., neutrophils) (Dong and Murphy, 1995, 1997; Ellerbroek *et al.*, 2004), interfere with cytokine secretion (Retini *et al.*, 1996; Villena *et al.*, 2008), directly inhibit T-cell proliferation (Yauch *et al.*, 2006), induce macrophage apoptosis mediated by Fas ligand (Villena *et al.*, 2008), and delay maturation and activation of human dendritic cells (Lupo *et al.*, 2008; Vecchiarelli *et al.*, 1994).

The cryptococcal capsule size varies depending on the environmental conditions and seems to be tightly regulated (Fig. 5.1A). In nature, cryptococcal cells rarely display the large capsule seen in clinical isolates. The infectious particles, in order to be inhaled and penetrate the small airway, have to be smaller than 4 μm in diameter with little or no capsule (Casadevall and Perfect, 1998). However, during infection, the capsule is dynamically enlarged and the size varies depending on the affected organ. For instance, the lung and brain environment appears to act as an active inducer of capsule growth (Rivera *et al.*, 1998). Capsule size can also be experimentally modulated by growing *C. neoformans* in diluted Sabouraud broth in the presence of serum, or in a CO_2 rich atmosphere in DMEM media with low iron concentration (Vartivarian *et al.*, 1993; Zaragoza and Casadevall, 2004). These conditions are present in host environment and may thus promote capsule production during infection.

Although the presence of capsule significantly contributes to the virulence of *C. neoformans*, it is not the only requirement. Many non-*neoformans* cryptococcal species possess a capsule, but are not pathogenic. Also in one study acapsular *C. neoformans* was found to cause persistent infections in the brains of nude mice, but not in mice with defects only in innate immunity (Casadevall and Perfect, 1998), suggesting that when mammalian immunity is sufficiently impaired, even noncapsular strains retain their virulence potential.

B. Melanin

The ability of *C. neoformans* to produce melanin was discovered by Staib in the 1960s (Polacheck, 1991) (Fig. 5.1B). Melanin is a negatively charged, hydrophobic pigment of high molecular weight that is formed by the oxidative polymerization of phenolic compounds (Casadevall *et al.*, 2000). Melanin synthesis in *C. neoformans* is catalyzed by laccase in the presence of certain o-diphenolic compounds, such as 3,4-dihydroxyphenylalanine (L-Dopa) (Williamson, 1997). In the environment, melanin protects yeast from UV light, high temperatures, freezing and thawing (Rosas and Casadevall, 1997; Wang and Casadevall, 1994). Nosanchuk and colleagues have demonstrated that *C. neoformans* cells recovered from human brain tissue are melanized (Nosanchuk *et al.*, 2000a) and gene disruption studies indicate that wild type melanin-producing *C. neoformans* are more virulent (Casadevall *et al.*, 2000). Compared to nonmelanized *C. neoformans* cells, melanized cells are less susceptible to oxidants (Emery *et al.*, 1994), and killing by antifungal drugs (e.g., caspofungin and amphotericin B) (van Duin *et al.*, 2002). Since production of an oxidative burst after phagocytosis is an important mechanism by which immune effector cells mediate antimicrobial action, these results suggest that melanin may enhance virulence by protecting fungal cells against attack by the immune system. This is further supported by the observation that melanized cells were more resistant to phagocytosis and cell death caused by phagocytic effector cells (Huffnagle *et al.*, 1995). It is important to note that some non-*neoformans* cryptococci are able to form melanin as well, such as *Cryptococcus podzolicus* (Petter *et al.*, 2001), although they are not pathogenic.

The importance of melanin production to the virulence has motivated studies to define components of this pathway. Two laccase genes: *LAC1* (Torres-Guererro and Edman, 1994) and *LAC2* (Missall *et al.*, 2005; Zhu and Williamson, 2004) were identified as central enzymes in melanin biosynthesis. Other genes including *VPH1*, *CLC1*, *CCC2*, *ATX1*, and *MBF1* have also been found to be essential (Erickson *et al.*, 2001; Walton *et al.*, 2005; Zhu and Williamson, 2003), although in most cases the mode of action of these components is not well characterized.

C. Ability to grow at physiological temperature

The ability to grow at physiological temperatures is essential for the virulence of *C. neoformans* and *C. gattii*. Although some cryptococcal species also possess capsules and/or produce melanin (e.g., *C. podzolicus*), only rarely are they capable of *in vitro* growth at 37 °C, and thus none of them cause consistent infection in mammals (Perfect, 2005). *C. neoformans* is enriched in bird guano, but birds do not become infected, probably because *C. neoformans* does not live well at the avian body temperature

of 40–42 °C (Mitchell and Perfect, 1995). Therefore, this temperature restriction is an important determinant of *C. neoformans* pathogenicity.

Early studies identified over a dozen genes as being necessary for high-temperature growth (summarized in (Perfect, 2005)). One such gene, CNA1, encodes the *C. neoformans* calcineurin A (CNA1). When CNA1 was disrupted in H99, the resulting mutant strain was found to be viable at 24 °C but not at mammalian physiological temperature. Correspondingly, the mutant strain was avirulent in an immunocompromised rabbit model of cryptococcal meningitis (Odom *et al.*, 1997). Therefore, a role for the regulation of growth at elevated temperatures by signaling cascades involving calcineurin has been proposed. Many cryptococcal genes are known to be regulated by temperature, although they are not necessarily required for high-temperature growth. A microarray transcriptional profiling of *C. neoformans* genes showing altered expression at 37 °C versus 25 °C described 49 genes induced at 37 °C, including *MGA2,* which showed significantly higher expression during growth at 37 °C, and was also important for normal growth at high temperature (Kraus *et al.*, 2004). Similarly, a recent study using an alternative approach called representational difference analysis (RDA) has revealed 29 genes that are upregulated at 37 °C, with some overlaps with the genes identified by Kraus *et al.* (Rosa *et al.*, 2008). These newly defined genes seem to have a variety of functions, ranging from stress signaling, cell wall assembly, membrane integrity, and basic metabolism (Kraus *et al.*, 2004; Rosa *et al.*, 2008; Steen *et al.*, 2002). Functional studies of genes identified in these work by targeted gene disruption followed by validation in animal models may contribute to a better understanding of their role in virulence and pathogen-host interactions.

D. Degradative enzymes

Proteinase

Both environmental and clinical isolates of *C. neoformans* have proteinase activity (Casadevall and Perfect, 1998). They have been shown to degrade host proteins including collagen, elastin, fibrinogen, immunoglobulins, and complement factors (Chen *et al.*, 1996). Tucker and Casadevall also proposed that replication of *C. neoformans* inside macrophages is accompanied by the production of enzymes including proteinases and phospholipases to damage the phagosomal membrane (Tucker and Casadevall, 2002). Therefore, cryptococcal proteinases can cause tissue damage, providing nutrients to the pathogen and protection from the host.

Phospholipases

Phospholipases are a heterogeneous group of enzymes that are able to hydrolyze one or more ester linkages in glycerophospholipids. The action of phospholipases can result in the destabilization of membranes,

cell lysis, and release of lipid second messengers (Ghannoum, 2000; Santangelo *et al.*, 1999). *C. neoformans* secrets a phospholipase enzyme that demonstrates phospholipase B (PLB), lysophospholipase hydrolase, and lysophospholipase transacylase activities. As with proteinases, phospholipases contribute to the degradation of host cell membrane and thus cell lysis. There is a correlation between phospholipase expression and virulence in a dose-dependent manner among the strains used to infect mice (Chen *et al.*, 1997; Ghannoum, 2000). Disruption of *PLB1* gene led to reduced virulence *in vivo* and growth inhibition in a macrophage like cell line (Cox *et al.*, 2001). Phospholipase can also cleave dipalmitoyl phosphatidylcholine, one of the main components of lung surfactant, and thus assists fungal spread (Steenbergen and Casadevall, 2003). Furthermore, recent studies demonstrated that phospholipase B of *C. neoformans* enhances adhesion of *C. neoformans* to a human lung epithelial cell line (Ganendren *et al.*, 2006) and dissemination of cryptococcosis in a murine model (Santangelo *et al.*, 2004).

Urease

Urease catalyzes the hydrolysis of urea to ammonia and carbamate and is an important pathogenic factor for certain bacteria (Steenbergen and Casadevall, 2003). The cryptococcal urease, Ure1, is an important virulence factor and mice infected with a *ure1* mutant strain live longer than mice infected with the wild type strain H99 (Cox *et al.*, 2000). Although urease was not required for growth in the brain, the dissemination patterns in the brain, spleen, and other organs after intravenous inoculation differed from the wild type strain, leading to the proposal that Ure1 is important for CNS invasion by enhancing yeast sequestration within microcapillary beds (such as within the brain) during hematogenous spread, thereby facilitating blood-to-brain transmission (Olszewski *et al.*, 2004).

E. Mating type

Most clinical and environmental cryptococcal isolates have been observed predominantly as vegetative haploid yeast. Like other basidomycetes, traditional mating can occur when opposite mating types (a and α) recognize and fuse with one another to produce a filamentous dikaryon, resulting in a transient a/α diploid state that immediately undergoes meiosis and sporulation producing a and α haploid progeny (Kwon-Chung, 1975, 1976). *Cryptococcus* can also undergo same-sex mating (monokaryotic fruiting), especially between two α cells to form stable α/α diploids and also α haploid progeny (Lin *et al.*, 2005). Mating without a partner of the opposite mating type might provide a survival advantage, particular under harsh or changing conditions (Lin *et al.*, 2007).

Several interesting observations implicate mating type as a virulence factor. Firstly, MATα cells are much more prevalent than MATa cells. For instance, in a survey of natural and clinical isolates, the *MAT*α mating type was 40-fold more abundant in environmental isolates and 30-fold more abundant in clinical isolates than its MATa counterpart (Kwon-Chung and Bennett, 1978). In addition, most of the Vancouver isolates are α mating type (Fraser *et al.*, 2003). Secondly, when congenic α and a strains (JEC21) of serotype D (genetically identical except at the mating type locus) were studied in a murine model of cryptococcosis, the MATα strain was found to be significantly more virulent than the MATa strain (Kwon-Chung *et al.*, 1992). Congenic α and a cells in the serotype A H99 background show the same pathogenicity level in various mammalian models (Nielsen *et al.*, 2003), but α cells have an enhanced predilection to penetrate the CNS during coinfection with a cells, which provides an explanation for the prevalence of α stains in clinical isolates (Nielsen *et al.*, 2005).

The finding that MATα cells are more prevalent and virulent than MATa cells has promoted molecular analysis of the *MAT*α mating type locus. Initially, an ~50 kb region presented only in MATα strains was defined as the *MAT*α locus, and it contains many α-specific genes including STE12α (Karos *et al.*, 2000). However, the actual size of the *MAT* locus appears to be much larger than that. It is more than 100 kb in length for both *C. neoformans* and *C. gattii*, containing>20 genes, including those involved in pheromone production and sensing, establishing cell type identity, components of a MAP kinase pathway, and those do not seem to have a function in mating (Fraser and Heitman, 2004; Lengeler *et al.*, 2002). There is still much to be learned about the linkage of sex and pathogenesis, especially at the genetic level. Detailed reviews on life cycle and mating type locus can be found in (Hull and Heitman, 2002; Idnurm *et al.*, 2005).

F. Phenotypic switching

Phenotypic switching has been observed in both prokaryotes and eukaryotes and involves stochastic switching between two or more alternative and heritable phenotypes. It occurs by spontaneous tuning in gene expression in order to escape recognition by the immune system and to adapt to a new host environment. Phenotypic switching is reversible and readily detectable in a fraction of cell population (D'Souza and Heitman, 2001).

The first detection of phenotypic switching in *C. neoformans* was reported by Fries and Casadevall in 1998 (Fries and Casadevall, 1998), in which they demonstrated that *C. neoformans* was able to undergo microevolution during chronic infection. Subsequently, in 2001, Fries

et al. showed for the first time that *C. neoformans* was able to undergo phenotypic switching *in vivo* during serial passage in mice (Fries *et al.*, 2001). So far, phenotypic switching has been observed in serotype A, B, and D strains (Guerrero *et al.*, 2006; Jain *et al.*, 2006), and always leads to changes in virulence by causing changes in capsule or cell wall morphology. For example, a *C. gattii* strain was found to switch reversibly between two colony morphologies. Switching to mucoid colonies (with a thicker layer of capsule) was observed during pulmonary infection and resulted in enhanced intracellular survival due to a larger capsule. However, only smooth colonies (with a thin layer of capsule) could be grown from brain homogenates in infected mice, probably because the thin capsule permits better crossing of the blood–brain barrier (Jain *et al.*, 2006). Phenotypic switching of *C. neoformans* was also shown to influence the outcome of the human immune response. For example, the mucoid colony phenotype elicits a macrophage- and neutrophil-dominated immune response, while the smooth colony phenotype elicits a lymphocyte-dominated immune response (Pietrella *et al.*, 2003). The ability of this organism to cause chronic infections even after prolonged antifungal therapy may be, in part, attributable to phenotypic switching (Guerrero *et al.*, 2006).

G. The origin and maintenance of virulence factors

C. neoformans and *C. gattii* are environmental saprophytes, mainly found in soil and trees, so humans probably represent an inadvertent host species rather than a primary niche. There is much evidence supporting the hypothesis that cryptococcal virulence originated due to environmental selective pressure. Firstly, many environmental isolates of *C. neoformans* are virulent in animals, indicating that these virulence factors have been developed without previous interaction with host animals. Secondly, a broad range of animals are susceptible to this organism and these hosts are not required for replication or viability of the pathogen (Casadevall *et al.*, 2003). Thirdly, many virulence factors appear to have ''dual use'' capacities that allow survival advantages in both animal hosts and in the environment. For instance, in bird excreta, the primary role of urease may be to enable *C. neoformans* to convert urea to the usable nitrogen source ammonia (Levitz, 2001). Decaying wood contains large amount of the aromatic polymer lignin, a substrate of laccases. Thus, it has been hypothesized that cryptococcal laccase helps the organism establish an ecological niche in rotting wood (Lazera *et al.*, 2000). The capsule can protect the fungus against dehydration and thus provide a survival advantage in conditions of low humidity (Aksenov *et al.*, 1973). Melanized *C. neoformans* cells, as mentioned earlier, are more resistant to UV

radiation, temperature extremes, and heavy metals (Rosas and Casadevall, 1997). In addition, phospholipase and protease can serve important nutritional roles (Chen *et al.*, 1996). Hence these virulence factors are not solely developed for survival inside mammalian hosts.

Finally, *C. neoformans* is a facultative intracellular parasite, surviving both inside and outside of phagocytes. Infection of macrophages and amoebae by *C. neoformans* was found to be very similar, and it has therefore been postulated that mammalian virulence factors in *C. neoformans* evolved as a defense mechanism against environmental predators (Malliaris *et al.*, 2004; Steenbergen and Casadevall, 2003; Steenbergen *et al.*, 2001). The observation that *C. neoformans* can be ingested by living amoebae was first reported by Bunting and colleagues nearly 30 years ago (Bunting *et al.*, 1979). Steenbergen *et al*. then demonstrated that incubation of *C. neoformans* and the amoeba *Acanthamoeba castellanii* results in phagocytosis of yeast cells and intracellular proliferation in a phagocytic vacuole followed by killing of amoebae; a process that is identical to that seen to occur in mammalian macrophages infected with this pathogen (Steenbergen *et al.*, 2001). Another amoeba, *Dictyostelium discoideum,* is also susceptible to infection with *C. neoformans* and the interactions are similar to those described previously for this fungus with macrophages. In addition, *C. neoformans* virulence was enhanced after growth in *D. discoideum*, and this enhancement correlated with increased capsule size and melanization (Steenbergen *et al.*, 2003). Both studies support the idea that pathogenicity of *C. neoformans* towards macrophages and vertebrate hosts may result from evolutionary pressure exerted by environmental predators. Similarly, Mylonakis *et al.* have demonstrated that soil-dwelling nematodes may also exert strong selective pressure on *Cryptococcus* species (Mylonakis *et al.*, 2002). Whilst nonpathogenic cryptococcal species (*C. laurentii* and *Cryptococcus kuetzingii*) are killed by the nematode *Caenorhabditis elegans,* wild type strains of *C. neoformans* are lethal to the worms. Furthermore, the interaction involves a number of genes that are also important during the host pathogen interaction in mammals, including *GPA1*, *PKA1*, *RAS1*, and *PKR1* (Mylonakis *et al.*, 2002). Virulence might also be maintained through infection of small rodents or other mammals that, after death, reintroduce virulent strains back to the environment (Idnurm *et al.*, 2005).

In conclusion, it appears increasingly likely that many virulence factors in *C. neoformans* and *C. gattii* are ''ready made'' (Casadevall *et al.*, 2003) due to environmental selective pressure rather than ''specially made'' in order to colonize mammalian hosts. There are many existing environmental reservoirs that are expected to affect the fitness of fungal cells in that environment and to provide selective pressures for virulence attributes leading to differences in fitness during mammalian infection.

III. SIGNALING PATHWAYS REGULATING PATHOGENICITY

Six major signaling pathways have been demonstrated to modulate morphological differentiation, virulence, and stress responses. They are the cAMP-PKA pathway, three MAP kinase pathways involving Cpk1, Hog1, and Mpk1, the Ras specific pathway and the Ca^{2+}-calcineurin pathway. These pathways are also responsible for regulating differentiation and pathogenicity in other fungi and are largely structurally and functionally conserved in serotype A and D strains, although there are serotype-specific differences.

A. cAMP-PKA

There is conservation of function in cAMP signaling pathways in fungi since a large and diverse group of fungi (including *C. albicans* and *A. fumigatus*) employ similar signaling elements (Alspaugh *et al.*, 1998; Liebmann *et al.*, 2003; Rocha *et al.*, 2001). In *C. neoformans*, cAMP signaling is triggered by environmental stimuli (mainly nutritional, such as starvation) through a G-protein-coupled receptor (e.g., Gpr4 (Xue *et al.*, 2006)) and the Gα protein called Gpa1 (Alspaugh *et al.*, 1997). Gpa1 activates a conserved cAMP pathway through the enzyme adenylyl cyclase (Cac1), which generates cAMP and leads to activation of Protein Kinase A (PKA) by causing the release of the regulatory subunits (Pkr1) from the two catalytic units of PKA (Pka1 and Pka2) (Pukkila-Worley and Alspaugh, 2004). In serotype A strains, Pka1 plays a major regulatory role, while in serotype D strains, Pka2 does so (Hicks *et al.*, 2004).

The cAMP-PKA pathway regulates several important processes in *C. neoformans*, including capsule production, melanin formation, and mating. *gpa1* mutants, *cac1* mutants, and *pka1* mutants all display similar defects in mating, capsule, and melanin production (Alspaugh *et al.*, 1997, 2002; D'Souza *et al.*, 2001). For instance, *C. neoformans gpa1* mutant strains could not produce melanin, showed markedly attenuated capsule production in response to the normal inducing condition of severe iron starvation, and were sterile. Correspondingly, in a rabbit model of cryptococcal meningitis, the mutant strain was markedly impaired in the ability to maintain CNS infection compared to the isogenic *wild type* strain (Alspaugh *et al.*, 1997). Disruption of *PKR1* suppresses the capsule and melanin defects of the *gpa1* mutant, causes cells to display an enlarged capsule phenotype, and results in hypervirulence (D'Souza *et al.*, 2001). In addition, a recent microarray study comparing the transcriptome of mutants (*pka1* and *pkr1*) to a *wild type* strain revealed a novel relationship between cAMP signaling and the secretory pathway in *C. neoformans* (Hu *et al.*, 2007). In the *pka1* and *pkr1* mutants, transcriptional

changes occur to many key components important for the secretory pathway, such as those responsible for translocation (Sec61 and Hsp70/Kar2), vesicle formation and fusion (Bet1, syntaxin), Golgi transport (α-1,6-mannosyltransferase), and vesicle delivery to the plasma membrane (e.g., Ypt3). This study along with the observation that *C. neoformans* secretes vesicles containing many of its well-defined virulence factors, suggests a model in which PKA regulates the expression of secretory pathway components to control the elaboration of virulence factors at the cell surface (Hu *et al.*, 2007; Rodrigues *et al.*, 2008).

B. MAP kinase pathway

The pheromone-activated MAP kinase pathway is another conserved pathway, in which the G protein β subunit (Gpb1) activates the transcriptional regulator Ste12α, whose downstream targets include *STE20*, *STE11*, and *STE7* (Lengeler *et al.*, 2000). *gpb1* mutants are sterile, defective in haploid fruiting and exhibit a severe defect in cell fusion assays (Wang *et al.*, 2000). Although studies disrupting the *STE12α* gene found that in both serotype A and D, Ste12α is absolutely required for monokaryotic fruiting, it seems only to augment virulence in serotype D, but not serotype A strains. In serotype D strains, Ste12α was found to control the expression of many virulence-associated genes, and disruption of the *STE12α* gene resulted in a significant reduction in virulence in a mouse model (Chang *et al.*, 2000), whereas earlier studies by Yue *et al.* demonstrated that the *STE12α* homolog is largely dispensable for virulence in a number of serotype A strains (Yue *et al.*, 1999).

More recently, the Pbs2-Hog1 MAP kinase pathway has been shown to have a significant impact on virulence of serotype A and some serotype D strains (Bahn *et al.*, 2005). The fungal Hog1 MAPK mediates responses to a plethora of environmental cues, including osmotic shock, UV irradiation, oxidative damage, and high temperature. Intriguingly, Hog1 is regulated in an opposite fashion in a majority of *C. neoformans* strains (especially highly pathogenic isolates, e.g., H99), compared to some of the serotype D strains and other model yeasts. In *S. cerevisiae,* MAPK Hog1 is dephosphorylated in normal conditions and following osmotic shock, a two component system can activate MAPK kinase Pbs2 through activation of Ssk1, which subsequently phosphorylates MAPK Hog1 (Bahn *et al.*, 2006). Phosphorylated Hog1 then translocates to the nucleus where it activates expression of target genes (Hohmann, 2002). A similar pathway has been observed for some *C. neoformans* serotype D strains, such as JEC21. However, in most *C. neoformans* strains, the Hog1 MAPK is constitutively phosphorylated by Pbs2 MAPK kinase under normal *in vitro* growth conditions, and upon osmotic shock, Hog1 is rapidly dephosphorylated (Bahn *et al.*, 2005). It was proposed that phosphorylated

Hog1 under normal conditions is mainly responsible for negatively regulating virulence factors, including capsule and melanin, and sexual development. In addition, the phosphorylated Hog1 concentrates in the nucleus, where it can interact with other transcription factors resulting in cross-talk with signaling cascades that regulate virulence factor expression in *C. neoformans* (Bahn *et al.*, 2006). For example, experimental data demonstrated that Hog1 negatively regulates melanin production by acting on PKA downstream targets for melanin synthesis, whilst Hog1 also negatively regulate capsule production by acting on upstream of Gpa1 or PKA itself. Furthermore, it is possible that Hog1 represses factors such as Ste12 that modulate melanin and capsule production (Bahn *et al.*, 2005). Under stress conditions, Hog1 is rapidly dephosphorylated, which could be the active form of the MAPK to induce stress defense genes in *C. neoformans*. For instance, it was reported that fludioxonil treatment can activate the HOG pathway by rapid dephosphorylation of the Hog1 MAPK in the majority of *C. neoformans* strains (Kojima *et al.*, 2006).

The Mpk1 MAP kinase pathway regulates cell-wall integrity and growth at high temperature. It is well studied in *S. cerevisiae*, and the function of Mpk1 in promoting growth at 37 °C in *S. cerevisiae* is conserved in *C. neoformans*. In this pathway, upstream components such as membrane sensors that detect stresses to the cell wall (Gray *et al.*, 1997; Verna *et al.*, 1997) and the Rho1 GTPase are responsible for activating protein kinase C (PKC), which in turn activates the Mpk1/Slt2 MAPK cascade (Kamada *et al.*, 1996; Lee *et al.*, 1993). *C. neoformans* mutants lacking Mpk1 are attenuated for virulence in the mouse model of cryptococcosis (Kraus *et al.*, 2003) and become more sensitive to antifungal drugs like fludioxonil (Kojima *et al.*, 2006).

C. Ras pathway and the Ca^{2+}-calcineurin pathway

The Ras-Cdc24 pathway and Ca^{2+}-calcineurin pathway independently control *C. neoformans* growth at high temperature. *C. neoformans ras1* mutant strains are viable, but they are unable to grow at 37 °C and thus less virulent in rabbit and murine models of cryptococcosis (Waugh *et al.*, 2002). The high temperature growth defect of *C. neoformans ras1* mutant strains was associated with a failure of actin polarization at elevated temperature (Waugh *et al.*, 2002). Similarly, calcineurin mutant strains are found to be viable, but do not survive *in vitro* conditions that mimic the host environment and are no longer pathogenic in a murine model of cryptococcal meningitis (Odom *et al.*, 1997).

Ras also plays a dual role to activate a MAP kinase cascade and to regulate cAMP production in *C. neoformans*. Initial experiments defining the Ras pathway in a serotype A strain indicated that Ras1 mediates MAP kinase, cAMP, and Ras-specific signal transduction pathways (Alspaugh

et al., 2000). By northern blot analysis, Ras1 was demonstrated to play a major role in the transcriptional regulation of genes in the pheromone response pathway. It also controls pheromone-independent signaling mechanisms which are essential for filamentation, development, and pathogenicity (Waugh *et al.*, 2003). Ras2 is expressed at a very low level compared to Ras1, and a *ras2* mutant showed no differences in vegetative growth rate, differentiation, or virulence factor expression, nor was it attenuated in the murine inhalational model of cryptococcosis. However, when overexpressed, Ras2 was able to restore mating and high temperature growth of a *ras1* mutant, indicating Ras1 and Ras2 may share overlapping functions (Waugh *et al.*, 2002).

The calcineurin pathway is well characterized. Besides its importance for growth at high temperature, it is also essential for cell integrity, monokaryotic fruiting, and mating (Cruz *et al.*, 2001; Fox *et al.*, 2001; Kraus *et al.*, 2003, 2005; Odom *et al.*, 1997). In this pathway, both calcineurin A and B subunits were found to be essential for virulence (Fox *et al.*, 2001), by binding to Cbp1 (calcineurin binding protein 1) (Gorlach *et al.*, 2000), and activating as-yet unidentified downstream transcription factors.

IV. *CRYPTOCOCCUS* AND THE HOST RESPONSE

Exposure to *C. neoformans* is thought to be common, but in a normal host the infection is usually self-limiting. In contrast, in immunocompromised individuals, the infection is not restricted to the primary site of infection, but frequently disseminates to the CNS. This suggests that phagocytes *in vivo* are able to dispose of *C. neoformans* effectively (or at least maintain the pathogen in a latent stage), only when T-cell defenses are intact. This probably involves activation of macrophages by T-cell derived cytokines (mainly Th1 type, including TNFα, IFNγ, IL-2, and IL-12) and granuloma formation to contain replicating organisms. In other words, phagocytes are ''temporary protectors'' until the acquired immune response is established. This part of review will focus on the interaction between phagocytic effector cells and *C. neoformans* in the presence and absence of a secondary immune response.

A. Immunocompromised host

In immunocompromised individuals, the innate immune response is the major barrier to cryptococcal infection. Although many studies have identified several innate factors such as serum, complement, and saliva that discourage infections (Baum and Artis, 1961, 1963; Hendry and Bakerspigel, 1969; Igel and Bolande, 1966; Nassar *et al.*, 1995; Szilagyi

et al., 1966), the outcome of the infection is largely dependent on the interaction between the pathogen and phagocytic effector cells (Shao *et al.*, 2005).

1. Neutrophils

An *in vivo* study on cryptococcal infection in mice by Feldmesser *et al.* (2000) noted that macrophages and neutrophils are the only inflammatory cells in contact with *C. neoformans* in the lung. Many *in vitro* studies also demonstrated that neutrophils could phagocytose and kill *C. neoformans* (Chaturvedi *et al.*, 1996; Kozel *et al.*, 1984; Mambula *et al.*, 2000; Miller and Mitchell, 1991). However, *in vivo*, neutrophils were only found to occasionally ingest *C. neoformans* for the first few days after infection, indicating that they predominate only in the early stage of an experimental infection (Feldmesser *et al.*, 2000). Furthermore, neutrophil-depleted mice had significantly higher levels of IL-4/IL-10 (Th2 cytokines) and IL-12/TNFα (Th1 cytokines), and they lived longer than control mice, suggesting neutrophil depletion is protective against *C. neoformans* pulmonary infection. The enhanced survival observed in neutrophil-depleted mice may be a result of a more effective killing of the pathogen triggered by IL-12 and TNFα, and reduced damage to the host moderated by IL-4 and IL-10 (Mednick *et al.*, 2003). Therefore, neutrophils probably do not contribute significantly to direct killing of invading *C. neoformans*, but rather play an important role in balancing the Th1/Th2 cytokine profile in the late stage of infection.

2. Dendritic cells

Recent studies also show that dendritic cells phagocyte *C. neoformans in vitro* (Kelly *et al.*, 2005; Syme *et al.*, 2002) and *in vivo* (Wozniak *et al.*, 2006). Dendritic cells are antigen-presenting cells that act as sentinels in the peripheral tissues, constantly sampling the antigens in their environment. During cryptococcal infection, dendritic cells are thought to be more important in the initial presentation of antigens to the naive T cells to induce an adaptive immune response. Indeed, they induce a stronger T-cell response to *C. neoformans* than alveolar macrophage or monocyte-derived macrophage cells (Mansour *et al.*, 2006; Syme *et al.*, 2002). Several major antigens (e.g., mannoproteins) known to drive T cell responses to *C. neoformans* were also found to be mainly presented by dendritic cells (Levitz and Specht, 2006).

3. Macrophages

Macrophages, also involved in antigen presentation and cytokine production (Casadevall and Perfect, 1998), have long been regarded as the phagocyte that initially encounters inhaled *C. neoformans* and act as the primary phagocytic cell at all times of infection in both murine and rat

models of infection (Bolanos and Mitchell, 1989; Feldmesser *et al.*, 1998, 2000; Goldman *et al.*, 2000; Levitz, 1994). Phagocytosis of *C. neoformans* by macrophages can be mediated by receptors such as the mannose receptor, β-glutan receptor, antibody receptors, and complement receptors. Phagocytosis via the latter two receptors is efficient (Casadevall and Perfect, 1998). Depending on the environment they adapt to, *C. neoformans* cells can actively ''choose'' to avoid being phagocytosed to a certain extent by regulating their antiphagocytic factors. For instance, *C. neoformans* was found to switch reversibly between two colony morphologies which were associated with changes in capsule (Jain *et al.*, 2006). Capsule is a major anti-phagocytic factor in the absence of opsonins (Kozel and Gotschlich, 1982; Kozel and Mastroianni, 1976). It inhibits phagocytosis partly by lessening presentation of phagocytic ligands to alveolar macrophages (Vecchiarelli *et al.*, 1994). In addition, encapsulated *C. neoformans* have a more negatively charged surface than acapsular cells, which causes electrostatic repulsion between the cryptococci and negatively charged phagocytic cells and thus reduces cell-cell interaction (Nosanchuk and Casadevall, 1997). However, in the presence of opsonins including antibody and complement components (*in vivo*), the antiphagocytic property of the capsule is usually diminished (Feldmesser *et al.*, 2000). App1 (antiphagocytic protein 1) is another factor found to regulate phagocytosis. It was first identified as a regulator of complement-mediated phagocytosis (Luberto *et al.*, 2003). App1 inhibits phagocytosis through a specific and novel mechanism without affecting other cryptococcal anti-phagocytosis factors, such as capsule and melanin. Without App1, *C. neoformans* is more likely to be ingested by macrophages. Interestingly, it was found that the App1 mutant strain is less virulent than the wild type strain in A/J, CBA/J and C57BL/J mouse models, which are immunocompetent, whereas in a T-cell and natural killer (NK) cell deficient mouse model, the *app1* mutant strain exacerbated the infection as compared with the infection caused by a wild type strain. These results indicate that when the cellular immune response is impaired, phagocytosis can be an advantage for *C. neoformans* infection because *C. neoformans* grows faster intracellularly than it does extracellularly (Feldmesser *et al.*, 2000) and also it can be transported more efficiently by macrophages from organ to organ (Del Poeta, 2004; Luberto *et al.*, 2003). Therefore, modulation of the expression of antiphagocytic factors by *C. neoformans* may play a key role in the outcome of infection.

Following particle internalization by macrophages, the resulting intracellular vacuole (known as the phagosome) is subsequently fused with lysosomes to form the phagolysosome. This process is called phagosome maturation and the newly formed phagolysosome possesses a number of complementary degradative properties including a very low pH, hydrolytic enzymes for particle digestion, bactericidal peptides, and the ability

to generate toxic oxidative compounds (Vieira *et al.*, 2002). Usually the phagolysosome is very efficient at digesting internalized microorganisms. However, for *C. neoformans*, four outcomes have been observed after phagocytosis. They are: (1) The yeast is killed by the macrophage (Brummer and Stevens, 1994; Casadevall and Perfect, 1998); (2) the yeast remains latent inside the macrophage (Alvarez and Casadevall, 2007; Del Poeta, 2004; Ma *et al.*, 2007); (3) the yeast grows within the phagosome, eventually causing macrophage lysis (Feldmesser *et al.*, 2000, 2001; Tucker and Casadevall, 2002); and (4) the yeast exits the macrophage by a novel expulsive process without causing death of either the yeast or the host macrophage (Alvarez and Casadevall, 2006; Ma *et al.*, 2006) (Fig. 5.3). Therefore, *Cryptococcus* can manipulate macrophages in various ways. Currently it is unclear as to what decides the outcome of the intracellular yeast/macrophage interaction, but it is generally established that *in vitro*,

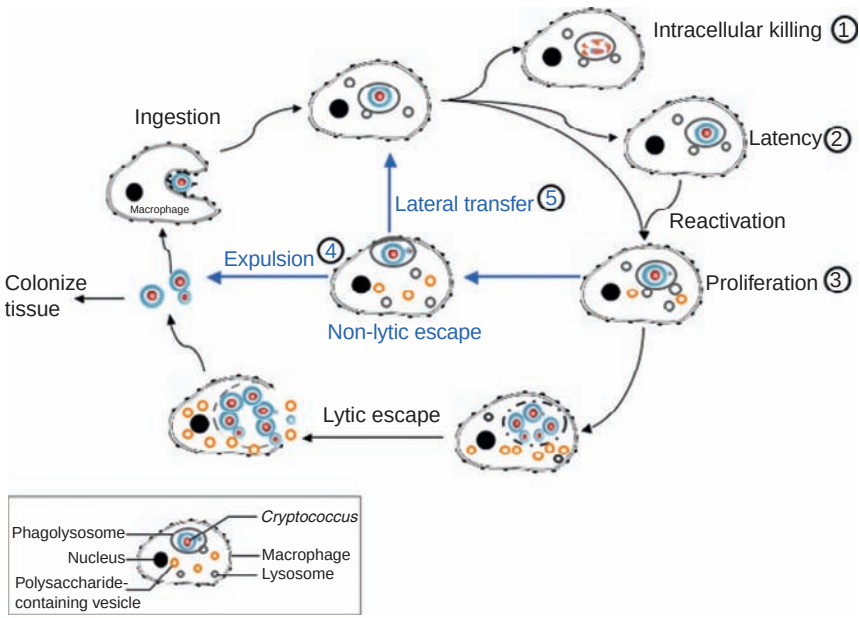

FIGURE 5.3 Macrophage parasitism by *C. neoformans*. Following phagocytosis, the internalized cryptococci can be killed by macrophages (1) or remain latent (2). When the host becomes immunocompromised, some of the cryptococci or latent population can reactivate and proliferate intracellularly (3), followed by the lytic burst of the host cells and release of the intracellular yeast cells into the extracellular environment. The released yeast cells can then carry on infecting more macrophages or establish extracellular dominance. Nonlytic escape pathways were also observed for *C. neoformans*, during which the yeast cells are expelled by macrophages without causing death of either party (4) or the intracellular yeast cells are directly delivered to a neighboring macrophage via lateral transfer (5).

macrophages activated with Th1 cytokines (secreted by CD4+ T cells) are more efficient at eradicating intracellular cryptococci than those activated with Th2 cytokines (Brodie *et al.*, 1994; Chen *et al.*, 1994; Kawakami *et al.*, 1995; Mody *et al.*, 1991; Weinberg *et al.*, 1987). In addition, the fate of intracellular cryptococci varies with strain ((Zaragoza *et al.*, 2007); (Ma *et al.*, unpublished data)) and local environment (e.g., oxygen availability) (Voelz *et al.*, unpublished data).

In the absence of a T-cell mediated immune response, intracellular survival and proliferation of *Cryptococcus* is very common. This intracellular behavior is important for pathogenicity, because it provides a basis for dissemination and latency: intracellular cryptococci are carried by infected macrophages to different parts of the body without being exposed to any extracellular hazards, such as complement components or antifungal agents present in the blood. This so-called ''Trojan horse'' mechanism of dissemination (Chretien *et al.*, 2002; Santangelo *et al.*, 2004) is supported by the observation that *C. neoformans* was found almost exclusively in macrophages in chronic and latent infection (Feldmesser *et al.*, 2000; Goldman *et al.*, 2000). Intracellular parasitism of macrophages by *C. neoformans* was reported in the early 1970s, when most ingested *C. neoformans* were found to be resistant to intracellular killing by either peritoneal exudate cells from Lewis rats or monocyte-derived macrophages (Diamond and Bennett, 1973; Mitchell and Friedman, 1972).

Unlike many other intracellular pathogens which persist within the phagosome by either affecting phagolysosome maturation (e.g., *Legionella pneumophila*) (Nguyen and Pieters, 2005) or by escaping from the phagosome and then proliferating in the host cytosol (e.g., *Listeria monocytogenes*) (Cossart *et al.*, 2003), *C. neoformans* has been demonstrated to persist inside apparently normal mature phagosomes in human monocyte-derived macrophage (MDM) *in vitro* (Levitz *et al.*, 1999). The pH of *C. neoformans*-containing phagosomes was similar to that observed following uptake of dead fungi over 24 h, and these phagosomes also colocalized with LAMP-1, a highly glycosylated protein found in endosomal and lysosomal compartments that is commonly used as a late mature phagosome marker, indicating that *C. neoformans* does not interfere with phagosome-lysosome fusion. In fact, *C. neoformans* grows more rapidly in acidic media than in neutral or alkaline media and appears to be able to resist the action of the macrophage lysosomal enzymes, which function optimally at acid pH (Levitz *et al.*, 1999). *In vivo*, intracellular persistence was associated with replication and residence in a membrane bound phagosome (Feldmesser *et al.*, 2000, 2001). Recent electron microscopy studies by Tucker and Casadevall revealed that intracellular residence by *C. neoformans* is accompanied by the accumulation of polysaccharide-containing vesicles, which originated from the phagosome, followed by macrophage dysfunction and lysis (Tucker and Casadevall, 2002).

Many virulence factors required for cryptococcal intracellular survival have already been identified, including capsule and melanin synthesis proteins, proteinases, and phospholipases, an alternative oxidase (*AOX1*) (Akhter *et al.*, 2003), inositol phosphosphingolipid-phospholipase C1 (*ISC1*) (Shea *et al.*, 2006), *SKN7* (Coenjaerts *et al.*, 2006), and vacuole protein *VPS41* (Liu *et al.*, 2006), most of which contribute to defense against exogenous oxidative stress. However, the detailed intracellular survival mechanism needs further investigation.

Interestingly, live *C. neoformans* and *C. gattii* were also found to be expelled by macrophages as individual cells or extruded as biofilm-like microcolonies (Alvarez and Casadevall, 2006; Alvarez *et al.*, 2008; Ma *et al.*, 2006). This novel expulsive process has never been observed with any other pathogens parasitizing inside macrophages. Remarkably, expulsion does not kill either the expelled yeast or the host macrophage. The process is extremely rapid and yet can occur many hours after phagocytosis of the pathogen. The frequency of expulsion is both strain and host cell dependent (Alvarez and Casadevall, 2006; Ma *et al.*, 2006). Although the underlying molecular mechanism is unknown, the process seems to be cytoskeleton dependent, and is independent of phagosome maturation (Alvarez and Casadevall, 2006; Ma *et al.*, 2006). Compared with earlier studies showing intracellular proliferation followed by a lytic burst, this novel expulsive mechanism allows the pathogen to reemerge from the host cell in a more subtle way. Therefore, it may represent an important mechanism by which pathogens are able to escape from phagocytic cells without triggering host cell death and thus inflammation.

Although the expulsion event is independent of the initial route of the phagocytic uptake (Ma *et al.*, 2006), the outcome of the expulsion was affected by the mode of opsonization (Alvarez *et al.*, 2008). Extrusion of antibody-opsonized *C. gattii* and *C. neoformans* resulted in the release of a clump of yeast cells that remained attached to one another and continue to replicate extracellularly as a biofilm. In contrast, complement-opsonzed *C. neoformans* cells were released from macrophages dispersed as individual cells, which then continued to divide in the extracellular milieu as single cells. Therefore, the biofilm-like microcolony exit strategy of *C. neoformans* and *C. gattii* following antibody opsonization reduced fungal cell dispersion, suggesting that antibody agglutination effects persist even inside the phagosome to attach nascent daughter cells together and may thus contribute to antibody-mediated protection (Alvarez *et al.*, 2008).

Finally, *C. neoformans* has also been shown to spread from one macrophage to another directly without being exposed to the extracellular environment (Alvarez and Casadevall, 2007; Ma *et al.*, 2007). This so-called ''lateral transfer'' event needs further investigation, although it appears to be actin dependent (Alvarez and Casadevall, 2007) and

superficially resembles cryptococcal expulsion (Ma *et al.*, 2007). Despite the low rate of lateral transfer, it is possible that this process may have significant clinical implications, since it allows the pathogen to spread whilst remaining concealed from the immune system, and to move from weak to healthy phagocytes to ensure intracellular persistence even if the host cells starts to die (Ma *et al.*, 2007). Furthermore, it may represent a novel mechanism for *C. neoformans* to cross the blood–brain barrier (discussed in more detail in Section V).

B. Immunocompetent host

The host defense against *C. neoformans* is critically regulated by cell-mediated immunity (Lim and Murphy, 1980), especially T lymphocytes, which play a central role in eradicating this infection (Hill and Harmsen, 1991; Huffnagle *et al.*, 1991; Mody *et al.*, 1990). The mechanisms by which the lymphocytes facilitate elimination of cryptococci have not yet been elucidated. It is generally thought that lymphocyte clearance of *C. neoformans* acts indirectly through production of cytokines to enhance clearance of the organism by natural effector cells, particularly macrophages (Brodie *et al.*, 1994; Chen *et al.*, 1994; Kawakami *et al.*, 1995, 2000; Lindell *et al.*, 2005; Weinberg *et al.*, 1987; Zhang *et al.*, 1997).

Exposure to various pathogens can stimulate at least two patterns of cytokine production mainly by CD4+ T cells: Th1 and Th2. For *C. neoformans*, the balance between Th1 and Th2 cytokines markedly influences the outcome of infection. The predominant synthesis of Th1 cytokines over Th2 protects mice from infection was observed, whereas infection is exacerbated under Th2 dominant conditions (Hoag *et al.*, 1995; Huffnagle, 1996; Koguchi and Kawakami, 2002; Snelgrove *et al.*, 2006; Uicker *et al.*, 2005). Mice depleted of Th1 type cytokines are highly susceptible to cryptococcal infection (Huffnagle, 1996; Kawakami *et al.*, 1996), while the infection is less severe in mice lacking Th2 cytokines than in control mice (Blackstock and Murphy, 2004; Decken *et al.*, 1998).

During cryptococcal infection, the Th1/Th2 balance is maintained mainly by phagocytic effector cells (e.g., dendritic cells and neutrophils as discussed earlier) (Mednick *et al.*, 2003; Wozniak *et al.*, 2006) and some primary lymphocytes (e.g., natural killer T (NKT) cells and γδ antigen receptor-bearing T cells) (Kawakami, 2004; Nanno *et al.*, 2007; Zhang *et al.*, 1997). A remarkable feature of NKT cells is the abundant production of IFNγ and IL-4 upon stimulation via their antigen receptors. After cryptococcal infection, NKT cells were found to be recruited to the lung, and trigger a Th1-mediated, but not Th2-mediated, immune response (Kawakami *et al.*, 2001). In contrast, γδ T cells play a down-modulatory role in the development of Th1 responses and host resistance against *C. neoformans* (Uezu *et al.*, 2004). Therefore, γδ T cells may act to keep

the balance of Th1–Th2 responses in a proper manner by suppressing the exaggerated Th1 response caused by NKT cells (Kawakami, 2004). The contrasting roles of NKT and γδ T cells, and the fact the neutrophil depletion can enhance both Th1 and Th2 cytokines (Mednick *et al.*, 2003), suggest that these innate immune response are not only important for induction of proper host defense but also to balance the level of defense.

1. Antifungal effect of activated macrophages

When a T-cell mediated immune response is present, the majority of the intracellular cryptococci are eradicated (Fig. 5.4). Properly activated macrophages have a variety of microbicidal mechanisms that are potentially active against *C. neoformans*, including both oxidative and nonoxidative mechanisms and granuloma formation. The oxidative microbicidal mechanism involves the generation of reactive oxygen- and nitrogen-derived intermediates (ROI and RNI). ROI, such as

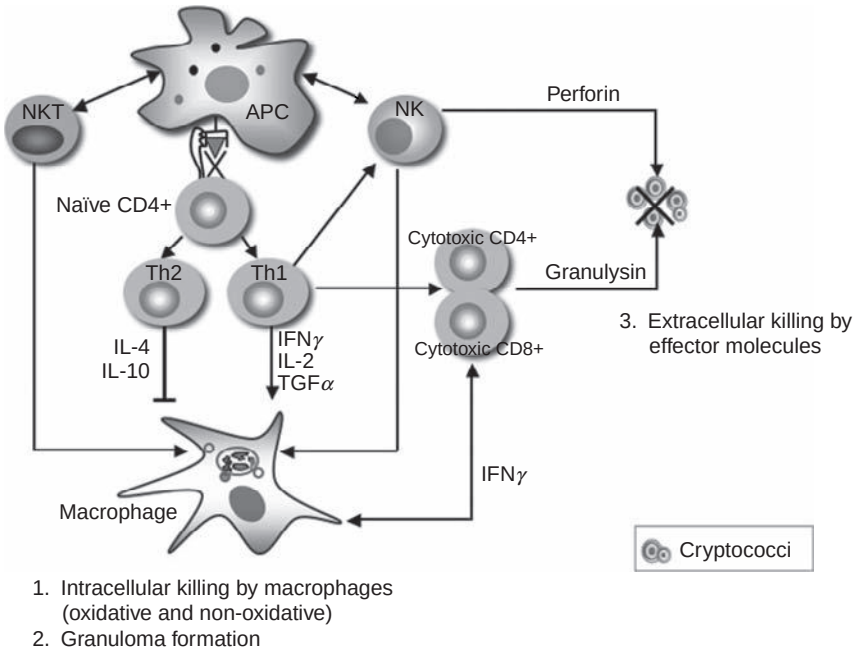

FIGURE 5.4 Killing of *C. neoformans* by the immune response in immunocompetent individuals. Killing can occur either intracellularly, when macrophages are activated by Th1 type cytokines, or extracellularly by effector molecules secreted by cytotoxic T lymphocytes (CD4+ and CD8+) and NK cells. Many other cells from the immune system also contribute to elimination of the cryptococci directly or indirectly by triggering and balancing Th1 type cytokine release.

superoxide anions, hydroxyl radicals, and hydrogen peroxide are generated as a result of the incomplete reduction of oxygen during respiratory metabolism (Turrens and Boveris, 1980). Nessa *et al.* showed that *C. neoformans* induced a markedly higher increase of oxidative metabolism in macrophages than did inert silica particles in an *in vivo* rabbit model of infection (Nessa *et al.*, 1997a). Such an increase was also observed with rat alveolar macrophages and *Candida* and *Aspergillus* species in *in vitro* studies (Nessa *et al.*, 1997b), indicating that production of ROI is a general mechanism of intracellular killing employed by macrophages. Cryptococcal strains lacking proteins (e.g., Aox1 and Skn7) that protect against reactive oxygen species inside macrophages, show reduced intracellular survival of *C. neoformans* and thus reduced virulence in animal models of infection (Akhter *et al.*, 2003; Coenjaerts *et al.*, 2006). RNI, produced by several mammalian cells, are also powerful antimicrobial molecules against intracellular *C. neoformans*. Nitric oxide, one of the key RNI molecules, is produced by macrophages through the action of inducible nitric oxide synthase on L-arginine (Tripathi *et al.*, 2007) and acts to suppress cryptococcal growth (Tohyama *et al.*, 1996). In addition, NK cells promote anticryptococcal activity of macrophages through enhancing nitric oxide activity (Kawakami *et al.*, 2000). Resistance to oxygen- and nitrogen-derived oxidants has been found to be a major factor in determining the outcome of infection with *C. neoformans* (Xie *et al.*, 1997), implying the importance of ROI and RNI in intracellular killing by macrophages.

In the presence of intact T cell function, macrophages also often form a histiocytic ring around *C. neoformans* cells and may fuse to form giant multinucleated cells in order to engulf heavily encapsulated yeast. This is called granuloma formation and has been demonstrated to be the most effective host response to localize the infection and prevent dissemination (Casadevall and Perfect, 1998; Hill, 1992). Furthermore, resolution of infection, when it occurs, almost always follows granuloma formation. For instance, intratracheal infection of rats with *C. neoformans* was found to elicit a strong granulomatous response and resulted in minimal or no dissemination (Goldman *et al.*, 1994, 1996; Kobayashi *et al.*, 2001). Granulomatous inflammation is more likely to be reported in non-HIV-associated cryptococcosis (Lee *et al.*, 1996; Mohanty *et al.*, 2003; Shibuya *et al.*, 2005), and there is evidence that a strong granulomatous response is dependent on intact T cell function (Clemons *et al.*, 1996; Hill, 1992), indicating a mechanism by which abnormalities of cell-mediated immunity can translate into poor inflammatory responses.

The fungistatic activity of macrophages can be enhanced by the presence of antibody. For example, antibody against capsular GXM seems to promote nitric oxide production in macrophages (Rivera *et al.*, 2002). Antibody-treated mice have been shown to have a more intense granulomatous

response than control mice, further supporting the concept that macrophage killing is enhanced in the presence of antibody (Casadevall and Perfect, 1998).

Nevertheless, a small number of cryptococci are able to survive and remain latent inside macrophages in immunocompetent individuals, despite the presence of Th1 cytokines and antibody. This latency is probably due to a combination of frequent lateral transfer events, the presence of cryptococcal anti-ROI/RNI factors and virulence factors (such as capsule, melanin, *AOX1*, *SOD1*, *CCP1*, *ISC1*, and *SKN7* (Akhter *et al.*, 2003; Alvarez and Casadevall, 2007; Coenjaerts *et al.*, 2006; Cox *et al.*, 2003; Giles *et al.*, 2005; Liu *et al.*, 2006; Ma *et al.*, 2007; Missall *et al.*, 2004; Zaragoza *et al.*, 2008)), rapid changes in virulence mediated by phenotypic switching and Th2-polarised responses later in infection to avoid tissue damages caused by the early Th1 response (Kawakami, 2004; Mednick *et al.*, 2003). This latent population is then able to trigger cryptococcosis later on in life when the host immune system becomes compromised (Garcia-Hermoso *et al.*, 1999).

2. Direct antifungal effects of T lymphocytes

Much evidence suggests that NK cells and T lymphocytes function as both regulators (by secreting cytokines, e.g., CD4+ T helper cell) and effectors (cytotoxic cells) during the immune response against *C. neoformans*. Hence, direct inhibition of cryptococcal cells by these host cells may be another important means of host defense against *C. neoformans*. Early studies by Levitz *et al.* demonstrated the competence of freshly isolated human CD4+, CD8+ lymphocytes, and CD16/56+ NK cells (but not B cells) to directly bind and inhibit the growth of *C. neoformans* in the absence of MHC restriction (Levitz and Dupont, 1993; Levitz *et al.*, 1994). These findings are in agreement with several previous studies (Hidore *et al.*, 1991; Murphy *et al.*, 1991, 1993; Nabavi and Murphy, 1985). Recent studies have improved our understanding of the underlying detailed mechanisms. These studies found that direct anticryptococcal activities of CD4+ and CD8+ cytotoxic cells are dependent on expression of granulysin after activation by CD4+ T helper cell (or IL-2/IL-15, which can substitute T cell helper) (Ma *et al.*, 2002; Zheng *et al.*, 2007), whereas NK cells used perforin instead (Ma *et al.*, 2004) (Fig. 5.4). Granulysin, a novel host defense protein, is able to increase membrane permeability of bacteria and fungi, and thus cause osmotic lysis (Ernst *et al.*, 2000). Granulysin expression in CD4+ cytotoxic T cells is controlled by PI3K and STAT5 signaling pathways through promoting IL-2Rβ induction (Zheng *et al.*, 2008). CD4+ cytotoxic T cells from HIV patients fail to induce granulysin expression due to defective PI3K and STAT5 pathways, resulting in inefficient killing (and growth inhibition) of *C. neoformans* (Zheng *et al.*, 2007). Similarly, CD8+ T cells express granulysin in the presence of IL-15 and CD4+ T cells, and the upregulation of granulysin correlated with the acquisition of

anticryptococcal activity (Ma *et al.*, 2002). Perforin, stored in secretory vesicles (granules) of T lymphocytes and NK cells, is another pore-forming effector molecule that acts by inserting into the target cell's plasma membrane, triggering lysis (Voskoboinik *et al.*, 2006). Perforin-mediated anticryptococcal killing is essential for NK cells, although both granulysin and perforin are constitutively expressed by this cell type (Ma *et al.*, 2004), and the killing is accompanied by activation of PI3K-ERK1/2 signaling pathway (Wiseman *et al.*, 2007).

C. Conclusion

C. neoformans is a facultative intracellular pathogen, capable of living both outside and inside cells. The current model of cryptococcal infection is based on five steps: internalization, dormancy, reactivation, proliferation, and dissemination (Fig. 5.3). In the initiation stage, *C. neoformans* interacts with and is internalized by lung phagocytes (mainly macrophages). Normally, in an immunocompetent individual, a T-cell mediated immune response (driven especially by CD4+ cells) develops. This leads to activation of macrophages via cytokine release and granuloma formation, resulting in either destruction of the intracellular fungus or containment in a latent state, which is probably maintained by lateral transfer of the yeast between host cells. Direct antifungal activity of lymphocytes also improves the host defense. Subsequently, when the individual becomes immunocompromised, *C. neoformans* can start proliferating inside the macrophage, followed by macrophage lysis and release of *C. neoformans*. The released organism can then enter other phagocytes, causing dissemination and increased proliferation. Long-term intracellular growth leads to enlargement of the capsule, which probably sequesters available complement proteins. The unopsonised organisms are poorly recognized by phagocytes and thus establish extracellular dominance. During prolonged infections, the yeast population can undergo microevolution, which results in both phenotypic and genotypic changes in order to be better adapted to local organs or environments (Lortholary *et al.*, 1999). The identification of genes and factors that contribute to either extra or intracellular proliferation of this pathogen may lead to the development of novel prevention and treatment strategies for cryptococcosis.

V. CURRENT UNDERSTANDING ON HOW *CRYPTOCOCCUS* CROSSES THE BLOOD–BRAIN BARRIER

Cryptococcal meningoencephalitis develops as a result of hematogenous dissemination of inhaled *Cryptococcus* from the lung to the brain. In order to penetrate into the brain, the yeast must cross the endothelium of the

blood–brain barrier, which is composed of brain microvascular endothelial cells connected by tight junctions between the cells (Rubin and Staddon, 1999).

Although the mechanisms of entry into the CNS for the majority of meningoencephalitis-causing microorganisms are not clear, three potential models have been described. Pathogens may cross the blood–brain barrier paracellularly (e.g., *Trypanosoma* species) (Grab *et al.*, 2004), transcellularly (e.g., *Streptococcus pneumoniae*) (Ring *et al.*, 1998), and by means of infected immune cells (Trojan horse mechanism, e.g., HIV) (Dallasta *et al.*, 1999; Erlander, 1995). In the case of *Cryptococcus*, several lines of evidence support the hypothesis that the yeast crosses the blood–brain barrier transcellularly. In 1995, an *in vitro* study by Ibrahim *et al.* demonstrated that *C. neoformans* (especially acapsular strains) was able to adhere to and then be internalized by endothelial cells, subsequently causing damage to endothelial cells. Furthermore, they found that internalization required the presence of a heat-labile serum factor, which could be one of the components of the classical complement pathway (Ibrahim *et al.*, 1995). Chretien and colleagues then reported for the first time that *in vivo*, *Cryptococcus* was phagocytosed by endothelial cells of leptomeningeal capillaries (Chretien *et al.*, 2002). Subsequently, Chen *et al.* and Chang *et al.* further proposed that *C. neoformans* was capable of altering the cytoskeletal morphology of human brain microvascular endothelial cell (HBMEC) through the ROCK-LIMK-cofilin pathway, and crossing the HBMEC layer transcellularly without affecting the monolayer integrity. Importantly, the virulence factor Skn7 has been demonstrated to coregulate the adaptive strategy of *Cryptococcus*, allowing intraphagocytic survival by conferring resistance to phagolysosomal killing in endothelial cells (Coenjaerts *et al.*, 2006). However, compared to *C. albicans*, the efficiency of adhesion and invasion is low (Chang *et al.*, 2004; Chen *et al.*, 2003; Jong *et al.*, 2001).

Several studies also demonstrated indirectly that phagocytes might act as a means of allowing *Cryptococcus* to invade the brain (Trojan horse mechanism). For example, microscopy of the leptomeninges of a mouse with severe meningoencephalitis showed cryptococci internalized both within mononuclear cells circulating within meningeal capillaries and within host cells touching the outer membrane of the capillaries. However, it was not determined whether the latter cells were microglial cells that had phagocytosed free cryptococci in the meningeal spaces or were macrophages derived from *C. neoformans*-infected monocytes (Chretien *et al.*, 2002). Furthermore, although direct transfer of *C. neoformans* from infected phagocytes to endothelial cells has not been demonstrated, such events have been observed between two macrophages (Alvarez and Casadevall, 2007; Ma *et al.*, 2007). When traveling throughout the host circulatory and lymphatic system, macrophage cells interact intimately with one another and with other cell types through transient contacts. It is possible that

internalized *C. neoformans* may use such transient contact in order to cross the blood–brain barrier by direct cell-to-cell spread from adherent infected macrophages to microvascular endothelial cells. In fact, spreading from macrophages to other cell types during dissemination has been demonstrated for other pathogens *in vitro*. For instance, *L. monocytogenes* can infect neurons by cell-to-cell spread from adherent macrophages, a more efficient process than direct invasion of neurons (Dramsi *et al.*, 1998). Intriguingly, cell-to-cell spread of bacteria from adherent infected phagocytes to endothelial cells of the CNS has also been reported (Drevets and Leenen, 2000) and it will clearly be of the great interest to investigate whether a similar process may occur during cryptococcosis.

Since cryptococcosis is very common in HIV-infected patients, it is not implausible to suspect that the presence of HIV may enhance cryptococcal entry into the CNS. Numerous studies have demonstrated that HIV is able to cause damage to the endothelial cell layer and thus facilitates other microorganisms to enter and infect the CNS (Dallasta *et al.*, 1999; Ricardo-Dukelow *et al.*, 2007; Toborek *et al.*, 2005). The interaction between HIV and *C. neoformans* has not been well studied, but a recent study reported an interesting interplay between the yeast and the HIV-1 protein gp41. Jong *et al.* demonstrated that the binding of *C. neoformans* to HBMEC could be enhanced by HIV-1 gp41 *in vitro* and also in a murine model. Therefore, they speculated that HIV-1 gp41 may play a role as a trans-predilection factor for *C. neoformans* invasion, thus resulting in a deteriorating meningoencephalitis in HIV-infected patients (Jong *et al.*, 2007).

In summary, there are three possible ways by which *Cryptococcus* can cross the blood–brain barrier and enter the CNS (summarized in Fig. 5.5). They are: (1) direct transcellular crossing, during which free cryptococci are internalized by endothelial cells and exit through the abluminal surface of the cells; (2) Trojan Horse mechanism, which proposes that cryptococci are engulfed by phagocytic cells at an early stage of infection and then trafficked by these host cells into the CNS; and (3) direct transfer from infected phagocytes into endothelial cells followed by exit at the abluminal surface of the cells. Moreover, the presence of HIV-1 may facilitate *Cryptococcus* to cross the blood–brain barrier by destroying the integrity of blood–brain barrier and/or by acting as a trans-predilection factor.

VI. ANIMAL MODELS

Analysis of molecular mechanisms by which a pathogen interacts with the human host is most commonly performed using a mammalian model of infection. However, several virulence-related genes previously shown to be involved in mammalian infection with *C. neoformans* have also been shown to play a role in the interaction of the pathogen with invertebrates,

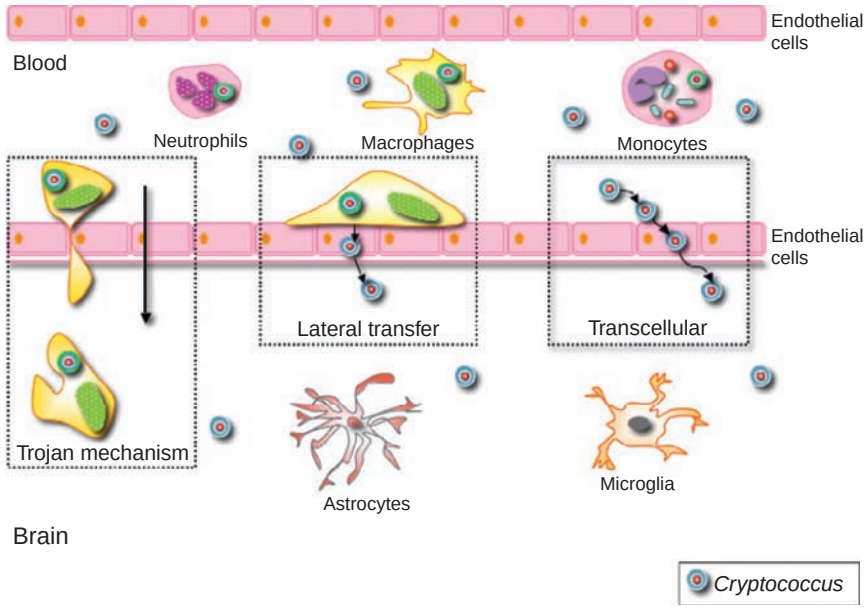

FIGURE 5.5 Possible routes for cryptococci to cross the blood–brain barrier: (1) Trojan horse mechanism; (2) Lateral transfer; and (3) Transcellular crossing.

including *C. elegans* (Mylonakis *et al.*, 2002; Tang *et al.*, 2005), *D. discoideum* (Steenbergen *et al.*, 2003), *D. melanogaster* (Apidianakis *et al.*, 2004), and *Galleria mellonella* (Mylonakis *et al.*, 2005). These organisms show promise for the study of *C. neoformans* pathogenesis, since they allow for high-throughput screening to identify novel loci related to mammalian pathogenesis (Tang *et al.*, 2005). Moreover, the interaction of these model organisms with *C. neoformans* suggest that the virulence factors important in human disease pathogenesis probably evolved through interactions with simpler organisms (discussed in Section II).

The routine mammalian systems used to study cryptococcal infection include mouse, rat, rabbit, and guinea pig (Barchiesi *et al.*, 2005; Clancy *et al.*, 2006; Cox *et al.*, 2000; da Silva *et al.*, 2006; Fries *et al.*, 2005; Perfect *et al.*, 1980; Torres-Rodriguez *et al.*, 2003; Zhou *et al.*, 2007). These models have been reviewed in detail recently (Carroll *et al.*, 2007), so they are not discussed here. It is important to point out, however, that although each model is robust and provides valuable insights into understanding of the host-pathogen interaction, there are many disagreements both between and within models. For instance, genes which are important for the virulence composite in one model are not in another (Cox *et al.*, 2000). Rats and mice are considered resistant and susceptible hosts respectively, partly due to difference in alveolar macrophage function in the two

species (Shao *et al.*, 2005). Within the murine model, there are several commonly used mouse laboratory strains including BALB/c, C57BL/6J, A/J, CBA/J, and DBA/J, and there is substantial variation in susceptibility to *C. neoformans* among these mouse strains (Huffnagle *et al.*, 1998). For instance, in an intranasal infection model with the same cryptococcal strains (WM276 and NIH444), WM276 was found to be more virulent in A/J mice (Fraser *et al.*, 2005), whereas the opposite was observed with BALB/c mice (Chaturvedi *et al.*, 2005). Early studies showed that the different resistance/susceptibility patterns of cryptococcal infection in various strains of mice were caused by differences in host factors (including genetic background, gender, and age) and were linked to differential phenotypes of the inflammatory responses (Hoag *et al.*, 1995; Huffnagle *et al.*, 1998; Lortholary *et al.*, 2002). A recent study exploring the immunological basis for differences in the susceptibility of BALB/c and CBA/J strains to *C. neoformans* infection demonstrated that the outcome of infection was also dependent on the route of infection: BALB/c mice are more resistant when infected intranasally, but both strains were equally susceptible when infected intravenously and, moreover, BALB/c mice were slightly more susceptible at higher intravenous infection doses. In addition, during intranasal infection, the resistance of BALB/c mice towards infection was associated with a stronger Th1 response (e.g., increased IL-12 but decreased IL-10), higher accumulation of CD4+ and CD8+ T cells in the lung and elevated antibody production during the early stage of infection. This may lead to the difference in macrophage activation profile, since significant differences in intracellular replication were observed between *C. neoformans* ingested by BALB/c versus CBA/J alveolar macrophages (Zaragoza *et al.*, 2007). Similarly, Chen *et al.* showed that BALB/c mice cleared infected *C. neoformans* more effectively compared to C57BL/6 mice, as a result of a protective Th1 response and greater numbers of CD4+ cells in the lungs following infection. Furthermore, they demonstrated that first-generation hybrid mice, unlike C57BL/6 mice, maintained the ability to clear cryptococci from the lungs, although their clearance was slower than that of BALB/c mice. Detailed analysis revealed F1 resistance was due to the inheritance of the propensity for a Th1 versus Th2 bias of the immune responses, but susceptibility versus resistance to *C. neoformans* infection was inherited in a complex fashion (Chen *et al.*, 2008a).

VII. PERSPECTIVES

The AIDS pandemic has resulted in a new wave of research on *C. neoformans* over the last 30 years, and our understanding of the biology of this pathogen has improved dramatically. As a result of technical improvements, such as

the development of three transformation techniques (electroporation (Chang *et al.*, 1996; Edman and Kwon-Chung, 1990), biolistic transformation (Alspaugh *et al.*, 1997; Odom *et al.*, 1997; Toffaletti *et al.*, 1993), and *Agrobacterium-mediated* transformation (McClelland *et al.*, 2005)) and the recent completion of several whole-genome sequences, many genes responsible for virulence in *C. neoformans* during infection have been identified and the mutants verified in robust animal models. Its clinical significance and well-defined virulence factors, along with advanced genome-wide analysis tools, have made *C. neoformans* an organism of choice for the study of fungal pathogenesis in general.

However, although the clinical management of cryptococcosis is possible, the morbidity and mortality remain high, and a critical challenge will be to develop novel treatments based upon advances in genomics, proteomics, and metabolomics. This requires a better understanding of host-pathogen interplay, and of the communication between the innate and adaptive immune response at the molecular level. The task for the future is to translate our growing biological understanding of this organism into real improvements in both therapeutic antifungals and preventative vaccines.

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CHAPTER 6

Molecular Networks in the Fungal Pathogen *Candida albicans*

Rebecca A. Hall, Fabien Cottier, and **Fritz A. Mühlschlegel**[1]

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Abstract

Candida albicans is an important opportunistic fungal pathogen of humans. Its success as a commensal and pathogen extends from its ability to switch between both yeast and hyphal growth forms. Therefore, extensive research on this fungus has also focused on the identification and understanding of the regulatory networks behind this morphological switch. Here we review established signaling pathways, including the mitogen-activated protein kinase cascades and the cyclic AMP-dependent protein kinase A signaling pathway. In addition, we focus on new developments in the rapidly growing area of fungal environmental sensing, but importantly also highlight exciting new developments in the expanding field of molecular networks involved in fungal–fungal and fungal–bacterial interkingdom communication.

I. INTRODUCTION

Candida albicans is a fungal pathogen of humans, and is a major concern for immunocompromized individuals. One factor that contributes to the organism's success as a pathogen is its polymorphic nature of growth. *C. albicans* grows in yeast and filamentous, both pseudohyphae and true hyphae, forms. The regulation of which is influenced by environmental factors the organism experiences, both in its natural environment and within the human host. These important environmental cues include serum, temperature, pH, CO_2, glucosamine, and nutrient limitation. Molecular biology approaches coupled with traditional microbiology have enabled scientists to dissect each pathway separately gradually building up a molecular network that regulates the response to the specific stimulus. This review aims to provide an overview of the recent developments for the major molecular networks that govern the *C. albicans* morphological switch. Notably during the infection process *C. albicans* will be in close contact with other organisms like *Escherichia coli* and *Pseudomonas aeruginosa*. Thus in addition to morphology, emerging molecular networks that function in quorum sensing and fungal bacterial interactions will also be discussed. Importantly when *C. albicans* is in contact with the human host it will not be exposed to just one of these cues, but many at any given time. In addition, being a commensal it will interact with other pathogens and organisms that comprise the natural flora. Therefore, understanding the cross talk between the identified networks will be critical for an enhanced understanding of *C. albicans* success as a pathogen and the development of novel antifungal therapeutics.

II. Ras1 Interrelated Networks

Center to the regulation of *C. albicans* morphology is the Ras1p interrelated networks. The two main pathways that have been studied in detail are the mitogen-activated protein kinase (MAPK) pathway and the cyclic AMP-dependent protein kinase A (PKA) pathway. The small GTPase Ras1p initiates both of these pathways. Notably, the signal and membrane receptors, which activate Ras1p in response to environmental signals, still remain elusive. However, Cdc24p and Cdc42p have been suggested to participate in the activation of Ras1p (see below) although direct evidence of the involvement in this activation is currently not available. In addition to initiating these two pathways, Ras1p can also influence other factors that function in morphology. For example, Cla4p and Crk1p are kinases that are influenced by Ras1p, but their activation is independent of either the MAPK pathway or cAMP-dependent PKA signaling (Chen *et al*., 2000a). In comparison to *Saccharomyces cerevisiae*, *C. albicans* only has one Ras encoding gene. The *ras1/ras1 C. albicans* strain is viable, although growth rates and morphology are affected. (Feng *et al*., 1999).

A. Mitogen-activated protein kinase signaling

MAPK pathways are cascades of phosphorylation from a MAPK kinase kinase (MAPKKK) to a MAPK and ultimately result in the activation of transcription factors. The five MAPKs that are well described for *S. cerevisiae,* consisting of Kss1p, Hog1p, Slt2p, Fus3p, and Smk1p, are involved in several processes from mating to stress resistance (Fig. 6.1). Proteins homologous to the *S. cerevisiae* MAPK have been identified in *C. albicans* and are designated Cek1p, Hog1p, Mkc1p, Cek2p, and Csk1p, respectively.

The Cek1p MAPK pathway was first studied in 1992 by Whiteway *et al.* and is renowned in *C. albicans* for its role in growth (Csank *et al*., 1998), mating (Chen *et al*., 2002) and cell wall construction (Eisman *et al*., 2006). This pathway is activated by the small GTPase Cst20p subsequently acting upon the MAPKKK Ste11p, followed by Hst7p, Cek1p, and finishing with the activation of the transcription factor Cph1p (Fig. 6.1). Mutants of all the proteins involved in this signaling cascade fail to produce hyphae upon induction on solid media, except in the presence of serum, suggesting that this cascade is essential for filamentation (Csank *et al*., 1998). However, the exact signal inducer for this cascade of phosphorylation remains elusive, but recent data suggest that two plasma membrane proteins, Sho1p and Msb2p, have a role in the pathway's activation (Roman *et al*., submitted paper), as has been described

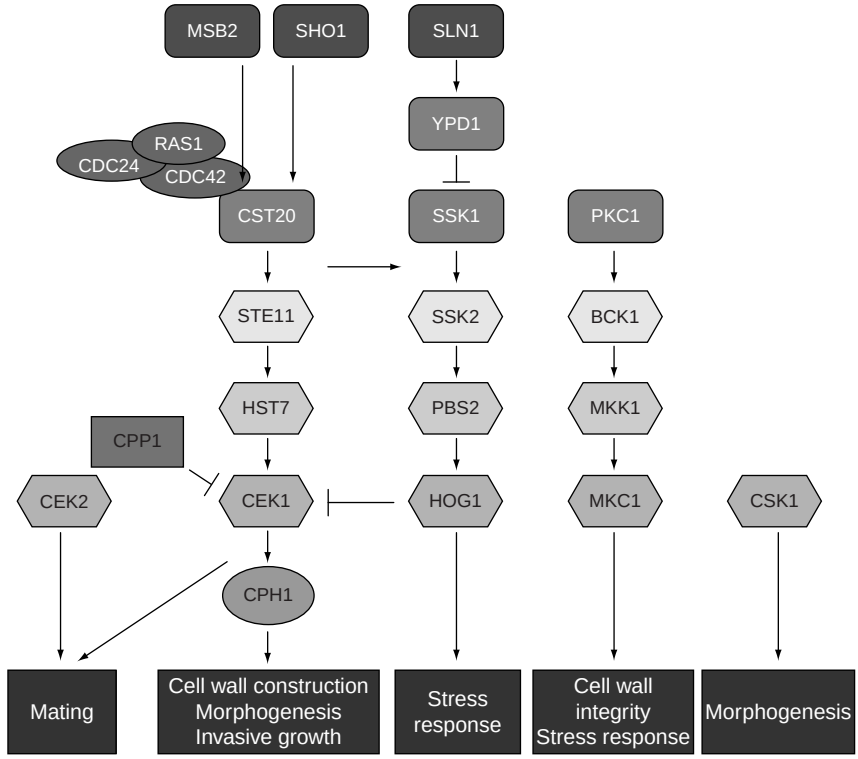

FIGURE 6.1 Mitogen-activated protein kinase signaling in *C. albicans.*

previously for *S. cerevisiae* (Cullen *et al.*, 2004). Indeed, these two proteins are involved in the phosphorylation of Cek1p during resumption of growth in *C. albicans* (Roman *et al.*, submitted paper). Furthermore, inactivation of *SHO1* and/or *MSB2* leads to defects in hyphae formation as shown for other members of this MAPK pathway (Roman *et al.*, submitted paper). Signal transmission between these putative receptors and the MAPK pathway in *S. cerevisiae* is carried out by Cdc42p, which can partially interact with the C-terminus of Msb2p (Cullen *et al.*, 2004) and also with the cytoplasmic protein Ste20p (homologue of Cst20p) (Peter *et al.*, 1996). In *C. albicans*, only the interaction between Cst20p and Cdc42p has been validated (Su *et al.*, 2005). However, Cdc24p (the exchange factor of Cdc42p) and Cdc42p have roles in hyphae formation (Bassilana *et al.*, 2003) where, similar to Ras1p, they were demonstrated to act with MAPK pathway members (Leberer *et al.*, 2001; Ushinsky *et al.*, 2002). Proposed models (Bassilana *et al.*, 2003) suggest that Cdc42p, with the help of Cdc24p, interacts with Ras1p to activate Cst20p, and the remaining members of the MAPK pathway. Finally this pathway is also

controlled by the phosphatase, Cpp1p (Csank *et al*., 1997) and by another MAPK, Hog1p (Eisman *et al*., 2006). These are implicated in the dephosphorylation and hence inactivation of Cek1p. Both *cpp1* and *hog1* single mutants are hyper-filamentous (Alonso-Monge *et al*., 1999), while the same mutant in a *cek1* background shows defects in hyphae formation, just like the *cek1* single mutant. These results demonstrate the repressing function of Hog1p and Cpp1p on Cek1p.

The second major MAPK pathway explored in *C. albicans* is implicated in several stress resistance mechanisms (osmotic, oxidative, temperature, and antifungal drugs) and utilizes the MAPK Hog1p. Similar to the situation found in *S. cerevisiae*, this pathway is activated by the plasma membrane protein Sln1p a sensor histidine kinase forming a two-component system with Ypd1p. It is important to emphasize that the two cytoplasmic proteins, Chk1p (Calera *et al*., 1998) and Nik1p (Nagahashi *et al*., 1998), are also histidine kinases involved in osmosensing, morphogenesis and cell wall biogenesis (Kruppa and Calderone, 2006). Under non-stress conditions Sln1p phosphorylates itself, which leads to the constitutive activation of Sln1p and Ypd1p. The phosphate is then transferred to Ssk1p, which inhibits the activation of Ssk2p and Ssk22p. Under stress inducing conditions, the initial step of phosphorylation in the cascade is inhibited, allowing phosphorylation of Ssk2p and Ssk22p and hence the activation of the MAPK pathway through Pbs2p and Hog1p. Phosphorylation of Hog1p results in its nuclear translocation. Once in the nucleus, activated Hog1p can activate the transcriptional activators Hot1p, Msn2p, Msn4p, and Skn7p, or the repressor Sko1p (Arana *et al*., 2005). Sln1p is dedicated to Hog1p activation, but Sho1p and Msb2p, involved in the Cek1p MAPK pathway, are also responsible in part for Hog1p activation. Indeed in *C. albicans*, *sln1*, or *ssk1* single mutants show growth deficiencies in some stress conditions (Román *et al*., 2005). However unlike the *hog1* or *pbs2* mutants, no dramatic phenotypes have been observed for these mutants on osmotic stress media (Arana *et al*., 2005). Nevertheless, double *ssk1 msb2*, or triple *ssk1 msb2 sho1*, mutants show a strong reduction in growth on media supplemented with 0.5 M NaCl, like the *hog1* mutant, when compared to single *sho1*, *msb2*, or *ssk1* mutants (Cottier, personal communication). Inactivation of both pathways (Sln1p and Sho1p) is necessary to obtain an equivalent phenotype of the *hog1* mutant. In *S. cerevisiae*, cross talk of the Sho1p and Hog1p pathways is mediated by the phosphorylation of Pbs2p by Ste11p (Posas and Saito, 1997). However, in *C. albicans* this interaction does not exist, as Ssk2p is the only activator of Pbs2p (Cheetham *et al*., 2007). These results suggest that the cross talk between the two pathways is up-stream of Pbs2p, because the *pbs2* mutant behaves like a *hog1* mutant, and mutants down-stream of Ssk1p. If not then the double mutant *ssk1 msb2*, should have an identical phenotype to the *ssk1* single mutant. However, it

remains to be seen which member of the Sho1p pathway is involved in this cross talk.

The final and relatively well-described MAPK pathway in *C. albicans* is implicated in cell wall integrity pathway and requires Mkc1p. Protein kinase C (Pkc1p) is responsible for the activation and phosphorylation of the MAPK core composed of Bck1p, Mkk2p, and Mkc1p. The first two kinases are homologues to *S. cerevisiae* proteins, but they have not yet been fully characterized in *C. albicans*. Mkc1p appears to be active in response to a number of stress conditions including osmotic, oxidative, antifungal drug, and temperature stress (Navarro-García *et al*., 2005). All of the latter partially depend on a functional *HOG1* pathway. Sensitivity of the *mkc1* mutant strain to stress could be due to the alteration of the disposition of the surface mannan compared to wild type (Navarro-García *et al*., 1998). Finally, Mkc1p is involved in biofilm formation (Kumamoto, 2005) and virulence (Diez-Orejas *et al*., 1997). Contrary to *S. cerevisiae*, the receptors and transcriptional activator involved in the Mkc1p MAPK pathway are not known.

The exact functions and signaling pathways involving Cek2p and Csk1p are poorly described in *C. albicans.* However, in contrast to *S. cerevisiae,* it appears that they do not interact with Hst7p or Cph1p (Chen and Chen, 2001; Elion *et al*., 1993; Errede *et al*., 1993). In *S. cerevisiae*, the Cek2p homologue is involved in mating (Farley *et al*., 1999) and the Csk1p homologue is implicated in spore formation (Krisak *et al*., 1994). Therefore, even if proteins involved in the different MAPK pathways are conserved between *S. cerevisiae* and *C. albicans*, their function and way of interaction can be highly diverse.

B. Cyclic AMP-dependent PKA pathway

Adenylyl cyclase is a nucleotide exchange factor that generates the secondary messenger, cAMP from ATP. Adenylyl cyclase proteins are wide spread throughout nature and are divided into different subgroups depending on tissue specific distribution and biochemical properties (Willoughby and Cooper, 2007 and references therein). Rocha *et al*. identified an adenylyl cyclase gene in *C. albicans*, which was subsequently named Cyr1p (or Cdc35p) (Rocha *et al*., 2001). Cyr1p lacks the 12 membrane spanning domains normally associated with adenylyl cyclases and is in fact predicted to be a soluble adenylyl cyclase, although the actual localization of Cyr1p is still unknown. The *cyr1/cyr1* deletion strain exhibits slower growth rates than the conventional wild-type strain, SC5314 (Rocha *et al*., 2001), and only exhibits hyphal development in embedded conditions (Cao *et al*., 2006). Measurement of intracellular cAMP levels in the *cyr1/cyr1* mutant indicates that Cyr1p is the only functional adenylyl cyclase in *C. albicans* (Rocha *et al*., 2001).

Serum is a potent inducer of hyphal development in *C. albicans*. Although many different approaches have been used to identify the active component of serum, these attempts have largely been unsuccessful. Feng *et al*. report that the active component has a molecular weight below 1 KDa (Feng *et al*., 1999). However, whether it is a single component or a combination of small molecules/proteins remains to be investigated. In response to serum Ras1p is activated (Fig. 6.2). This activation is proposed to be due to Gpr1p, a G-protein coupled receptor, and Gpa2p, an alpha subunit regulated by Gpr1p (Miwa *et al*., 2004; Sánchez-Martínez and Pérez-Martín, 2002). Signal transduction from Ras1p to Cyr1p

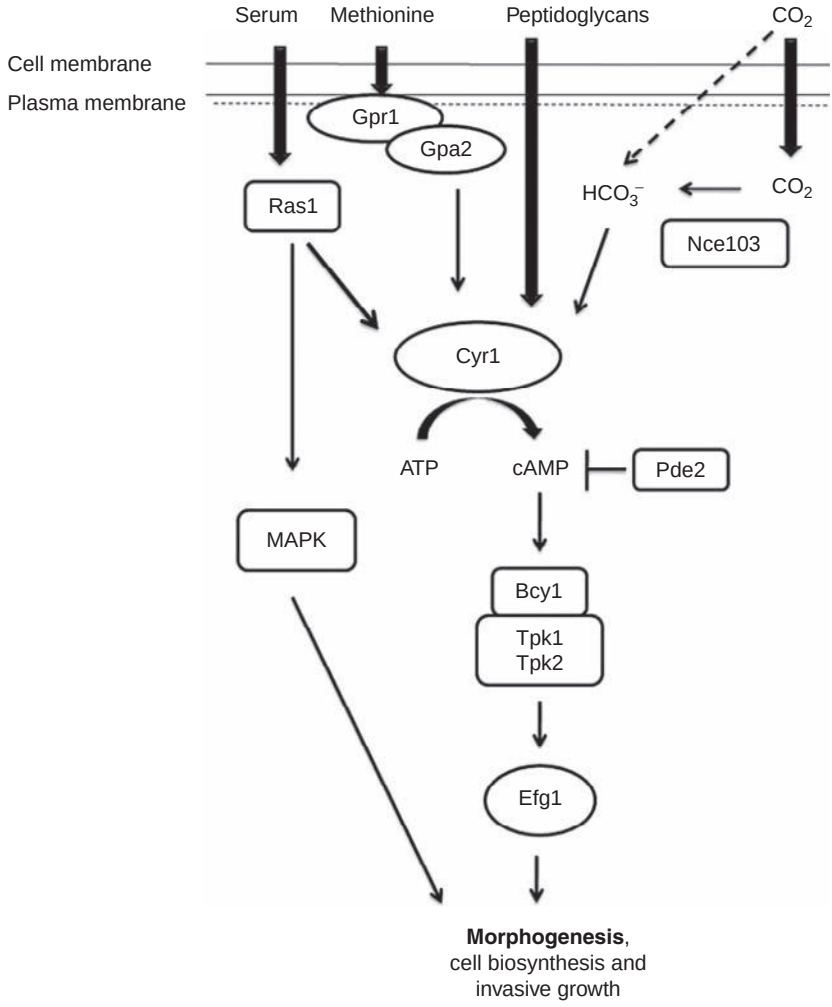

FIGURE 6.2 cAMP-dependent protein kinase A signaling.

requires a protein known as CAP (adenylyl cyclase-associated protein) in many species (Bahn *et al*., 2004; Noegel *et al*., 2004; Shima *et al*., 2000). Bahn and Sundstrom identified a CAP homologue in *C. albicans*, which was subsequently named Srv2p (homologous to *S. cerevisiae* Srv2p) (Bahn and Sundstrom, 2001). Deletion of *SRV2* prevents the yeast to hyphal switch, which has been attributed to prevention of the intracellular increase in cAMP levels normally observed after stimulation of Cyr1p (Bahn and Sundstrom, 2001). Upon activation Ras1p binds to the N-terminal Ras Activating (RA) domain of Cyr1p (amino acid residues 304–394) (Fang and Wang, 2006). Removal of this domain prevents filamentation in serum containing media, but does not interfere with CO_2 induced filamentation (see below), which is regulated via the C-terminal catalytic domain (amino acid residues 1166–1571) (Klengel *et al*., 2005). This association promotes cAMP production. Signaling via cAMP is controlled by phosphodiesterases, which hydrolyse cAMP. *C. albicans* possesses two phosphodiesterases, Pde1p and Pde2p, with Pde2p being the most active. The *pde2/pde2* mutant displays elevated intracellular cAMP levels and is constitutively filamentous (Bahn *et al*., 2003).

The local increase in cAMP levels activates Protein Kinase A (PKA). PKAs are comprised of regulatory domains and catalytic domains. In general, cAMP binds to the regulatory domain of PKA, which results in a conformational change and the release of the catalytic domain. Subsequently, the catalytic domain is free to activate downstream effectors. In *C. albicans* the catalytic domain of PKA is encoded by two genes designated *TPK1* (Bockmühl *et al*., 2001) and *TPK2* (Sonneborn *et al*., 2000), and the regulatory domain by *SRA1* (also known as *BCY1*) (Cassola *et al*., 2004). Tpk1p appears to be essential for filamentation on solid medium, but dispensable for filamentation in liquid media, while Tpk2p is essential only for filamentation in liquid medium (Sonneborn *et al*., 2000). Overexpression of Tpk2p can restore filamentation in a *cek1/cek1* background, but not in an *efg1/efg1* background (Sonneborn *et al*., 2000). Therefore, it is highly likely that Tpk2p functions in the cAMP–Efg1p pathway and not the MAPK signaling cascade.

The cAMP pathway activates the transcription factor Efg1p and it has been suggested that Tpk2p directly phosphorylates Efg1p (Tebarth *et al*., 2003). Detailed and systematic microarray analysis confirms that Efg1p is responsible for regulating a large number of genes that participate in the morphological switch (Lane *et al*., 2001). As expected the *efg1/efg1* strain is nonfilamentous under the majority of conditions, and can only form pseudohyphae in the remaining conditions (Stoldt *et al*., 1997). Considering that overexpression of Efg1p in a *tpk2/tpk2* background can restore filamentation, it is likely that other kinases contribute to the activation of Efg1p, which compensate for the loss of Tpk2p. This compensation has been suggested to arise from increased phosphorylation activity of Tpk1p

(Harcus *et al*., 2004). In addition to functioning as a lone transcription factor, Efg1p works in concert with other transcriptional regulators, namely Flo8p, Czf1p, and Efh1p (Cao *et al*., 2006; Doedt *et al*., 2004; Kumamoto, 2005). Interactions of Efg1p with these regulators are evidenced by yeast two-hybrid assays (Cao *et al*., 2006; Giusani *et al*., 2002). Furthermore, Efg1p also influences the expression of other transcription factors. For example, *TEC1* expression levels are regulated in an Efg1p-dependent manor (Lane *et al*., 2001). The ability of Efg1p to regulate a vast number of genes, in conjunction with its ability to work in concert with other transcription factors and to regulate gene expression in response to a plethora of signals, positions Efg1p toward the center of the *C. albicans* molecular network map.

III. CARBON DIOXIDE SENSING

The CO_2 concentration of blood is 150-fold higher than of air (5.5% compared to 0.033%) and is the most powerful filament inducing signal identified so far. Carbon dioxide was first shown to influence *C. albicans* morphology by Persi *et al*. (1985) and is now termed CO_2 sensing. Since then, CO_2 sensing has been brought to the forefront of *C. albicans* research due to its significance in diagnosis of fungal disease caused by Candida species. Although *C. albicans* is the most common cause of candidiasis, proper diagnosis is required to ensure that the correct treatment is provided. Typically diagnosis of Candida species is achieved through a combination of phenotype and biochemical tests, which can take up to 48 hours to process. However, phenotypically screening Candida isolates in environments elevated in CO_2 (5.5%) significantly reduces diagnostic time ($<$ 24 hours), as *C. albicans* is the only Candida species that forms true hyphae under these conditions (Sheth *et al*., 2005). The molecular network identified in CO_2 sensing involves cAMP signaling (Fig. 6.2) as detailed below.

A. Adenylyl cyclase (Cyr1) senses environmental CO_2

The cAMP signaling cascade has implications for many morphological changes that have been observed for *C. albicans*. Elements of this pathway have been tested for their involvement in CO_2 sensing. Mutants of downstream elements, including Efg1p and Cyr1p remain in the yeast form during exposure to CO_2, confirming the involvement of cAMP signaling (Klengel *et al*., 2005). However, in *ras1/ras1* backgrounds pseudohyphae are observed, stimulating invasion into the agar during exposure to CO_2, an effect not observed for *cyr1* or *efg1* mutants (Klengel *et al*., 2005). In addition, recombinant Cyr1p is directly activated by bicarbonate ions

(Klengel *et al.*, 2005), suggesting that Cyr1p is the major sensor of CO_2. Mammalian soluble adenylyl cyclases along with other fungal and bacterial soluble adenylyl cyclases are also responsive to bicarbonate concentrations (Chen *et al.*, 2000b; Gewiss Mogensen *et al.*, 2006; Klengel *et al.*, 2005; Masuda and Ono, 2005; Rojas *et al.*, 1992; Wuttke *et al.*, 2001), suggesting that this activation is evolutionary conserved.

B. CO_2 sensing: A role for carbonic anhydrase

Deletion of carbonic anhydrase (CA) has been shown to result in high CO_2 requiring phenotypes for both bacterial and fungal species (Aguilera *et al.*, 2005; Bahn *et al.*, 2005; Götz *et al.*, 1999; Merlin *et al.*, 2003). *NCE103* encodes a β-carbonic anhydrase. Mutations within the *NCE103* open reading frame prevent growth of *C. albicans* in aerobic conditions, which can be complemented by supplementation of 5% CO_2 into the atmosphere (Klengel *et al.*, 2005). Growth under aerobic conditions can also be restored through complementation by carbonic anhydrases from other species (Clark *et al.*, 2004), suggesting that the phenotype results from loss of CA activity rather than as a result of stress inducing conditions. It is hypothesized that under elevated CO_2 conditions environmental CO_2 diffuses through the fungal cell wall and cell membrane where it is spontaneously converted into its impermeable form, bicarbonate. The local increase in intracellular bicarbonate concentrations is thought to be sufficient to integrate into intermediate metabolism to permit growth of the fungus. Support for this comes from *S. cerevisiae* and *Cryptococcus neoformans* where growth of carbonic anhydrase mutants in air can be restored through the supplementation of metabolic intermediates (Aguilera *et al.*, 2005; Bahn *et al.*, 2005). However, Nce103p is not essential for filamentation under elevated CO_2 conditions. Increased incubation of *nce103* mutants in 5% CO_2 (48 hours) induces filamentation (Hall and Eaton unpublished observation). Therefore, in the case for *nce103* mutants, CO_2 signaling is hierarchal. Initially the CO_2 is converted into bicarbonate, which then feeds into metabolism. Once the metabolic demand for bicarbonate has been fulfilled, the increase in bicarbonate then activates Cyr1p, to increase intracellular cAMP concentrations and active downstream components of the PKA pathway.

IV. QUORUM SENSING AND ITS EFFECTS ON *C. albicans* MORPHOLOGY

Quorum sensing (QS) is described as the regulation of genes in a density dependent manor and is established as contributing to pathogenicity of certain bacterial species through the regulation of essential virulence factors (Smith and Iglewski, 2003; Williams *et al.*, 2000; Winzer, 2001).

However, establishment of QS in eukaryotic organisms is still in its infancy. *C. albicans* was the first eukaryotic organism identified with quorum sensing properties. Hornby *et al.* confirmed that inhibition of filamentation observed when *C. albicans* was inoculated into sterilized spent media (supernatants from stationary phase cultures) and at high cell densities, was due to the presence of the sesquiterpene, farnesol (Hornby *et al*., 2001). Supplementation of mixed or purified farnesol isomers indicated that the QS properties were specifically attributed to the *E*, *E* isoform (Hornby *et al*., 2001). However, until recently the molecular networks through which farnesol exerts its effects have remained elusive. The following sections provide an overview of recent developments that lay down the foundations to the establishment of molecular networks that respond to quorum sensing.

A. Farnesol exerts its effects through cAMP signaling cascades

The inhibition of filamentation by farnesol has mainly been investigated under conditions where induction of filamentation was mediated via the Ras1–cAMP–PKA signaling pathway (described earlier in this chapter). Therefore, to identify key regulatory networks affected by farnesol, investigation into cAMP signaling was a logical step. Indeed, supplementation of exogenous cAMP into media containing farnesol completely restored filamentation (Davis-Hanna *et al*., 2008 and our own observation), suggesting that inhibition of cAMP signaling was one of the major targets of farnesol. Furthermore, transcriptional analysis of cAMP target genes including *HSP12* and *CTA1* confirmed that in the presence of farnesol, in cAMP inducing conditions, both genes were de-repressed (Davis-Hanna *et al*., 2008). In addition, the 12 carbon alcohol dodecanol, a common substitute for the QS molecule 3-oxy-C12-homoserine lactone (3OC12HSL) secreted by *P. aeruginosa*, mediated similar effects on the cAMP signaling cascade as farnesol (Davis-Hanna *et al*., 2008). Although Efg1p targets are de-repressed in the presence of farnesol this is not a result of increased Efg1p levels, as transcript analysis confirmed that *EFG1* mRNA levels were unaffected by farnesol (Kebaara *et al*., 2008).

As exogenous cAMP is sufficient to restore filamentation in the presence of farnesol and dodecanol, these QS molecules must mediate their effects through upstream components of the pathway including either Ras1p or Cyr1p. As Ras1p is associated with the membrane and farnesol has been shown to localize to membranes (Shchepin *et al*., 2005), interactions between Ras1p and farnesol seem likely (as suggested in Fig. 6.3). However, farnesol and dodecanol were able to inhibit filamentation of strains containing dominate active Ras1p ($Ras1^{G13V}$) (Davis-Hanna *et al*., 2008), suggesting that constitutive expression of Ras1p is not sufficient to by-pass the effects of these QS molecules. However, from experiments

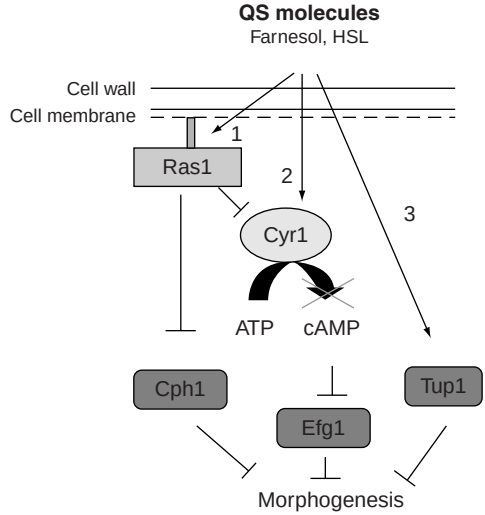

FIGURE 6.3 Hypothetical networks mediating quorum sensing in *C. albicans*. 1, the lipid soluble QS molecules interact with Ras1p, anchoring it to the membrane (shown by the orange bar). This association prevents Ras1p from binding to the RA domain of Cyr1p and hence inhibits cAMP signaling and potentially the MAPK cascade. 2, The QS molecules directly interact with Cyr1p and inhibit the generation of cAMP. Therefore, the expression of hyphal specific genes is inhibited. 3, The QS molecules, through an as yet unidentified pathway, activate the transcriptional repressor Tup1p, which subsequently inhibits the expression of hyphal specific genes.

performed so far it is not possible to deduce whether farnesol and dodecanol exert their effects through preventing Ras1p interactions with Cyr1p. Support for this comes from a recent publication where it is proposed that Ras1p and Cyr1p form a more complex molecular sensor, which only functions fully when both are present (Xu *et al.*, 2008). As discussed earlier, CO_2 sensing stimulates the cAMP–PKA pathway, but does so without stimulating Ras1p. Therefore, if QS molecules could inhibit filamentation in the presence of CO_2 then this would suggest that the target is not Ras1p. However, the effects of QS molecules on CO_2 sensing have not been reported so far.

Cyr1p has been identified as an environmental sensor, which is directly able to sense and respond to changes in CO_2 concentrations (Klengel *et al.*, 2005). Therefore, it may be plausible that Cyr1p is the direct target of QS molecules like farnesol and dodecanol. In fact, Cyr1p has recently been shown to bind peptidoglycan-derived molecules from bacterial species that are located within serum of the mammalian host (Xu *et al.*, 2008). In comparison to bicarbonate, which is believed to bind directly to the catalytic domain of Cyr1p, the peptidoglycans bind to the

leucine rich repeat (LRR) domain located within the center of the protein (Xu *et al*., 2008). Once bound, the peptidoglycan derivatives stimulate cAMP production, resulting in filamentation. Therefore, Cyr1p is a multi-domain enzyme, and these multiple domains are essential to the function of Cyr1p as the central molecular sensor. Removal of the LRR domains from Cyr1p prevents filamentation of *C. albicans* in hyphal inducing conditions, but restores wild-type growth rates when transformed into the *cyr1* null (Xu *et al*., 2008). Therefore, it is plausible that other QS molecules like farnesol and dodecanol, which inhibit filamentation could mediated their effects through inhibition of the LRR domain function, resulting in reduced filamentation, but exerting no effects on growth rates.

B. Tup1p is involved in quorum sensing in *C. albicans*

Tup1p along with the transcription factors Nrg1p and Rfg1p negatively regulates genes that are essential for filamentation. Consequently strains carrying null mutations in these genes produce hyphae in the absence of inducing stimuli. Recent data suggest that farnesol can still inhibit filamentation in the *rfg1/rfg1* strain, but not in the *tup1/tup1* and *nrg1/nrg1* strains, suggesting that these two transcription factors are important for QS (Kebaara *et al*., 2008). Transcriptional studies identify enhanced mRNA levels for *TUP1* and the of Tup1p regulated genes (Cho *et al*., 2007), but recent data suggest that farnesol does not impact on *NRG1* mRNA levels (Kebaara *et al*., 2008). Therefore, Tup1p plays a key role in the regulation of hyphal specific genes in response to QS molecules, which may involve Nrg1p. Upstream effects of Tup1p remain unknown. Considering the important role(s) Tup1p has in regulating *C. albicans* filamentation, identifying the molecular network behind Tup1p regulation will be an important avenue for future research.

C. Transcriptional analysis of quorum sensing in *C. albicans*

With the view to further characterize the effects of QS molecules on *C. albicans*, and to identify how QS molecules mediate their effect, multiple transcriptional analysis approaches have been taken (Cao *et al*., 2005; Cho *et al*., 2007; SATO *et al*., 2004). However, so far these extensive projects have produced a large set of conflicting data. For example, in one study only genes specifically regulated via the MAPK pathway were differentially regulated in response to farnesol, suggesting that QS in *C. albicans* is mediated predominantly via the MAPK signaling pathway (Sato *et al*., 2004). However, well-known cAMP regulated genes have been identified in other global transcriptional projects, and by other alternative experimental approaches, as being influenced by the presence of farnesol

(Cao *et al*., 2005) (Davis-Hanna *et al*., 2008). Therefore, the transcriptional data currently do not provide sufficient information to confirm which molecular networks QS molecules specifically affect. The differences observed are most likely due to the number of different experimental parameters identified in each study, suggesting that the mechanism in which farnesol exerts its effects may be multifactorial.

V. pH Regulation of Cell Morphology

Among the most extensively studied environmental factor in fungal and yeast molecular biology is pH. However, the effects of pH are now being explored on a broader scale including the nematode model system, *Caenorhabditis elegans* (Hall *et al*., 2008). In 2002 a comprehensive review covering over a decade of research focusing on the development of the Rim101 (Candida and Saccharomyces species), or PacC (Aspergillus species) signaling cascade was published (Penalva and Arst, 2002). Therefore, this chapter will not reiterate the full details of the signaling cascades, but will provide a brief summary of the Rim101 cascade with specific focus to recent developments.

The zinc finger transcription factor, Rim101p undergoes proteolytic activation in alkaline conditions (Porta *et al*., 2001). This processing removes approximately 100 amino acid residues from the C-terminus of the transcription factor and is regulated by a cascade of proteins (reviewed in Penalva and Arst, 2002). Initially the environmental pH is sensed by the transmembrane proteins, Rim9p, Rim21p, and Dfg16p (Barwell *et al*., 2005; Penalva and Arst, 2002). The arrestin like protein Rim8p, is modified in alkaline conditions through phosphorylation and ubiqutination, which is essential for signal transduction. Although the exact roles of Rim8p in Rim101 signal transduction are unknown, functions in endocytosis have been proposed (Peñalva *et al*., 2008). Rim20p binds to consensus sequences located either side of the processing site and recruits Rim13p (the processing protease (Li *et al*., 2004)) to the site (Xu and Mitchell, 2001). In addition to the Rim proteins, members of endosomal sorting complexes required for transport (ESCRT) also play a role in environmental pH sensing. Rim20p and Rim13p homologues have been identified as binding vascular sorting proteins (VPS) Vps32p and Vps4p in the multivesicular body (MVB) (Boysen and Mitchell, 2006; Cornet *et al*., 2005; and reviewed in Penalva and Arst, 2002). This interaction was initially thought to act as a molecular scaffold, forming a platform for the processing of Rim101p. However, deletion of *VPS32* results in alkaline sensitive phenotypes that are more serverer than mutations in *RIM101*, suggesting that members of the MVP may play a more significant role in pH regulation than first anticipated (Cornet *et al*., 2005).

Once processed, Rim101p binds to Rim101p consensus sequences within the promoter region of alkaline induced genes, and acts as a transcriptional activator to induce expression. The promoter region of Rim101p contains multiple Rim101p consensus sequences and is itself alkaline induced, leading to amplification of the signal (Ramon *et al*., 1999). Although the mechanism in which Rim101p acts as an inducer of alkaline expressed genes is well documented, the mechanism in which Rim101p acts as a transcriptional repressor is not well known. In *Aspergillus nidulans* the Rim101p orthologue PacCp is thought to compete with the relative transcriptional activator for overlapping consensus sites (Espeso and Arst, 2000). As PacCp levels are elevated in alkaline conditions (Tilburn *et al*., 1995), it out-competes the activator resulting in repression of the target gene. This form of regulation has not so far been described for *C. albicans*. However, Baek *et al*. have recently demonstrated that *C. albicans* Rim101p binds to the promoter region of the alkaline repressed gene *PHR2* (Baek *et al*., 2006). Moreover, Rim101p was shown to bind promoter sequences in the absence of processing, although binding is enhanced in alkaline conditions (Baek *et al*., 2006). Therefore, Baek *et al.* conclude that Rim101p activity may be regulated by an as yet unidentified pathway, in addition to the well-characterized Rim101 network (Baek *et al*., 2006).

VI. OTHER PATHWAYS AFFECTING MORPHOLOGY

During yeast growth, genes that function in filamentation are suppressed. Three transcriptional repressors (Tup1p, Nrg1p, and Rfg1p) have been identified in *C. albicans* that specifically repress genes that are essential for filamentation. Inactivation of these repressors results in constitutively filamentous phenotypes (Braun and Johnson, 1997; Khalaf and Zitomer, 2001; Murad *et al*., 2001). Deleting *EFG1* in parallel to *TUP1* can relieve filamentation in the *tup1/tup1* strain. Microarray data using *tup1/tup1*, *nrg1/nrg1*, and *rfg1/rfg1* demonstrate that these genes are responsible for the repression of a vast amount of genes that function in the yeast to hyphal switch, but also in virulence (Kadosh and Johnson, 2005). Comparisons of the different data sets confirm that many genes that are regulated by Nrg1p and Rfg1p, are also dependent on Tup1p (Kadosh and Johnson, 2005). However, Tup1p also appears to regulate a number of genes independently of Nrg1p and Rfg1p (Kadosh and Johnson, 2005). Considering the importance Tup1p, Nrg1p, and Rfg1p have in regulating the yeast to hyphal switch, virulence-associated genes and their involvement in *C. albicans* quorum sensing, surprisingly little is known about the upstream signalling networks that these repressors function in.

VII. CONCLUSIONS

C. albicans responds to a vast number of environmental cues, which in turn regulate a large number of genes that function in morphological switching, stress resistance, virulence and cell growth and differentiation, through a variety of molecular signaling networks. Extensive research has identified a number of kinases, phosphatases and other signaling molecules, which form parts of these cascades. However, initially research was focused on identifying effectors of single cues, while in reality *C. albicans* will not just be responding to one cue, but is more likely to be responding to a variety of combinations at any given time, dependent on its immediate environment. As a result a large amount of cross talk between the different signaling networks would be expected, and indeed is beginning to immerge from more recent data. With the immergence of interplay between different networks it is intriguing to hypothesize which gene(s) will lay at the heart of such a map. Reviewing the data already available suggests that two genes, the soluble adenylyl cyclase Cyr1p and the transcription factor Efg1p are key components in the network implicated in morphology.

There are several experimental lines of evidence that suggest that Cyr1p could be one center of signaling networks. For one, the *cyr1/cyr1* is locked in the yeast form under all environmental conditions tested, except under embedded conditions. Therefore, even when there is no direct implication of cAMP in the proposed signaling cascade cAMP is actually required (Rocha *et al*., 2001). For example, stimulation of the MAPK signaling pathway in a *cyr1/cyr1* background does not result in filamentation, even if components of the MAPK pathway are overexpressed (Rocha *et al*., 2001). Secondly, Cyr1p is a multi-domain protein. So far, the N-terminal domain has been shown to contain a Ras-activating domain that is essential for the response to serum and the C-terminal catalytic domain is sufficient to mediate CO_2 signaling, although the exact domain boundaries and responsible elements are still to be determined. Moreover, recent data suggest that the leucine rich regions are responsible for the response to bacterial peptidoglycans (Xu *et al*., 2008). With Cyr1p spanning over 1,600 amino acids, with a further two predicted domains of unknown function (G-protein binding domain and the Pp2C domain), it is highly possible that other domains of Cyr1p will be associated with other responses. For example, quorum-sensing molecules are known to affect cAMP signaling (Davis-Hanna *et al*., 2008). Although the exact mechanism by which cAMP signaling is affected remains to be seen, this effect is proposed to occur from Cyr1p and up. Therefore, it is plausible that these QS molecules directly or indirectly, through an as yet unidentified protein, interact with Cyr1p. In favor of this, QS in bacterial species is mediated through cAMP receptor proteins (Liang *et al*., 2007a,b).

Evidence that Efg1p should be in the center of all regulatory networks extends from the fact that Efg1p regulates a plethora of genes either directly or in concert with other transcription factors. Therefore, it appears as if Efg1p acts as funnel to channel all signaling inputs from the various signaling cascades and convert the signal into transcriptional outputs.

In summary, continued research on signaling cascades in *C. albicans* will lead to the production of a complicated interrelated signaling network, which is required to further increase our understanding of this remarkable opportunistic pathogen.

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CHAPTER 7

Temperature Sensors of Eubacteria

Wolfgang Schumann

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Abstract In their natural habitats, bacteria are frequently exposed to sudden changes in temperature. It has been shown that bacteria use different strategies to cope with temperature changes. These strategies are genetically determined and start with registration of the temperature followed by the induction of a subset of genes allowing them to adapt to the stressful situation. Four different mechanisms have evolved termed the high and the low temperature response and the heat and the cold shock response. These temperature changes are registered by three different thermosensors: DNA, RNA and protein.

I. INTRODUCTION

Bacteria have successfully colonized every niche on our planet which provides nutrients. In these vast varieties of environments, bacteria are exposed to a multitude of fluctuating stress factors, including sudden changes in temperature (heat and cold), changes in their external pH (acid and alkaline shock), changes in osmolarity (hypo- and hyperosmotic shock), oxidative stress by reactive oxygen species, changes in the concentration of nutrients and toxins and starvation to mention the most prominent ones (see the book edited by Storz and Hengge-Aronis (2000) for a recent compilation). To cope with stress factors, bacteria can try to move by, for example, their molecular motor, the flagellum (Mitchell and Kogure, 2006) or to adapt to changes in their vicinity. Bacteria use different adaptation strategies to varying environmental conditions allowing them to thrive in a wide range of niches. Stress factors typically induce a stress response resulting in a characteristic change in the pattern of gene expression. This stress response helps the bacterial cells to protect vital processes, to restore cellular homeostasis and to increase the cellular resistance against subsequent stress challenges. Different stress factors induce different but often overlapping stress responses. Research carried out over the last 25 years revealed that bacteria code for genetic programs allowing them to deal with stressful conditions. These programs consist roughly of three different steps: (1) the stress factor is registered either directly or indirectly by a sensor (often through a damaged molecule), (2) the sensor leads to the induction of a subset of genes (called stress genes) involved in the adaptation to the new situation, and (3) in many cases, expression of the stress genes is turned off after adaptation through feedback inhibition defining the classical stress response. But some

genes stay turned on as long as the bacterial cells are exposed to the new situation. This is, for example, used by pathogenic bacteria to identify their presence in their mammalian host. Most research has been carried out to elucidate the mechanisms underlying adaptation to a sudden increase and decrease in temperature. Here, four different mechanisms can be distinguished, the high temperature response (HTR) and the low temperature response (LTR) and the heat shock response (HSR) and the cold shock response (CSR). How bacterial cells sense these temperature changes will be the content of this review article.

II. THERMOSENSORS

Three different thermosensors have been described so far: (1) DNA as part of the nucleoid termed nucleoid modulator, (2) RNA, and (3) proteins.

A. Nucleoid modulators

The nucleoid-associated proteins, also called architectural proteins and formerly designated as histone-like proteins, constitute a superfamily of proteins that exert genome structuring functions in bacteria. Binding of these proteins to DNA does not only influence its conformation, but also DNA replication, recombination, and transcription (Dame, 2005; Dorman, 2004). Characteristics of this class of proteins are the low molecular mass of their monomeric subunits (below 20 kDa) and their ability to dimerize or even to oligomerize. By their association with DNA, they are able to constrain DNA supercoils *in vitro*, thus maintaining the appropriate level of chromosome supercoiling to specific environmental conditions (Thanbichler *et al.*, 2005; Travers and Muskhelishvili, 2005). Well known nucleoid-associated proteins from *Enterobacteriaceae* are H-NS (heat-stable nucleoid-structuring), HU (heat unstable), IHF (integration host factor), and FIS (factor of inversion stimulation) (Luijsterburg *et al.*, 2006).

Best characterized in different enteric bacteria such as *Escherichia coli* or *Salmonella enterica* serovar Typhi, the H-NS protein serves as the paradigm of a globular modulator exerting its effects, mostly negative, in response to different environmental signals, such as osmolarity or temperature (for a recent review see Dorman, 2004). H-NS is a DNA-binding protein of about 15 kDa molecular mass (Atlung and Ingmer, 1997; Bertin *et al.*, 1999). It is a major component of the bacterial nucleoid and is present in concentrations of up to 20,000 monomers per cell (Williams and Rimsky, 1997). DNA binding by H-NS appears to be largely independent of the nucleotide sequence at the binding site. DNA structure, particularly curvature, plays an important role in determining the affinity of H-NS for a particular site (Bracco *et al.*, 1989; Owen-Hughes *et al.*, 1992). H-NS can condense

DNA (Dame *et al.*, 2000) and constrain supercoils in DNA *in vitro* (Tupper *et al.*, 1994) and *in vivo* (Higgins *et al.*, 1988). It affects transcription of many genes, usually negatively (Hommais *et al.*, 2001). The ability of this protein to oligomerize is central to its function. While the oligomerization domain is located in the N-terminal part, the DNA binding domain is present in the C-terminal part, and both domains are connected by a flexible linker (Dorman *et al.*, 1999; Smyth *et al.*, 2000; Ueguchi *et al.* 1996).

When interacting with DNA, H-NS binds preferentially to curved DNA as mentioned above (Owen-Hughes *et al.*, 1992; Yamada *et al.*, 1990). To repress transcription, H-NS binds in many instances to two distal curved DNA sequences which flank a promoter region. Binding to DNA is followed by oligomerization and often interaction between the two oligomers thus looping out the DNA located between the two binding sites. H-NS is able not only to generate homo-oligomers but to interact with other proteins. Examples are its paralogue StpA (Williams *et al.*, 1996) and members of a family of small proteins, the Hha/YmoA family (Nieto *et al.*, 2000, 2002). Both Hha and YmoA were initially described in *E. coli* and *Yersinia enterocolitica* as thermomodulators of the expression of virulence genes (Cornelis *et al.*, 1991; Mourino *et al.*, 1996; Nieto *et al.*, 1991). The YmoA protein is a temperature-dependent modulator of the expression of different virulence factors in *Y. enterocolitica* (Cornelis *et al.*, 1991). Other members of the Hha/YmoA family of proteins include RmoA and YdgT. The RmoA protein is encoded by plasmid R100 and may participate in the modulation of plasmid transfer in response to some environmental factors, such as osmolarity (Nieto *et al.*, 1998). YdgT, a hypothetical 8.4-kDa protein has been identified as part of the *E. coli* K-12 genome.

H-NS and related proteins can engage in both homologous and heterologous protein–protein interactions. The genes that encode H-NS-like proteins can be carried on mobile genetic elements allowing their distribution by horizontal and lateral gene transfer. How does supercoiling affect promoter activity and what is the temperature sensor in this process? The ability of an effector protein, such as H-NS and YmoA, to dock on the nucleic acid target is influenced by a temperature-mediated change in the topology of that particular stretch of DNA. Alternatively, H-NS may itself respond to temperature and release bound DNA, which can then be altered with respect to supercoiling.

B. RNA

It is becoming interestingly clear that certain mRNA molecules are not simply a substrate for ribosomes but contain control elements that modulate their own expression in a condition-dependent fashion. Structural changes in such sensory RNAs are induced by specific environmental changes. Two principally different classes can be distinguished: *cis*-acting

RNA elements that bear their regulatory potential embedded within the mRNA sequence and *trans*-acting small, noncoding RNAs that function by base-pairing with complementary mRNA sequences encoded elsewhere in the genome; *cis*-acting RNAs change their conformations in response to physical or chemical signals.

Two different classes of *cis*-acting elements have been described located almost exclusively in the 5′-untranslated region (UTR): riboswitches and RNA thermometers. Riboswitches monitor the metabolic state of a cell by binding to metabolites with high specificity and affinity. Extended secondary structures serve as binding sites for metabolites and the mRNAs code for the biosynthesis, uptake or degradation of small metabolites and provide feedback control to these pathways. In almost all cases, binding of the metabolite causes transcription termination, rarely it prevents translation initiation when the Shine-Dalgarno sequence is part of the secondary structure or even activates a ribozyme (Mandal and Breaker, 2004; Nudler and Mironov, 2004). In a few cases, binding of the metabolite causes transcription attenuation. In contrast, RNA thermometers respond to the absolute temperature as a physical parameter.

RNA thermometers have evolved to sense and transduce ambient temperature signals to the translation machinery. They operate posttranscriptionally by the formation of secondary structures that mask the ribosomal binding site at low temperature; elevated temperatures disrupt base pairing and thereby facilitate ribosome binding and translation initiation. This simple regulatory principle can be realized by quite different RNA structures, but only a few distinct types of RNA thermometers have been discovered so far. RNA thermometers register even subtle changes in temperature and adjust gene expression accordingly. RNA thermometers control various cellular processes such as the HSR (Morita *et al,*. 1999b; Nocker *et al*., 2001a), the CSR (Yamanaka and Inouye, 1999), λ phage development (Altuvia *et al*., 1989), and expression of virulence genes (Johansson and Cossart, 2003). In summary, RNA proved to be a simple, yet diverse, flexible, and versatile molecule that offers sufficient chemical complexity to have broad functional capacity.

C. Proteins

Theoretically, both transcriptional and translational repressors and activators could act as thermosensors. In the case of repressors, these proteins will bind to their operators (DNA) or recognition sites (RNA) reducing the transcription or translation rate. At high temperatures, the repressor proteins dissociate from their binding sites allowing a high level transcription or translation rate. In the case of activators, these are also unable to bind to their target sites at high temperature, but become active at low temperature. So far, only one temperature-sensitive transcriptional

activator has described the NIFA protein (see below). That the binding is really temperature-dependent can be shown by several experimental approaches. Transcription can be analyzed *in vitro* with purified components, and addition of the repressor protein should prevent transcription at low, but not at the high temperature. Furthermore, in gel mobility shift assays, the repressor protein should be able to shift a DNA fragment containing its binding site to a lower mobility at the low, but not at the high temperature. In addition, the secondary structure of such a thermosensor protein should change depending on the temperature as analyzed by CD spectroscopy. The first thermosensor repressor to be described was the TlpA protein encoded by a virulence plasmid present in some pathogenic *Salmonella* spp. strains (Hurme *et al.*, 1997).

III. RESPONSES TO SUDDEN CHANGES IN THE GROWTH TEMPERATURE

Temperature is one of the crucial environmental parameters that restrict growth and other biological activities in any organism. Temperature sets physiological limits for a wide variety of biological activities and, therefore, the ability to sense and respond to changes in temperature is of vital importance for many different kinds of organisms. The temperature range in which growth of organisms has been described reaches from −12 °C to approximately 112 °C. Within this temperature range, the velocity of chemical reactions changes dramatically, and different bacterial species have adapted to a small temperature range. This resulted into a general classification of microorganisms into psychrophiles, mesophiles, thermophiles, and hyperthermophiles. Numerous processes are temperature-regulated in bacteria such as the expression of heat and cold shock genes and of virulence genes of pathogenic microorganisms (Narberhaus *et al.*, 2006). Temperature changes represent signals sensed and processed by the biochemical machinery of the bacterial cell to allow optimal adaptation and survival of the microorganisms when the environment changes (Hurme and Rhen, 1998).

Why bacteria need thermosensors? There are four major reasons: (1) sudden increases in temperature can cause denaturation of their proteins, and denatured proteins are prone to form aggregates, and large aggregates will kill the cell. Therefore, cells have to prevent the formation of aggregates to survive. The amount of denatured proteins formed is solely dependent on the temperature increment and is not dependent on the absolute temperature. The larger the increment, the higher the amount of denatured proteins. In the experimental setup, a temperature increase of 12 °C is normally used, where it is independent whether cells are first grown at 20 °C and then shifted to 32 °C, or whether they are first adapted at 30 °C and then shifted to 42 °C; (2) pathogenic bacteria make use of

thermosensors to detect the inside world of their mammalian host to induce their virulence genes the products of which are involved in pathogenicity. Since the temperature is always 37 °C (or slightly higher when the host is experiencing fever), this class of thermosensors detect the absolute temperature rather than temperature increments; (3) a sudden decrease in temperature reduces the fluidity of the cytoplasmic membrane and causes formation of secondary structures mainly in mRNAs. Here, genes have to be expressed or proteins activated to cope with this stressful situation; and (4) bacteria are also able to sense low temperature to induce, for example, conjugation or become motile by the synthesis of flagella.

How do thermosensors function which detect temperature increases and decreases and the absolute temperature? Four different responses to sudden changes in temperature have evolved: The HTR and the HSR deals with high temperature and the LTR and CSR with low temperature. The HTR and LTR are responses to the absolute temperature. They are constitutive which means that the expression of the genes is continued at a high level as long as the cells are exposed to the high or low temperature. I propose to call these genes high and low temperature genes coding for high and low temperature proteins. On the contrary, the HSR is the answer to temperature increments independent of the absolute temperature. It is transient leading to a shut-off by feedback inhibition when the denatured proteins have been removed from the cell. Accordingly, the CSR is induced by sudden temperature decreases and is also turned off by feedback inhibition when cells have adapted to the stressful situation. In all cases, sensors register an increased or decreased temperature leading to the induction of the appropriate subset of genes. The essential characteristics of the four responses are summarized in Table 7.1 and the thermosensors described here as summarized in Table 7.2.

TABLE 7.1 The four temperature responses

Response	Characteristics
HTR	Constitutive expression of high temperature genes at high temperatures and strongly reduced expression at low temperatures
HSR	Transient expression of heat shock genes after a sudden temperature upshift
LTR	Constitutive expression of low temperature genes at low temperatures and strongly reduced expression at high temperatures
CSR	Transient expression of cold shock genes after a sudden temperature downshift

HTR, high temperature response; HSR, heat shock response; LTR, low temperature response; CSR, cold shock response.

TABLE 7.2 Thermosensors

Temperature response	Thermosensor	Examples
HTR	Nucleoid modulator	*virF* promoter of *S. flexneri*
		promoter of the *E. coli* hemolysin gene
		synthesis of pili (fimbriae)
		virulence genes encoded by *Yersina* plasmids
	RNA	*rpoH* transcript of *E. coli*
		ROSE element
		FourU element
		lcrF transcript of *Y. pestis*
		prfA transcript of *L. monocytogenes*
		hspA of *S. vulcanus*
		hspA transcript of *S. albus*
		group II intron of *A. vinelandii*
	Proteins	TlpA repressor protein of S. enterica
		RheA protein of *S. albus*
		ClpXP and Lon proteins of *Y. pestis*
		translational repressor of *T. elongatus*
		sensor kinases
		replication initiation protein
HSR	Molecular chaperons	σ^{32}—DnaK system of *E. coli*
		HrcA-GroEL system of *B. subtilis*
		HspR-DnaK system of *S. coelicolor*
	Proteases	DegS of *E. coli*
LTR	*cis*-acting RNA	lysis-lysogeny decision of phage λ
	Trans-acting RNA	DsrA RNA
	Protein	VirA sensor kinase of *A. tumefaciens*
		NIFA activator of *K. pneumoniae*
		Reponse regulator DegU of *L. monocytogenes*
		Conjugation
CSR	RNA	*cspA* transcript of *E. coli*
		pap transcript of *E. coli*
	Protein	DesK sensor kinase of *B. subtilis*

HTR, high temperature response; HSR, heat shock response; LTR, low temperature response; CSR, cold shock response.

A. The high temperature response

High temperature genes are usually expressed around 37 °C, the mammalian host temperature, but are turned off at 30 °C or below (Harel and Martin, 1999; Konkel and Tilly, 2000). Five different types of HTR thermosensors have been described: (1) bent DNA, (2) supercoiling of DNA, (3) chemical modification of DNA, (4) DNA binding transcription factors, and (5) secondary structures of mRNA molecules (Drlica and Perl-Rosenthal, 1999; Finlay and Falkow, 1997; Heithoff *et al.*, 1999; Hurme and Rhen, 1998).

The important example of high temperature sensing is that of pathogenic bacteria. Here, temperature controls the transcription of genes encoding virulence factors, and this thermoregulatory response has been demonstrated in many bacterial genera including *E. coli* (Jordi *et al.*, 1992; Roosendaal *et al.*, 1986), *Shigella* (Maurelli *et al.*, 1984), *Bordetella* (Coote, 1991), *Yersinia* (Cornelis *et al.*, 1989), and *Listeria* (Leimeister-Wächter *et al.*, 1992). Bacteria sense temperature and other environmental signals to determine whether they are inside or outside of a mammalian host. Low temperature may thus serve as an environmental signal to the bacterial cell that it is outside of a host. The shift to high temperature that bacteria encounter at host entry is believed to be one of the central clues triggering virulence factor expression (Konkel and Tilly, 2000; Maurelli, 1989; Skorupski and Taylor, 1997; Tobe *et al.*, 1991). Thermosensing by pathogenic bacteria registers the absolute temperature, is constitutive and results in the induction of the high temperature genes as long as the bacterial cells are exposed to that temperature. Besides temperature, expression of many virulence factors is regulated by osmolarity, pH, and oxygen availability (Falconi *et al.*, 1998; Gardel and Mekalanos, 1994; Mekalanos, 1992).

B. The heat shock response

The HSR is the stress condition to which most attention has been devoted over the last 30 years. It is a universal, adaptive, and homeostatic cellular response against stress-induced conformational damage of proteins caused by a sudden increase in temperature or by other stresses causing denaturation of proteins such as ethanol. It is induced when cells growing at a low temperature are exposed to a high temperature independent of the absolute temperature. The HSR results in the transient induction of the heat shock genes coding for heat shock proteins (HSPs). The major function of HSPs is to assist in protein folding, assembly, transport, and degradation during normal growth and especially under stress conditions. The larger the temperature increment, the stronger the induction of the heat shock genes. What triggers the HSR? The heat shock results in

the appearance of partially or totally unfolded proteins collectively termed nonnative proteins. These nonnative proteins expose hydrophobic regions which are normally buried within the interior of the folded polypeptide chain and tend to form aggregates. Since large protein aggregates can become life-threatening, cells have to prevent their formation by either allowing their refolding, or degradation or a mixture of both. These nonnative proteins are sensed by either molecular chaperones or by proteases and will lead to the induction of the HSR. Major HSPs are molecular chaperones and ATP-dependent proteases which will deal with these nonnative proteins to prevent the formation of protein aggregates. While molecular chaperones are involved in the refolding of nonnative proteins, proteases catalyze their degradation.

The HSR occurs in four consecutive steps: (1) unfolding of proteins where the extent of denaturation strictly depends on the temperature increment, (2) sensing of nonnative proteins by molecular chaperones or proteases, (3) removal of the nonnative proteins by refolding and degradation, and (4) shut-off of the HSR upon removal of the nonnative proteins.

What process leads to the formation of denatured proteins after a heat shock? This, of course, might be dependent on the absolute temperature. It has been suggested that certain proteins are thermolabile and become denatured after a heat shock. While this can be envisaged after a temperature shock to 42 °C and higher in *E. coli*, it is difficult to understand after a heat shock from 20 to 30 °C. I would like to suggest an alternative mechanism which is independent of the absolute temperature. I assume that a subfraction of the translating ribosomes dissociate upon a sudden increase in temperature and release nascent polypeptide chains. These are unable to fold correctly and are prone to aggregate formation and thus trigger the HSR. For recent reviews on the HSR see (Narberhaus, 1999; Schumann, 2003; Servant and Mazodier, 2001; Yura *et al.*, 2000).

C. The low temperature response

Many bacterial species are exposed to low temperatures in various situations. It has been estimated that the temperature of more than 80% of our biosphere is below 5 °C (Graumann and Marahiel, 1996). One important example for exposure to low temperatures is food-related bacteria, which are repeatedly exposed to low temperatures during food handling and storage (Wouters *et al.*, 2000). Genes expressed at low, but not at high temperatures are those involved in conjugation and synthesis of flagella among others. Why conjugative transfer of DNA and being motile at high temperatures could be a disadvantage for some bacterial species? In the case of mammalian pathogens, flagella are not needed any more after having entered their host and act as an excellent antigen. This could

explain turning off of flagella synthesis at 37 °C. It is more difficult to explain why conjugation should be inhibited at high temperatures. Smith (1974) suggested that plasmids that transfer at low temperatures may be important vectors in the dissemination of antibiotics resistance genes in soil and aquatic environments. But this idea does not really explain the absence of conjugation at high temperatures.

D. The cold shock response

The CSR is classically exhibited when an exponentially growing culture is shifted from the optimum growth temperature to a lower growth temperature (Phadtare, 2004). Such a sudden decrease in temperature causes a transient arrest of cell growth observed in many bacterial species including *E. coli*. A cold shock affects multiple levels of cellular physiology such as: (1) decrease in cytoplasmic membrane fluidity affecting membrane-associated functions including active transport and protein secretion, (2) slow or inefficient folding of some proteins, (3) stabilization of secondary structures of RNA and DNA, leading to reduced efficiency of mRNA transcription and translation, and (4) adaptation of ribosomes to the low temperature to function properly.

If *E. coli* cells are shifted from the optimal growth temperature, 37 °C, to the lower temperature of 20 °C–15 °C, cells stop growth immediately and the synthesis of most proteins is repressed. It takes about 2 h to resume full translation and during this acclimation phase, the synthesis of about 15 proteins increases, and these proteins have been termed cold shock proteins (CSPs) encoded by cold shock genes (Weber and Marahiel, 2003). *E. coli* CSPs are essentially constituted by nucleic acid binding proteins involved in different cellular processes like RNA degradation, transcription, DNA replication and supercoiling, translation and ribosome maturation as well as five members of the CspA (for cold shock protein A) family which represents the major CSP and accumulates up to 10% of the total protein synthesis (Goldstein *et al.*, 1990). CspA and other CSPs are among the fasted proteins discovered to fold in response to large temperature changes (Leeson *et al.*, 2000). Besides CspA, *E. coli* codes for two additional proteins, CspB and CspG. All three proteins can bind to RNA and destabilize secondary structures thereby acting as RNA chaperones (Jiang *et al.*, 1997).

IV. SENSORS OF THE HTR

So far, three classes of macromolecules have been identified as thermosensors: DNA, mRNA, and proteins. The underlying principles for all three sensor types are alternative conformations. While one conformation

(present below 37 °C) prevents expression of the high temperature genes, the alternative conformation (present at 37 °C) leads to the induction of these genes.

A. Nucleoid modulators as thermosensor

Besides acting as storage for proteins and RNA, the structure of the DNA itself can influence access to the genes. Here, DNA bending, either intrinsic or protein-induced or both, directly affects transcription. Intrinsic bends are caused solely by innate sequences, such as homopolymeric adenine:thymine tracts (also called AT-tracts) and lead to curved DNA (Ohyama, 2001). Intrinsic DNA bends in the upstream region of a promoter can activate virulence-associated genes by increasing the binding affinity of the RNA polymerase (RNAP) to the promoter (Nickerson and Achberger, 1995) or, alternatively, can repress virulence-associated genes by excluding or trapping the RNAP with a bacterial nucleoid-associated silencer protein such as H-NS (Dame *et al.*, 2002).

1. The virF promoter of *Shigella flexneri*

S. flexneri and closely related bacteria are the etiological agents of bacillary dysentery in humans. The bacterium is a facultative intracellular pathogen, and the genes required for virulence are located within a 31 kb region of the 230 kb plasmid pINV (Maurelli *et al.*, 1985; Sasakawa *et al.*, 1988). An important characteristic of *Shigella* pathogenicity is the ability of the cells to penetrate into and replicate within human colonic epithelial cells. Infection results in destruction of the colonic epithelium and ulcerative lesions, producing the bloody diarrhea characteristic of bacillary dysentery. Both chromosomal virulence (*vir*) genes and a 230 kb plasmid pINV are involved in expression of the virulence phenotype in *S. flexneri* (Formal and Hornick, 1978; Sansonetti *et al.*, 1982, 1983). Furthermore, expression of the invasive phenotype is regulated by the growth temperature (Maurelli *et al.*, 1984). It follows that bacteria growing at 37°C are virulent and invade epithelial cells, whereas the same cells are noninvasive when grown at 30 °C. Loss of invasive ability at 30 °C reflects a reversible phenotypic change completely restored after shifting the growth temperature back to 37 °C.

Using a strain carrying a transcriptional fusion between a temperature-regulated *vir* gene promoter and *lacZ* (Maurelli and Curtiss, 1984), a transposon mutant was isolated exhibiting high β-galactosidase activity both at 30 °C and 37 °C (Maurelli and Sansonetti, 1988). This gene was termed *virR* and later shown to be identical to *hns*. Further experiments have shown that the primary event following the upshift of *S. flexneri* to the host temperature is the synthesis of VirF, a transcriptional activator, which in turn triggers a regulatory cascade involving the activation of

virB and *virG* (Adler *et al.*, 1989; Tobe *et al.*, 1991). Besides mutations in *hns*, addition of novobiocin, a known inhibitor of the gyrase, led to decreased expression of *virF* at 37 °C resulting in an avirulent phenotype indicating that in addition to *hns* superhelicity influences expression of *virF* (Durand *et al.*, 2000).

At the *virF* promoter, H-NS binds to two sites separated by a region of DNA curvature. Binding to these sites occurs cooperatively at temperatures below 32 °C but not at 37 °C, the permissive temperature for virulence gene transcription. The resulting repression complex is sensitive to supercoiling of the DNA, and bent DNA itself might act as a sensor of temperature (Falconi *et al.*, 1998). Additional experiments have shown that the intrinsic bend located between the two H-NS binding sites melts abruptly around 32 °C resulting in replacement of the transcriptionally inactive DNA architecture by a more relaxed conformation which no longer hinders the formation of productive transcriptional complex (Prosseda *et al.*, 2004). All experimental data support the hypothesis that the curved DNA tract within the *virF* promoter operates as a thermosensor and its structural alternations affect the binding of H-NS molecules to their target sites, thereby controlling the transcriptional activity. The temperature increase produces a progressive downstream sliding of the bending center. The extent of this sliding is not linearly related to the temperature, but undergoes a marked acceleration around the transition temperature around 32 °C as mentioned.

2. The promoter of the *E. coli* hemolysin operon

The toxin α-hemolysin (Hly), produced by many uropathogenic *E. coli* (UPEC) strains, is one of many virulence factors whose expression is thermoregulated (Mourino *et al.*, 1994). Hemolytic *E. coli* strains contain the *hlyCABD* operon located either in the chromosome or on conjugative plasmids (Goebel and Schrempf, 1971; O'Hanley *et al.*, 1993). The *hlyA* gene codes for a 110 kDa protein that is activated by the *hlyC* gene product directing fatty acylation to render the mature toxin (Hardie *et al.*, 1991). Secretion of the active toxin HlyA to the external medium is dependent upon proteins HlyB and HlyD (Braun *et al.*, 1993; Wagner *et al.*, 1983). Synthesis of the α-hemolysin is repressed both under conditions of high osmolarity and at low temperature (Mourino *et al.*, 1994). *hha* mutants exhibit a derepression of the hemolysin expression when cells are grown at low temperature (Mourino *et al.*, 1996). Essential regulatory sequences are spread upstream from the promoter and include two H-NS binding sites in the *hly* regulatory region. One of them partially overlaps the promoter region, and the other is located about 2 kb upstream.

Based on *in vitro* and *in vivo* data, the following model has been proposed to explain temperature-dependent regulation of the *hly* operon. At low temperature, the increased affinity of H-NS for the two identified

binding sites generates nucleoprotein complexes including Hha. Because of the increased flexibility of the DNA at low temperature being a consequence of a higher degree of supercoiling, H-NS molecules at both sites may contact leading to the formation of a larger nucleoprotein complex. That complex would occlude the promoter region, thus repressing transcription. An increase in the growth temperature may have two different consequences: (1) reduction of the affinity of H-NS for its target sequences and (2) reduction of the flexibility of the DNA (Madrid *et al.*, 2002). Growth at 37 °C does not result in complete derepression of *hly* expression, which was measured in *hns* mutants growing at 37 °C (Nieto *et al.*, 2000).

3. Synthesis of pili (fimbriae)

Pathogenic bacteria are characterized by their ability to express a number of special functions which nonpathogenic bacteria lack including adherence factors (pili, also called fimbriae), hemolysins, specific O and K antigens, and iron-sequestering systems. The ability of pathogenic bacteria to adhere to certain host tissues is of primary importance in diseases such as diarrhea, gonorrhea, and urinary tract infections. UPEC frequently express filamentous appendages called P-fimbriae or Pap (pyelonephritis-associated pili) pili (Baga *et al.*, 1985). These pili facilitate binding to host epithelial cells and subsequent colonization of the host upper urinary tract. Pap pili transcription of the structural gene for the pilus subunit *papA* is regulated in response to the growth temperature (Goransson and Uhlin, 1984). Optimal expression occurs at 37 °C, with a 52-fold reduction in *papBA* transcription at 23 °C (White-Ziegler and Low, 1992), and this regulation occurs at the level of transcription (Blyn *et al.*, 1989). The two proteins H-NS and RimJ have been identified to be important in the regulation of *papBA* transcription in response to temperature. H-NS prevents expression at the low temperature (Goransson *et al.*, 1990; White-Ziegler *et al.*, 1990) by binding within the *pap* regulatory region at 23 °C but not at 37 °C (White-Ziegler and Low, 1992). RimJ is the N-terminal acetyltransferase of the ribosomal protein S5 (Cumberlidge and Isono, 1979), and deletion of the *rimJ* gene leads to a loss of thermoregulation so that *papBA* transcription levels are equivalent at both 37 °C and 23 °C (White-Ziegler *et al.*, 2002). The mechanism by which RimJ represses *papBA* transcription is unknown.

In *E. coli*, several fimbriae in addition to the Pap fimbriae are not transcribed at low temperature due to repression by H-NS including type I (Dorman and Ní Bhriain, 1992), 987P (Edwards and Schifferli, 1997), CFA/I (Jordi *et al.*, 1992), S pili (Schmoll *et al.*, 1990), K88 (Nagy *et al.*, 1986), and Bfq (Puente *et al.*, 1996).

4. Expression of virulence genes encoded by *Yersinia* plasmids

Yersinia pestis is the etiologic agent of plague, an invasive and often fatal disease of animals and humans. The virulence genes are encoded by a 70 kb plasmid termed pCD1 present in *Y. pestis* KIM (Perry *et al.*, 1998). This plasmid encodes a set of secreted antihost proteins termed Yops (*Yersinia* outer proteins) and a type III secretion system (T3SS) (Hueck, 1998). The transcription of pCD1 operons-encoding components is regulated by temperature (Straley and Perry, 1995). Expression of these operons requires the transcriptional activator LcrF (Hoe and Goguen, 1993; Lambert de *et al.*, 1992). Synthesis of LcrF is regulated at the level of transcription by changes in DNA topology and at the level of translation by temperature-dependent sequestration of the *lcrF* transcript ribosome binding site (Hoe and Goguen, 1993; Rohde *et al.*, 1994, 1999). Multiple intrinsic DNA bends that melt at 37 °C are located upstream of *lcrF* and in other regions of the plasmid pCD1 (Rohde *et al.*, 1999).

Y. enterocolitica is a psychrotolerant, peritrichously flagellated human pathogen that can cause gastrointestinal disorders, such as enterocolitis and mesenteric lymphadenitis (Bottone, 1999). Its pathogenicity is determined by a number of virulence factors, many of which are encoded by pYV (plasmid of *Yersinia* Virulence) (Snellings *et al.*, 2001). The expression of many plasmid-encoded virulence genes (Lambert de *et al.*, 1992), as well as some genes of the flagellar regulon (Kapatral and Minnich, 1995; Kapatral *et al.*, 1996), and certain chromosomal virulence genes are tightly regulated by temperature. Genes of the flagellar regulon and early virulence genes are expressed below 30 °C, whereas plasmid-encoded virulence genes are expressed at 37 °C.

When *Y. enterocolitica* cells are cultured at 30 °C or below, they are flagellated and motile. In contrast, cells cultured at 37 °C or above lack flagella and are nonmotile. It has been shown that temperature affects the synthesis of flagella of *Y. enterocolitica* by affecting transcription of *fliA*, *fleA*, *fleB,* and *fleC* (Kapatral and Minnich, 1995; Kapatral *et al.*, 1996). While wild-type *Y. enterocolitica* cells exhibit increased expression levels of flagellar genes and decreased expression levels of plasmid-encoded virulence genes at 25 °C (as compared to 37 °C), temperature-dependence of gene expression is much reduced in *fliA* mutants. The *fliA* gene codes for an alternative sigma factor (Iriarte *et al.*, 1995). It was concluded that FliA contributes to the inverse temperature regulation of flagellar and plasmid-encoded virulence genes (Horne and Prüss, 2006), and the exact mechanism how FliA is involved in temperature sensing remains elusive.

Y. enterocolitica seems to use changes in DNA topology as a regulatory means. In *Y. enterocolitica*, chromosomally encoded YmoA is a histone-like protein with properties similar to H-NS but no shared similarity at the amino acid sequence level (Mikulskis and Cornelis, 1994). YmoA seems to

be a global repressor of the *yop* virulence regulon in *Yersinia*, implicating supercoiling as one putative mechanism of control (Lambert de *et al.*, 1992). One study proposes that elevated temperature, which has been shown to affect reporter plasmid supercoiling, would dislodge YmoA and allow the VirF activator in *Y. enterocolitica* to activate genes (Rohde *et al.*, 1994). Furthermore, it has been shown that the plasmid pYV undergoes a conformational transition between 30 and 37 °C based on multiple regions of intrinsic curvature, including *virF*. These bends are detectable at 30 °C but melt at 37 °C (Rohde *et al.*, 1999). This regulation is reminiscent to the *virF* promoter of *S. flexneri* (see above).

B. RNA as thermosensor

Eight examples will be discussed where mRNA molecules sense the absolute temperature. They block the Shine-Dalgarno sequence at low temperature by base pairing, and destabilization of the structure at elevated temperature permits ribosome binding and translation initiation: (1) the *rpoH* transcript of *E. coli*, (2) the repression of heat shock gene expression (ROSE), (3) the FourU element, and the expression of the virulence genes (4) *prfA,* (5) *lcrF*, (6) the *hspA* transcript of *Synechococcus vulcanus*, (7) the *hsp18* transcript of *Streptomyces albus*, and (8) a group II intron.

1. The rpoH transcript of *E. coli*

The alternative sigma factor σ^{32} acts as a key regulator of the HSR (for review, see Yura and Nakahigashi, 1999). At low temperature, cells contain very little σ^{32} (10–30 molecules at 30 °C). By 5 min of temperature upshift, the amount of σ^{32} increases about 15-fold and thereafter declines to a new steady state level (Lesley *et al.*, 2003). Changes in the amount of σ^{32} following temperature upshift result from changes in both the stability and synthesis of σ^{32}. The cellular amount of ^{32} is regulated at the translational level, and using a large number of *rpoH-lacZ* fusions, it could be shown that two distinct 5′-proximal regions termed A and B present in the coding sequence of *rpoH* are responsible for translational control (Nagai *et al.*, 1991). Secondary structure formation between regions A and B was postulated to repress translation at low temperatures, whereas a temperature upshift would lead to the disruption of this structure. Experimental evidence for such an RNA structure was obtained by deletion analysis, point mutagenesis, and structural probing of the *rpoH* transcript. In addition, toeprinting experiments provided evidence that high temperature was indeed needed for ribosome binding (Morita *et al.*, 1999a, 1999b). A close inspection of *rpoH* sequences from other γ-proteobacteria strongly suggests that translational control of *rpoH* is conserved among these bacteria including *Citrobacter freundii*, *Enterobacter cloacae*, *Serratia*

marcescens, *Proteus mirabilis*, and *Pseudomonas aeruginosa* (Nakahigashi *et al.*, 1995).

2. The ROSE element

The ROSE element was discovered in *Bradyrhizobium japonicum* (Narberhaus *et al.*, 1998). Meanwhile, more than 40 ROSE-like elements have been described first in different *Rhizobium* species and then in *Agrobacterium tumefaciens* (Balsiger *et al.*, 2004; Nocker *et al.*, 2001b). Furthermore, potential ROSE-like RNA thermosensors upstream of small heat shock genes have been found in a wide variety of α- and γ-proteobacteria including the *E. coli ibpAB* genes (Waldminghaus *et al.*, 2005). Since the *ibpAB* genes belong to the σ^{32} regulon, combining of regulatory modules, the σ^{32} promoter and a ROSE-type element provides a stringent mechanism to control gene expression. All ROSE elements are located in the 5′-UTR of small heat shock genes, are 70–120 nucleotides long and contain a conserved RNA structure composed of three or four hairpins based on computer-assisted secondary structure predictions (Nocker *et al.*, 2001a). In all ROSE elements, the Shine-Dalgarno sequence and the start codon are masked by imperfect base pairing in the highly conserved 3′-proximal stem-loop. Mutational analyzes have demonstrated that a complex architecture including paired regions, internal loops, and a bulged G residue opposite the Shine-Dalgarno sequence is necessary for proper thermosensing (Chowdhury *et al.*, 2003; Nocker *et al.*, 2001a). Nucleotide exchanges predicted to disrupt base pairing increased basal expression of ROSE-*lacZ* fusions at 30 °C. On the contrary, mutations that improved base pairing further enhanced repression and abolished heat induction. A delicate balance between paired and unpaired nucleotides is critical to ensure repression at low temperature and induction at higher temperature.

3. The FourU element

A novel unusually short RNA thermometer consists of only 52 nucleotides, which folds into two hairpins and controls expression of the small HSP *agsA* in *Salmonella enterica* serovar *Typhimurium* T (Waldminghaus *et al.*, 2007). While hairpin I is not absolutely required for regulation, it might play a structural role during cotranscriptional folding of the RNA thermometer *in vivo*. The Shine-Dalgarno sequence in hairpin II is blocked by a consecutive stretch of four uridine residues, and hence designated as FourU element.

4. The lcrF transcript encoded by plasmid pCD1 of *Yersinia pestis*

The expression of virulence genes can also be controlled by RNA thermometers was first postulated in the early 1990s. *Y. pestis* is the etiologic agent of plague, an invasive and often fatal disease of animals and

humans. The virulence genes are encoded by a 70 kb plasmid termed pCD1 present in *Y. pestis* KIM (Perry *et al.*, 1998). This plasmid encodes a set of secreted Yops and a T3SS (Hueck, 1998). As mentioned above, transcription of pCD1 operons requires the transcriptional activator LcrF (low calcium response F) the cellular level of which is much higher at 37 °C than at 26 °C, though transcription was insensitive to temperature (Hoe and Goguen, 1993; Lambert de *et al.*, 1992). When the amount of LcrF protein produced per unit of transcript at 26 °C was compared to 37 °C, it turned out that the efficiency of *lcrF* mRNA translation increased with temperature. A temperature-labile stem-loop structure blocking the Shine-Dalgarno sequence was predicted upstream of the *lcrF* gene in *Y. pestis*. Thermal destabilization of this stem-loop allowing translation initiation was calculated to occur between 33 and 37 °C (Hoe and Goguen, 1993).

5. The prfA transcript of *Listeria monocytogenes*

Another RNA thermometer is encoded by the 5′-UTR of *prfA* in the food-borne pathogen *L. monocytogenes* (Johansson *et al.*, 2002). The virulence gene activator PrfA is not present at low temperatures, though its transcript is being made (Renzoni *et al.*, 1997). An extended stem-loop structure was predicted in the about 120 nucleotides 5′-UTR, and chemical probing experiments together with an extensive mutational analysis strongly suggested that this structure is responsible for thermoregulation of *prfA* expression (Johansson *et al.*, 2002).

6. The hspA transcript of *Synechococcus vulcanus*

The *hspA* gene of the thermophilic cyanobacterium *S. vulcanus* codes for a small HSP SK (Roy and Nakamoto, 1998). When this gene was fused to an IPTG-inducible promoter in *E. coli*, addition of the inducer did not result in induction of *hspA* at 30 °C (Kojima and Nakamoto, 2005). When the induction was carried out at 42 °C, the expression of *hspA* was greatly enhanced. Nucleotides between the fourteenth codon and the twenty-ninth codon are complementary to bases 1449–1499 in helix 44 of the *E. coli* 16S RNA. The authors speculate that base-pairing between the mRNA and the 16S RNA occurs at 30 °C largely preventing translation. At 42 °; this base-pairing is destroyed.

7. The hsp18 transcript of *Streptomyces albus*

The *S. albus* G protein HSP18 belongs to the small HSP family and can be detected only at high temperature. The *hsp18* gene is under negative control by the transcriptional repressor RheA (see below). Disruption of *rheA* led to the synthesis of the *hsp18* transcript at 30 °C, but this mRNA is not translated at this temperature (Servant and Mazodier, 1996). Translation requires high temperatures suggesting a posttranscriptional

mechanism which could involve a stem-loop structure preventing binding of the ribosomes as shown for *rpoH* mRNA and others containing the ROSE element (see above).

8. A group II intron present in groEL gene of *Azotobacter vinelandii*

The *groEL* gene of the nitrogen-fixing bacterium *Azotobacter vinelandii* was the first housekeeping gene where a group II intron has been described (Adamidi *et al.*, 2003). This group II intron interrupts the stop codon of the *groEL* gene within the *groESL* operon. It has been suggested that the thermodynamics of RNA folding might play an important role to induce self-splicing. Therefore, alternative secondary structures act as sensors of temperature.

C. Proteins as thermosensor

1. The TlpA repressor protein of *Salmonella enterica*

Entry from the ''cold'' environment into the ''warm'' host is believed to be one of the central cues triggering virulence factors in pathogenic bacteria (Miller *et al.*, 1989). TlpA was the first documented case of a temperature-sensing protein. It is a 371-amino-acid cytoplasmic protein, encoded on the 96 kb pSLT virulence plasmid of *S. enterica* serovar Typhimurium (Gulig and Curtiss, 1987) and characterized by a remarkably long α-helical coiled-coil motif (Hurme *et al.*, 1994; Koski *et al.*, 1992). The N-terminus of TlpA is a sequence-specific DNA-binding domain that acts as an autorepressor. The TlpA protein is present in a temperature-dependent two-state equilibrium between unfolded monomers and highly α-helical coiled-coil oligomers. At temperatures below 30 °C, transcription of *tlpA* is repressed by the autorepressor, which is present in its dimeric and folded coiled-coil conformation. Elevated temperatures lead to a shift in the equilibrium that favors the nonfunctional unfolded monomeric form leading to increased transcription (Hurme *et al.*, 1996, 1997).

2. The RheA repressor protein of *Streptomyces albus*

The second example is the RheA repressor identified in *S. albus* (Servant *et al.*, 1999). This transcriptional autorepressor negatively regulates expression of *hsp18* (codes for a small HSP; see above) and its own gene. Both genes form a divergon containing the two promoters and the operators for the RheA repressor. Using an *in vitro* transcription assay, it could be shown that the RheA repressor prevents transcription of both genes at 30 °C, while transcription occurred at 41 °C. Upon a downshift to 30 °C, transcription was blocked again suggesting that the repressor protein can regain its binding activity. When a bandshift assay was performed, RheA repressor was able to retard a DNA fragment with its operator at 30, but

not at 41 °C, and upon a downshift, the fragment was retarded again confirming the conclusion that the repressor protein regains activity at the low temperature (Servant *et al.*, 2000).

3. The ClpXP and Lon proteases of *Yersinia pestis*

A *Y. pestis* strain deficient in the production of ClpXP and Lon proteases does not express a functional T3SS partly because of high cytosolic levels of YmoA. YmoA is rapidly degraded at 37 °C in wild-type *Y. pestis*, but remains stable in a *clpXP* and *lon* double knockout strain. The stability of YmoA in wild-type *Y. pestis* increased as the growth temperature of the culture decreased. If follows that the ClpXP and Lon proteases contribute to thermoregulation of the *Y. pestis* T3SS through regulated proteolysis of YmoA (Jackson *et al.*, 2004). The YmoA protein is highly unstable during growth at 37 °C, shows intermediate stability at 27 °C and is relatively stable at 17 °C. The degradation of YmoA during growth at 37 °C appears to predominantly result from the activity of the Lon protease, whereas the ClpXP protease probably functions as a backup system. The mechanism by which YmoA is targeted for degradation by the ClpXP and Lon proteases at 37 °C, but not at 17 °C, is unknown. Degradation might include a conformational change in YmoA at 37 °C that increases the susceptibility of the polypeptide chain to ClpXP and/or Lon degradation. Alternatively, it might need induction or activation of an accessory protein at 37 °C that modifies or directly targets YmoA for degradation.

4. A translational repressor of *Thermosynechococcus elongatus*

The gene *hspA* of *T. elongatus* codes for a small HSP and is transiently heat-inducible. It could be shown that a protein(s) binds specifically to an AT-rich imperfect inverted-repeat region present in the 5′-UTR of the *hspA* gene (Kojima and Nakamoto, 2002). This protein looses its DNA-binding activity rapidly at the heat shock temperature and could thus act as a thermosensitive translational repressor.

5. Sensor kinases may act as thermosensor

The *lonD* gene of *Myxococcus xanthus* has been shown to be heat-inducible, and induction of transcription depends on the transcriptional activator protein HsfA (Ueki and Inouye, 2002). HsfA is a response regulator of the two-component signal transduction system, where HsfB acts as sensor kinase and is able to become phosphorylated and to transfer the phosphate to HsfA. Most interestingly, HsfB contains a receiver domain and does not function as temperature sensor. It has been suggested that another so far unknown protein acts as thermometer either another sensor kinase which transfers the phosphoryl group to the receiver domain of HsfB or a protein which directly activates HsfA after a heat shock (Ueki and Inouye, 2002). It should be possible to distinguish between these two

possibilities by removal of the receiver domain. In *B. subtilis*, the two HtrA-like membrane-bound proteases, HtrA and HtrB, have been shown to be heat-inducible, and the CssS–CssR two-component regulatory system plays an essential role in transcription activation (Darmon *et al.*, 2002). Here, it has been suggested that the sensor kinase CssS undergoes autophosphorylation when denatured polypeptides appear between the cytoplasmic membrane and the thick cell wall. Therefore, CssS would act as a temperature sensor.

6. The replication initiation protein of pE194

Plasmid pE194 is naturally temperature-sensitive for replication in *B. subtilis* above 45 °C (Sheer-Abramowitz *et al.*, 1981). It has been shown that the RepF protein is needed for initiation of replication (Villafane *et al.*, 1987). Therefore, this positively acting protein is naturally temperature-sensitive. What can be the advantage for a plasmid which such a phenotype? Two possibilities can be envisaged which are sustained by experimental data: (1) such a plasmid can act as a natural suicide vector for the delivery of transposons and (2) it can mobilize chromosomal markers. The first possibility has been analyzed by loading it with the transposon Tn*917*. Tn*917* was discovered as part of the nonconjugative *Streptococcus faecalis* plasmid pAD2 and encodes resistance to macrolides, lincosamides, and streptogramin B antibiotics (MLS^r) (Tomich *et al.*, 1980). Insertion of this transposon into pE194 has been isolated and used to obtain random Tn*917* insertion mutants into the *B. subtilis* chromosome (Youngman, 1987). In another attempt, a derivative of Tn*10* has been used in a similar way (Petit *et al.*, 1990). As to the second possibility, it has been shown that pE194 is able to integrate into the *B. subtilis* chromosome by a *recA*-independent Campbell-like recombination using several plasmid integration sites (Hofemeister *et al.*, 1983). The plasmid can excise from its chromosomal location either precisely or imprecisely with a chromosomal insert. If pE194 can be mobilized into recipient cells of the same or closely related species, it can introduce these genes.

V. SENSORS OF THE HSR

A. Molecular chaperones as thermosensors

1. The σ^{32}-DnaK system of *E. coli*

As outlined above, besides regulation at the level of translation, regulation of σ^{32} occurs in addition at the level of stability and activity. The cellular components involved in modulating the activity of σ^{32} and its stability have been identified. While the DnaK chaperone machine controls the activity, the ATP-dependent metalloprotease FtsH, an integral

cytoplasmic membrane protein with the active site located in a cytosolic domain, is mainly responsible for the degradation of σ^{32} (Tatsuta *et al.*, 1998). It has been observed that σ^{32} is highly unstable at 30 °C with a half-life below 1 min. After a heat shock, σ^{32} is transiently stabilized.

Any model to explain the concerted action of both components has to be based on the following observations. In a $\triangle$*dnaK* mutant, σ^{32} accumulation and HSP synthesis are strongly increased (Tatsuta *et al.*, 1998). Small increases in the chaperone levels resulted in decreased level and activity of σ^{32} and a faster shut-off of the HSR (Tomoyasu *et al.*, 1998). To explain these data, I would like to suggest the following model: σ^{32} is present within the cells in an active and an inactive conformation where the active conformation allows binding to the RNAP core enzyme to initiate transcription at σ^{32}-dependent promoters. The DnaK chaperone system is able to bind σ^{32} in its active conformation to either allow its folding into the inactive conformation or, alternatively, stabilizes the inactive conformation, which is subject to degradation, mainly by the FtsH protease. When cells are challenged to heat stress, denatured proteins appear which titrate the molecular chaperones present within the cell including DnaK. This will increase the amount of active σ^{32} which is further enhanced by the increased translation rate. This in turn will lead to the dramatically increased expression of all the genes belonging to the sigma-32 regulon. Important genes belonging to this regulon are molecular chaperones including DnaK and GroE and ATP-dependent proteases including FtsH. Both groups of proteins recognize and bind denatured proteins to refold or to degrade them. The more denatured proteins have been removed the more DnaK chaperone will become available to convert active σ^{32} in its inactive conformation followed by degradation—the HSR is gradually turned off even if the cells are kept at the high temperature.

Temperature directly controls functional properties of the DnaK/DnaJ/GrpE chaperone system. GrpE, which is an elongated homodimer both in solution and in crystalline form, has been found to undergo two well separated temperature-induced conformational transitions with midpoints at ~48 and 75–80 °C (Grimshaw *et al.*, 2001). The first transition, which occurs in the physiological temperature range, has proven to be fully reversible. The long helix pair in the GrpE dimer acts as a thermosensor that, by decreasing its ADP/ATP exchange activity, induces a shift of the DnaK-substrate complexes toward the high affinity R state and in this way adapts the DnaK/DnaJ/GrpE system to heat shock conditions.

2. The HrcA-GroE system of *Bacillus subtilis*

The second example is the GroEL chaperone team which modulates the activity of the HrcA transcriptional repressor. The *hrcA* gene has been discovered in *B. subtilis* controlling the expression of the heptacistronic *dnaK* and the bicistronic *groE* operon (Homuth *et al.*, 1997; Schmidt *et al.*,

1992). Meanwhile, the *hrcA* gene has been discovered in more than 120 bacterial species making it the most widespread system of heat shock regulation. In most species, different from *Bacillus* spp., HrcA seems to regulate only the *groE* operon. As it is assumed for σ^{32}, HrcA is also present in two conformations, an active and an inactive one, and the equilibrium between these two conformers is influenced by GroEL. But contrary to σ^{32}, GroE shifts this equilibrium toward the active conformation. This model is supported by three different observations: (1) while an increase in GroE reduces the basal level of expression of the two operons negatively regulated by HrcA, a decrease leads to an increase; (2) in a bandshift assay, purified HrcA retards more DNA in the presence of the GroE team; and (3) GroEL has been observed to specifically bind to immobilized HrcA (Mogk *et al.*, 1997; Reischl *et al.*, 2002). These observations led to the following model. *De novo* synthesized HrcA and HrcA dissociated from its operator is present in the inactive conformation. Interaction with the GroE system converts it in its active conformation. This is transiently prevented after heat challenge due to titrations of GroEL by nonnative proteins leading to the induction of the *dnaK* and *groE* operons.

3. The HspR-DnaK system of *Streptomyces coelicolor*

In *S. coelicolor*, the *dnaK* operon consists of the four genes *dnaK, grpE*, *dnaJ*, *hspR* (Bucca *et al.*, 1993). The *hspR* (for HSP regulation) gene codes for a repressor protein which specifically binds to the promoter region of the *dnaK* operon to an operator termed HAIR (for hspR-associated inverted repeat) (Bucca *et al.*, 1995). In an *hspR* disruptant mutant in *S. albus*, transcription of that operon was constitutively derepressed at all temperatures (Grandvalet *et al.*, 1997), and both results strongly suggested that HspR acts as the repressor of the *dnaK* operon. Additionally, HspR controls expression of the *clpB* gene belonging to the Clp ATPase family (Grandvalet *et al.*, 1999), and a HAIR element occurs upstream of the *lon* gene, another ATP-dependent protease (cited in Servant and Mazodier, 2001). The activity of the HspR repressor is modulated by the DnaK chaperone (Bucca *et al.*, 2000). This conclusion is based on four different observations: (1) in a gel retardation assay, the DnaK protein must be together with HspR to retard a DNA fragment containing the *dnaK* promoter region. This effect is independent of the cochaperones DnaJ and GrpE and does not depend on ATP; (2) addition of antiDnaK monoclonal antibodies to the retarded complex resulted in a supershift, showing that DnaK is a component of this complex; (3) HspR copurified with DnaK in column chromatography; (4) the induction of the *dnaK* operon is partially decreased by the overproduction of *dnaK* in *trans*. The authors concluded that DnaK functions as a transcriptional corepressor by binding to HspR at its operator site, and DnaK activates the HspR repressor. Under normal

growth conditions, native HspR binds DnaK, and this complex binds avidly to the *dnaK* promoter region to repress transcription of the operon efficiently. Upon a heat shock, the denatured proteins will titrate DnaK, leading to a derepression of the operon. Removal of the denatured proteins will lead to the reestablishment of repression (Bucca *et al.*, 2000).

B. Proteases as thermosensors

The transcriptional regulator of the periplasmic stress response is σ^E, which is normally held inactive by interaction with the cytoplasmic domain of the antisigma factor RseA. The RseA protein consists of three functional domains, where the central domain anchors it in the cytoplasmic membrane and the C- and N-terminal domains are exposed into the periplasm and cytoplasm, respectively (De Las Peñas *et al.*, 1997; Missiakas *et al.*, 1997). Upon appearance of nonnative proteins within the periplasm or outer membrane, the periplasmic domain of RseA is efficiently cleaved by the periplasmic DegS protease (Walsh *et al.*, 2003), and the remaining part of RseA is subsequently further degraded (Alba *et al.*, 2002; Kanehara *et al.*, 2002). It is assumed that these proteolytic events destabilize the cytoplasmic domain of RseA, releasing σ^E to activate transcription of the genes of the σ^E regulon (Alba *et al.*, 2001).

The DegS protease acts as the sensor. It has an N-terminal transmembrane domain, followed by a central protease domain and a C-terminal PDZ domain that extends into the periplasm (Alba *et al.*, 2001). PDZ domains are found in a large variety of proteins, and are known to recognize specific C-terminal polypeptide sequences (Doyle *et al.*, 1996). Interestingly, the PDZ domain of DegS also recognizes C-terminal peptides with the motif Y-X-F, common to a number of outer membrane porins which largely terminate in Y-X-F. Supplying DegS with polypeptides terminating in this motif caused DegS-mediated cleavage of RseA *in vitro* and *in vivo*, and resulted in σ^E activation *in vivo*. Because the C termini of the porins are normally folded inaccessibly into the native proteins, PDZ domain recognition and DegS activation would only occur when the native structures of the porins become disturbed, as during heat shock or overproduction (the export of polypeptides beyond the folding capacity of the periplasmic chaperones would produce free C termini to interact with DegS). Inhibition of the DegS protease by the PDZ domain is probably through direct contact between the domains. This mechanism provides a unique means of stress sensing, by detection of a specific unfolded protein sequence through the regulatory PDZ domain. The DegS activation mechanism is most likely supported by other regulatory steps. For example, RseB also interacts with unfolded periplasmic proteins (Collinet *et al.*, 2000) and might influence the accessibility of RseA to cleavage by DegS.

VI. SENSORS OF THE LTR

When *Y. enterocolitica* cells are cultured at 30 °C or below, they are small, motile, single coccobacilli with peritrichous flagella. When these cells are shifted to 37 °C, a pronounced change in physiology occurs including a loss in motility within 60–90 min (Cornelis *et al.*, 1987; Kapatral and Minnich, 1995). Motility is also repressed at 25 °C in the presence of subinhibitory concentrations of novobiocin suggesting that this gyrase inhibitor changes the DNA topology (Rohde *et al.*, 1994). This finding was corroborated by analyzing a class of novobiocin-resistant DNA gyrase mutants exhibiting the 37 °C phenotype at 25 °C. The three flagellin genes *fliABC* are coordinately expressed at low, but not at high temperature from a σ^F-dependent promoter. Transcription of *fliA* coding for σ^F is immediately arrested when cells are exposed to 37 °C (Kapatral *et al.*, 1996).

A. RNA as thermosensor

1. *cis*-acting RNA

The lysis-lysogeny decision of phage λ

The cIII gene product of phage λ plays an important role in the decision of whether it enters the lytic or lysogenic cycle after infection. It does so by binding to the ATP-dependent FtsH protease, which degrades the cII protein, a central regulator in the lysogenic pathway (Herman *et al.*, 1997; Shotland *et al.*, 1997). High concentrations of cIII result in the stabilization of cII thus promoting lysogeny. Two alternative structures were first predicted and then verified by structure probing *in vitro* and *in vivo* (Altuvia *et al.*, 1989). While one structure sequesters part of the Shine-Dalgarno sequence and the start codon, the alternative structure leaves the translation initiation region accessible to the ribosomes. The equilibrium between both structures is temperature-dependent. High temperatures (45 °C) favor the energetically more stable conformation thereby blocking translation of cIII. At 45 °C, the AUG start codon and the Shine-Dalgarno sequence are sequestered in a hairpin structure. This in turn leads to a decrease in the cII concentration and initiates the lytic pathway. Physiological temperatures (37 °C) shift the equilibrium toward the structure in which the ribosome binding site is available resulting in the synthesis of cIII and initiation of the lysogenic pathway. This RNA thermometer switches on translation with decreasing temperature and does not operate by gradual melting but alternates between two mutually exclusive conformations. What might be the physiological reason for temperature control of the cIII gene? Phage λ tends to proliferate effectively when host cells are healthy and is more likely to integrate into the

E. coli chromosome when growth conditions are poor. But under life-threatening conditions such as a severe heat shock, it might be beneficial for the phage to escape from the host.

2. *trans*-acting RNA

Binding of the small DsrA RNA to the *rpoS* leader mRNA favors its translation at low temperature

Expression of the general stress sigma factor RpoS of *E. coli* is controlled at the levels of transcription, translation, and protein stability. The amount of RpoS is adjusted in response to various environmental signals, and each step of RpoS expression can be affected by one or several environmental stimuli (reviewed in Hengge-Aronis, 2002). One environmental cue that increases RpoS synthesis is low temperature (below 37 °C), and two thermosensors have been identified controlling σ^S activity, the small noncoding RNA DsrA (Sledjeski *et al.*, 1996) and the small protein Crl (Olsen *et al.*, 1993) the amount of which increases at low temperature.

The small nontranslated RNA DsrA activates translation of the alternative sigma factor σ^S by pairing with the leader of its mRNA to allow more efficient translation (Majdalani *et al.*, 1998). Temperature affects both the rate of transcription initiation of the *dsrA* gene and the stability of its transcript (Repoila and Gottesman, 2001). The net affect is a 25- and 30-fold decrease in full-length *dsrA* transcript at 37 and 42 °C, respectively, compared to 25 °C. What mechanism is responsible for temperature regulation of the *dsrA* promoter? Temperature control of transcription initiation of *dsrA* requires only the minimal promoter of 36 bp (Repoila and Gottesman, 2001). While all of the elements of the *dsrA* promoter contribute to temperature-sensitive expression, the sequence of the -10 box and the spacer region are the essential elements for the thermal response of the *dsrA* promoter (Repoila and Gottesman, 2003).

The small protein Crl stimulates the activity of the alternative sigma factor σ^S of *E. coli* by directly interacting with the sigma factor (Bougdour *et al.*, 2004). The gene coding for the Crl protein is expressed at low temperature (30 °C) and stationary phase but not at 37 °C. The transcription factor involved in thermosensing remains elusive. Interacting of Crl with the σ^S holoenzyme ($E\sigma^S$) promotes transcription initiation at the *csgBA* promoter and another about 40 genes constituting the Crl regulon (Bougdour *et al.*, 2004; Lelong *et al.*, 2007). The *csgBA* operon codes for the subunits of the curli proteins that form fibers at the cell surface called fimbriae, which are involved in cell–cell attachment and adhesion to extracellular matrices. The synthesis of Crl is temperature-dependent and since transcription is not thermoregulated, its synthesis may be controlled posttranscriptionally.

In summary, the two mechanisms act in concert because at low temperature DrsA stimulates σ^S translation and Crl enhances the activity of σ^S. While the DsrA-mediated temperature control modulates the amount of σ^S in the cell, the temperature-dependent expression of Crl appears to fine-tune σ^S activity.

B. Proteins as thermosensors

1. The VirA sensor kinase of *Agrobacterium tumefaciens* is involved in temperature sensing

A. tumefaciens is a soil bacterium which infects plant wound sites and induces tumor formation. The bacterial cells harbor a large tumor-inducing plasmid (Ti-plasmid) encoding virulence genes and transfer DNA (T-DNA). The function of the virulence genes is the processing and transfer of the T-DNA from the Ti-plasmid into susceptible plant cells with subsequent integration (for recent review see Gelvin, 2000). The expression of the virulence genes is under the control of a two-component signal transduction system comprised of VirA and VirG. VirA is an inner membrane histidine kinase (Melchers *et al.*, 1989) which autophosphorylates in response to certain phenolic compounds released from wounded plant with subsequent transfer of the phosphate moiety to the response regulator VirG (Jin *et al.*, 1990) followed by transcription activation of a specific set of promoters. Virulence gene expression is specifically inhibited at temperatures above 32 °C. It could be shown that both the autophosphorylation of VirA and the subsequent transfer of phosphate to VirG are sensitive to high temperatures (Jin *et al.*, 1993). At temperatures of 32 °C and higher, the VirA protein undergoes a reversible inactivation. Why transfer of DNA is prevented at high temperature? Since several plant-specific proteins are involved in steps subsequent to T-DNA transfer, one or more of these proteins might be inactive at high temperature thus blocking successful integration of the T-DNA into the plant genome.

2. The transcriptional activator NIFA of *Klebsiella pneumoniae* exhibits temperature-sensitive DNA-binding activity

Synthesis of active nitrogenase requires transcription of a large number of nitrogen fixation (*nif*) operons. In diazotrophs belonging to the large Gram-negative phylum ''purple bacteria'' *nif* operons are transcribed by the alternative sigma factor σ^{54} in conjunction with the transcriptional activator NIFA (Merrick, 1993). NIFA binds to upstream activation sequences (UAS) that are located about 100 bp upstream of *nif* promoters to catalyze isomerization of closed complexes between $E\sigma^{54}$ and *nif* promoters to produce open complexes. In *K. pneumoniae*, expression of the *nif* genes is sensitive to high temperature (>37 °C), and this thermosensitive gene expression is correlated with a temperature-sensitive DNA binding

activity of the NIFA protein (Lee *et al.*, 1993). Since the DNA-binding motif of NIFA is not inherently heat-labile, it is possible that the failure of NIFA to bind DNA at 37 °C is due to the fact that the helix-turn-helix motifs in different subunits are not correctly oriented with respect to one another at 37 °C.

3. The response regulator DegU of *Listeria monocytogenes* is active only at low temperature

In *L. monocytogenes*, expression of flagella-based motility is regulated in response to the growth temperature with the permissive temperature being 30 °C and below (Peel *et al.*, 1988; Williams *et al.*, 2005), and it was demonstrated that the flagellin FlaA encoding the major subunit of the flagellum is not expressed at higher temperatures (Dons *et al.*, 1992). Temperature-dependent transcriptional regulation of flagellar genes in *L. monocytogenes* relies on three regulatory proteins, the repressor protein MogR, the response regulator DegU and GmaR acting as the antirepressor of MogR (Gründling *et al.*, 2004; Knudsen *et al.*, 2004; Williams *et al.*, 2005). The MogR protein was shown to directly bind to multiple recognition sites within the promoter regions of the motility genes, thus preventing their transcription at temperatures above 30 °C (Gründling *et al.*, 2004; Shen and Higgins, 2006). The response regulator DegU is required to relieve the MogR-mediated repression by enabling expression of GmaR at low temperatures. The antirepressor GmaR is both able to bind to soluble and to DNA-bound MogR, thus disrupting preformed repressor-DNA complexes (Shen *et al.*, 2006). Since the DegU protein is present at ambient temperatures, its activity has to be modulated in response to the growth temperature. Phosphorylation does not play a role (Mauder *et al.*, 2008). Either DegU is a temperature-sensitive protein being active at low and inactive at high temperatures, or the activity of DegU is regulated by another protein in a temperature-dependent way. Why synthesis of flagella should be prevented at 37 °C, the temperature of the mammalian host? It has been proposed that downregulation of *flaA* expression during *in vivo* infection of *L. monocytogenes* may serve as an adaptive mechanism to avoid host recognition and mobilization of the host innate immune response (Dons *et al.*, 2004; Way *et al.*, 2004).

4. Conjugation

Exchange of genes in bacteria promotes adaptation to environment challenges and the evolution of bacterial species. Conjugation is one of the main mechanisms responsible for horizontal (conjugative transfer among cells of the same species) and lateral (among different species) gene transfer. Bacterial conjugation is a DNA transfer event that requires three plasmid-encoded multiprotein complexes: (1) the membrane-spanning mating pair formation (Mpf) complex, also called transferosome,

which is composed of 12–15 membrane-associated proteins which span the inner and outer membrane of the donor cell; (2) the cytoplasmic nucleoprotein relaxosome complex, and (3) a homo-multimeric coupling protein that links the Mpf and relaxosome at the cytoplasmic membrane (for review see Cascales and Christie, 2004). Plasmids belonging to the HI incompatibility group (IncHI) of *Enterobacteriaceae* are large (>180 kb), encode multiple antibiotic resistances and are thermosensitive for transfer with conjugation occurring at optimal frequencies between 22 and 30 °C, but conjugation is negligible at 37 °C (Rodriguez-Lemoine *et al.*, 1975; Taylor and Levine, 1980). IncHI1 conjugative plasmids are the only known plasmids in human pathogens that are temperature-sensitive for transfer (Anderson, 1975; Smith, 1974). It has been suggested that transfer is optimized to occur in soil and water environments (Maher and Taylor, 1993).

Using the transmission electron microscope, no pili were observed on cells grown at 37 °C, whereas cells grown exclusively at 27 °C exhibited one to four pili indicating that temperature-sensitive DNA transfer result from lack of pili at 37 °C (Maher *et al.*, 1993). Transcriptional analysis of the gene *trhC* belonging to the Mpf complex and essential for plasmid transfer and H-pilus formation indicated that this and other proteins are not produced at 37 °C thus explaining the lack of H-pili (Gilmour *et al.*, 2001). Later, transcription of additional genes involved in conjugation has been shown to be decreased at 37 °C (Gunton *et al.*, 2005). It has been suggested that a putative R27-encoded repressor protein could be involved in the inhibition of promoters at 37 °C (Alonso *et al.*, 2005).

VII. SENSORS OF THE CSR

The mechanisms regulating the induction of cold-shock genes in *E. coli* act either at the level of transcription or posttranscriptionally, and most frequently both levels are involved. Two major mechanisms of posttranscriptional regulation have been identified: selective cold shock-induced stabilization of the transcripts of cold-shock genes and translational bias whereby at low temperature translation of cold shock mRNA is preferentially favored and that of noncold shock transcripts is disfavored.

A model has been proposed in which ribosomes are the prokaryotic sensors for a temperature downshift (VanBogelen and Neidhardt, 1990). The induction of the CSPs at low temperature does not require the synthesis of any new protein such as the specific cold shock sigma factor or a transcriptional regulator, as it takes place under conditions that completely block protein synthesis (Etchegaray and Inouye, 1999). It could be shown that several *cis* elements are implicated in the regulation

of the cold-inducible *csp* genes in *E. coli* and *B. subtilis* (for reviews, see Baker *et al.*, 2006; Sourjik, 2004; Szurmant and Ordal, 2004).

A. RNA as sensor

1. The *cspA* transcript

In *E. coli* and other bacteria, the expression of a set of genes, known as the cold-shock genes, becomes specifically enhanced or induced *de novo* during the growth lag following the lowering of the temperature from 37 °C to 10 °C (Jones *et al.*, 1987). Among the cold-shock genes is *cspA*, the structural gene for the major cold-shock protein CspA (Goldstein *et al.*, 1990), which was shown to act as a cold-shock transcriptional enhancer of the expression of at least some cold-shock genes (La Teana *et al.*, 1991). By which mechanism the *cspA* gene is turned on and off during the early stages of the cold-shock response? First, it could be shown that the *cspA* mRNA is not translated at 37 °C indicating that *cspA* mRNA is functionally unstable at elevated temperature (Tanabe *et al.*, 1992). Indeed, the *cspA* mRNA is extremely unstable at 37 °C with a half-life of about 10 sec (Goldenberg *et al.*, 1996). Upon a shift to 15 °C *cspA* mRNA becomes highly stable, but this stability is transient and is lost once the cells are adapted to the low temperature. Similar results have been published by another group demonstrating that both the chemical and functional half-lives of the *cspA* transcript were substantially longer at 10 °C compared to 37 °C (Brandi *et al.*, 1996). Three-base substitutions around the Shine-Dalgarno sequence in the 159-base 5′-UTR region of the *cspA* mRNA stabilizes the transcript 150-fold, resulting in constitutive expression of *cspA* at 37 °C. This stabilization is at least partially due to resistance against RNase E degradation (Fang *et al.*, 1997). Based on these results, it has been suggested that at 37 °C, the *cspA* transcript adopts a secondary structure which is recognized by RNase E, while it adopts a different secondary structure at 25 °C not recognized by the endoribonuclease. In summary, the *cspA* mRNA serves as a cold-shock sensor.

2. The *pnp* transcript

Polynucleotide phosphorylase (PNPase), one of the main 3′ to 5′ exonucleolytic activities involved in mRNA turnover and stability (Nierlich and Murakawa, 1996), has been identified as another CSP (Jones *et al.*, 1987). A more than 10-fold increase in the overall intensity of the *pnp* transcript signals have been described within the first hour of incubation at low temperature, where the induction is mainly due to an increased stability of the transcript (Zangrossi *et al.*, 2000). While the most abundant transcript detected at 37 °C is represented by the mature monocistronic *pnp* mRNA ending at the t_{pnp} terminator, at early times after cold shock including the coding region for the *deaD* encoding a DEAD-box RNA

helicase. There is a Rho-dependent termination site within *pnp*, and in the cold acclimation phase, termination at this site depends upon the presence of PNPase, suggesting that during cold shock *pnp* is autogenously regulated at the level of transcription termination.

B. Proteins as sensor

One of the essential processes in the cold-shock response is the adaptation of the membrane. After a temperature downshift, the physical properties of the membrane changes by undergoing a phase transition from its normal liquid–crystalline phase to a more rigid gel-like phase. Cells of *B. subtilis* are able to use two different mechanisms to maintain the membrane fluidity at low temperatures. One mechanism is based on the *de novo* synthesis of branched-chain fatty acids, which are incorporated into the membrane, to lower the melting point. Here, fatty acid branching is changed from iso to anteiso, the change being dependent on the presence of isoleucine or precursors of anteiso-branched fatty acids, which have a lower melting point than iso-fatty acids (Kaneda, 1977). The other mechanism involves desaturation of fatty acid moieties of the membrane. Here, the fatty acid desaturase converts already existing fatty acid moieties into $\triangle$5-unsaturated fatty acids, resulting in a higher membrane fluidity (Aguilar *et al.,* 1998). The transcription of the desaturase gene *des* is cold-induced and regulated by the two-component system DesK and DesR (Aguilar *et al.*, 2001). The histidine kinase DesK is responsible for sensing of low temperatures resulting in autophosphorylation and transduction of a phosphate group to the response regulator DesR. The phosphorylated DesR activates the transcription of the *des* gene by binding to a DNA segment from -28 to -77 positions relative to the start site of the *des* gene (Cybulski *et al.*, 2004). While the *des* transcript is hardly detected at 37 °C, its synthesis is transiently induced (10–15-fold) 4 h after cold shock (Aguilar *et al.*, 1999). DesK has four transmembrane domains, and one or more of these domains may propagate a conformational change across the membrane, which can be governed by the physical state of the membrane lipid bilayer. As shown by domain swap experiments, the membrane domain of DesK is the temperature-sensing element (Hunger *et al.*, 2004).

VIII. THE THERMOTACTIC RESPONSE

E. coli can sense a variety of chemotactic signals such as amino acids, dipeptides, and sugars, as well as pH, the redox state and temperature through transmembrane receptors also termed methyl-accepting chemotaxis proteins (MCPs). While major MCPs, such as those for aspartate

(Tar) and serine (Tsr), are present in several thousand molecules per cell, minor receptors, such as those specific for dipeptides (Tap), ribose and galactose (Trg), and redox potential (Aer) number to only a few hundred copies per cell (Li and Hazelbauer, 2004). The chemotaxis receptors form clusters at the cell poles in *E. coli* and other bacteria (Maddock and Shapiro, 1993).

Signaling in chemotaxis relies on protein phosphorylation. Changes in attractant or repellent concentrations are sensed by the protein assembly of receptors, the adaptor protein CheW and the histidine-kinase CheA forming a ternary complex. Two response-regulator proteins, CheY and CheB, compete for binding to CheA. While CheY is a single-domain protein binding to the flagellar motor, CheB consists of two domains, one of which functions as a methylesterase and controls the adaptation of MCPs. CheY∼P binds the switch protein FliM on the flagellar motor causing a reversal in the direction of motor rotation. The phosphatase CheZ increases the spontaneous dephosphorylation rate of CheY∼P allowing rapid signal termination. A decrease in the attractant concentration results in decreased attractant binding to the MCPs, which stimulates CheA autophosphorylation followed by an increase in the CheY∼P concentration. This in turn causes a switch to clockwise rotation resulting in cell tumbling and direction change. CheB is also phosphorylated by CheA∼P resulting in an increased methyltransferase activity and an increased demethylation of the MCPs. Demethylated MCPs have a reduced ability to induce CheA autophosphorylation, and the rate of direction returns to the prestimulus level. An increased concentration of attractant inhibits the autophosphorylation of CheA thereby reducing the concentration of CheY∼P and the frequency of motor switching. This causes the cells to swim in this positive direction for a longer time. Phosphorylation of CheB is also reduced, which allows the methyltransferase CheR to increase the methylation of the MCPs. Highly methylated MCPs stimulate CheA autophosphorylation, even in the continued presence of a chemoattractant returning CheA autophosphorylation to the prestimulus level and the cell to a normal frequency of direction changing (for reviews, see Baker *et al.*, 2006; Sourjik, 2004; Szurmant and Ordal, 2004).

E. coli has the ability to sense temperature changes as thermal stimuli and to respond to small changes in temperature by altering its swimming behavior to migrate in spatial temperature gradients termed thermotaxis (Maeda *et al.*, 1976). Four MCPs have been identified as thermosensors (Maeda and Imae, 1979; Nara *et al.*, 1991). The warm sensors Tsr, Tar, and Trg mediate attractant and repellent responses upon increases and decreases in temperature, respectively, and the cold sensor Tap mediates the opposite responses to the same stimuli (Nara *et al.*, 1991). The thermosensing mechanism is thought to involve temperature-dependent

changes in the structure of the receptor-CheW-CheA ternary complex and/or in the interactions among multiple ternary complexes. Among the components of the ternary complex, the receptor itself is thought to be the primary thermosensor because a strain devoid of receptors never gives a thermotactic response and each of the four receptors characteristically functions either as a warm or a cold sensor.

While a temperature increase suppressed tumbling, a temperature decrease induced tumbling. Next, it could be shown that L-serine acted as a potent inhibitor of the thermosensory transduction recognized by the Tsr receptor (Maeda and Imae, 1979). Cells with a defect in the Tsr protein exhibited a reduced thermoresponse (Mizuno and Imae, 1984). On the other hand, Tar-protein deficient mutants exhibited the inverted thermoresponse when cells were simultaneously adapted to L-aspartate and L-serine, a first indication that the Tar protein plays a key role in the inversion of the thermoresponse (Mizuno and Imae, 1984).

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CHAPTER 8

Deciphering Bacterial Flagellar Gene Regulatory Networks in the Genomic Era

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Abstract

Synthesis of the bacterial flagellum is a complex process involving dozens of structural and regulatory genes. Assembly of the flagellum is a highly-ordered process, and in most flagellated bacteria the structural genes are expressed in a transcriptional hierarchy that results in the products of these genes being made as they are needed for assembly. Temporal regulation of the flagellar genes is

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achieved through sophisticated regulatory networks that utilize checkpoints in the flagellar assembly pathway to coordinate expression of flagellar genes. Traditionally, flagellar transcriptional hierarchies are divided into various classes. Class I genes, which are the first genes expressed, encode a master regulator that initiates the transcriptional hierarchy. The master regulator activates transcription a set of structural and regulatory genes referred to as class II genes, which in turn affect expression of subsequent classes of flagellar genes. We review here the literature on the expression and activity of several known master regulators, including FlhDC, CtrA, VisNR, FleQ, FlrA, FlaK, LafK, SwrA, and MogR. We also examine the Department of Energy Joint Genomes Institute database to make predictions about the distribution of these regulators. Many bacteria employ the alternative sigma factors σ^{54} and/or σ^{28} to regulate transcription of later classes of flagellar genes. Transcription by σ^{54}-RNA polymerase holoenzyme requires an activator, and we review the literature on the σ^{54}-dependent activators that control flagellar gene expression in several bacterial systems, as well as make predictions about other systems that may utilize σ^{54} for flagellar gene regulation. Finally, we review the prominent systems that utilize σ^{28} and its antagonist, the anti-σ^{28} factor FlgM, along with some systems that utilize alternative mechanisms for regulating flagellar gene expression.

I. INTRODUCTION

The flagellum is the locomotion organelle for many species of bacteria from diverse phyla. Motility is required for several important microbial processes, including tactic responses (e.g., chemotaxis, aerotaxis, magnetotaxis), colonization of plant and animal hosts, biofilm formation, bacterial development, and dispersion of bacteria. Bacterial species differ with respect to the number of flagella they possess and their distribution across the cell surface. Despite these differences, all bacterial flagella consist of the following basic structures: (1) the basal body, which anchors the flagellum in the bacterial cell envelope and contains the motor that powers flagellar rotation; (2) the hook, which is a flexible, curved rod that converts rotary motion into waves; and (3) the filament, which propagates the waves initiated by the hook and pushes against the surrounding medium to propel the bacterium forward (Macnab, 1996). The basal body is the most complex of these structures, consisting of three ring structures in Gram-negative bacteria, a rod, a rotary motor, and a flagellar protein export apparatus. The export apparatus is a type III secretion system that is required for translocation of most of the flagellar proteins that localize outside the cell membrane (Minamino and Macnab, 1999).

More details on the structure and function of the bacterial flagellum can be found in a recent review (Chevance and Hughes, 2008).

Bacterial flagellar biosynthesis is a complex and ordered process requiring the coordinated and temporal regulation of dozens of genes via a transcriptional hierarchy. The organization of flagellar genes varies greatly among bacteria. In some bacteria the flagellar genes are arranged within a few operons that are clustered together within the chromosome (e.g., *Sinorhizobium meliloti*). In contrast, the flagellar genes in *Helicobacter pylori* are arranged in over 20 operons that are scattered around the chromosome.

Temporal regulation of flagellar genes ensures that the structural proteins of the flagellum are produced as they are needed for assembly of the nascent flagellum. In the assembly pathway, the basal body is generated first, followed by the hook and then the filament (Fig. 8.1). Sequential expression of flagellar genes is achieved through the integration of regulatory networks that control the expression of different sets of flagellar genes. These regulatory networks are responsive to specific checkpoints in flagellar biosynthesis which helps coordinate flagellar gene regulation with assembly. Temporal regulation of flagellar genes is also subject to developmental control in many bacterial species that exhibit a dimorphic lifestyle (Section II.B). These bacteria include *Caulobacter crescentus*, which is the paradigm for developmental control of flagellar gene expression, as well as the budding, prosthecate bacteria (which include *Hyphomonas* spp., *Hyphomicrobium* spp., *Pedomicrobium* spp., and *Rhodomicrobium vannielli*), *Kineococcus radiotolerans*, and several members of the phylum Planctomycetes. In addition, some bacteria elaborate different arrangements of flagella depending on environmental conditions. For example, *Vibrio parahaemolyticus* expresses a polar

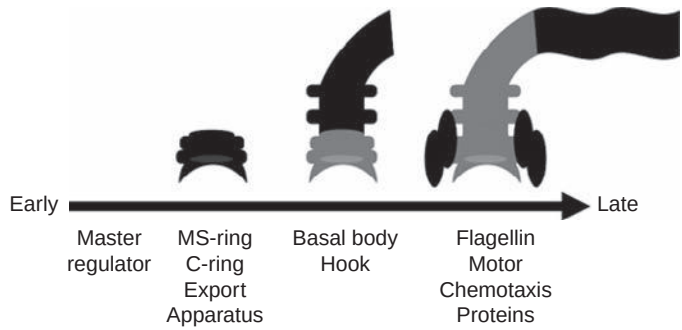

FIGURE 8.1 Assembly of the Gram-negative flagellum. Temporal control of flagellar genes ensures that flagellar genes are expressed as their products are required for assembly. Completion of the basal body-hook structure is a key checkpoint for coordinating assembly with gene expression in many bacteria.

flagellum for swimming motility and a separate set of lateral flagella for swarming motility across surfaces or in viscous medium (Section II.D.3). Examples of other bacteria that possess functional dual flagella systems include some *Aeromonas* spp., *Azospirillum brasilense*, and *Rhodospirillum centenum* (see for review Merino *et al.*, 2006).

As might be expected, considerable variation exists in the architecture of the regulatory networks that control flagellar biosynthesis in different bacterial species (Fig. 8.2). Here we review some of the prominent paradigms for flagellar gene regulation. In addition, we examine databases of bacterial genomic sequences to predict how flagellar regulatory networks might operate in select bacterial species where flagellar gene regulation has not been studied.

To identify genomes of flagellated bacteria for our analysis, homology searches were done for four key flagellar biosynthetic proteins: FliG, the flagellar rotor protein; FliM, the rotor-switch protein; FlgE, the hook protein; and FlhA, an essential component of the flagellar protein export apparatus. Bacterial genomes in the United States Department of Energy Joint Genome Institute Integrated Microbial Genomes database (DOE JGI, http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) were searched for the genes encoding these proteins. Bacterial species whose genomes

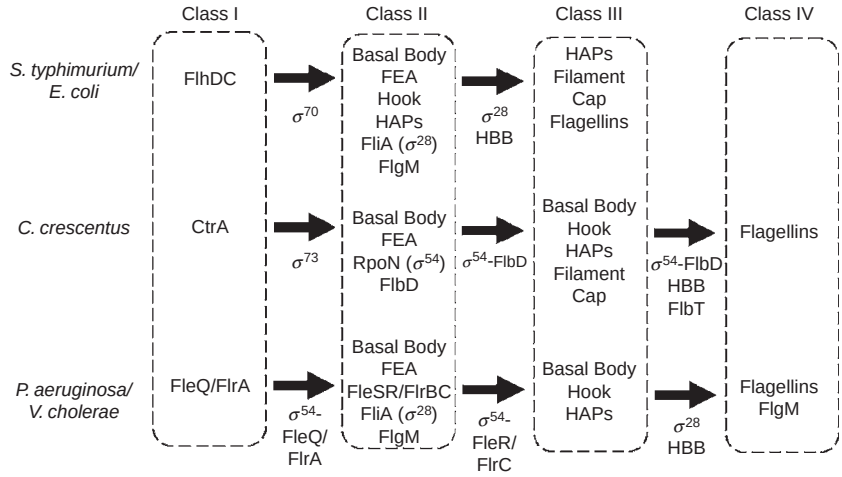

FIGURE 8.2 Paradigms for flagellar gene hierarchies. Flagellar gene regulatory networks are compared for three different systems. Class I genes encode the master regulators. Regulatory proteins encoded by Class II genes include the σ^{28} (FliA), the anti-σ^{28} factor (FlgM), and σ^{54}-dependent regulators FlbD in *C. crescentus*, FleRS in *P. aeruginosa* and FlrBC in *V. cholerae*. Abbreviations for class II genes are as follows: flagellar protein export apparatus (FEA) and hook associated proteins (HAPs). Regulation of Class III/IV genes is governed by completion of the hook-basal body complex (HBB).

contained orthologs (reciprocal best BLASTP hits) of at least three of these proteins were predicted to be flagellated for at least part of their life cycle. After examining 865 sequenced bacterial genomes, 442 genomes representing 303 unique species were predicted to be flagellated using this criterion. This estimation has certain pitfalls since some pathogenic organisms such as *Shigella*, *Bordetella pertussis*, and *Yersinia pestis* (which are not included in the above estimate) possess all four of these key flagellar genes yet do not produce flagella apparently due to the highly antigenic nature of the structure (Akerley and Miller, 1993; Parkhill *et al.*, 2001; Tominaga *et al.*, 1994).

II. MASTER REGULATORS

In traditional flagellar gene transcriptional hierarchies the first genes to be transcribed encode regulatory proteins that initiate transcription of the early structural genes. This regulator is referred to as the master regulator, and it recognizes elements in the promoter regulatory regions of genes whose products are required at the earliest steps of flagellar assembly. Traditionally, genes encoding the master regulator are termed class I genes. A variety of master regulators have been identified in different bacterial species (Table 8.1), but for many systems a master regulator is yet to be identified. The following sections focus on various master regulators that have been described to date.

TABLE 8.1 Master regulators of flagellar genes

Name	Group (Subgroup)	Representative Bacteria	Gene Locus[a]
FlhDC	Proteobacteria (β/γ)	*S. typhimurium* LT2	STM1925 STM1924.S
CtrA	Proteobacteria (α)	*C. crescentus* CB15	CC3035
VisNR Rem[b]	Proteobacteria (α)	*S. meliloti* 1021	SMc03015 SMc03016 SMc03046
FtcR	Proteobacteria (α)	*B. melitensis* 16M	BMEII0158
FleQ FlrA[c]	Proteobacteria (β/γ/δ)	*P. aeruginosa* PAO1 *V. cholerae* O395	PA1097 VC0395_A1721
SwrA	Firmicutes	*B. subtilis* 168	BSU35230
MogR	Firmicutes	*L. monocytogenes* EGD	Lmo0674

[a] Locus tag for genes in the representative bacteria are in the same order as the names.
[b] VisNR and Rem are two separate regulators that are both required for expression of downstream flagellar genes.
[c] FlrA and FleQ are orthologous σ^{54}-dependent activators.

A. FlhDC

FlhDC is the most extensively studied master regulator and is found in members of the phylum Proteobacteria including *Salmonella typhimurium* (Kutsukake *et al.*, 1990), *Escherichia coli* (Liu and Matsumura, 1994), *Serratia liquefaciens* (Givskov *et al.*, 1995), *Proteus mirabilis* (Furness *et al.*, 1997), *Yersinia enterocolitica* (Young *et al.*, 1999), *Xenorhabdus nematophilus* (Givaudan and Lanois, 2000), *Ralstonia solanacearum* (Tans-Kersten *et al.*, 2004), *Burkholderia glumae* (Kim *et al.*, 2007), *Erwinia carotovora* (Cui *et al.*, 2008), and *Azotobacter vinelandii* (Leon and Espin, 2008). Expression of *flhDC* is controlled by several regulators (Fig. 8.3) including but not limited to the heat shock proteins DnaK, DnaJ, and GrpE, which respond to changes in temperature (Li *et al.*, 1993; Shi *et al.*, 1993); H–NS, which responds to changes in pH (Soutourina *et al.*, 1999, 2002); OmpR, which responds to osmolarity (Shin and Park, 1995); and cAMP–CAP, which responds to the availability of carbon sources (Kutsukake, 1997; Soutourina *et al.*, 1999). Additional signals that affect *flhDC* expression include quorum sensing in *Y. pseudotuberculosis* and *E. coli* (Atkinson *et al.*, 2008; Sperandio *et al.*, 2002) as well as transition to a solid surface in *P. mirabilis* (Hatt and Rather, 2008). In addition to environmental inputs, expression of flagellar master regulators is also subject to cell cycle control. This is most apparent in *C. crescentus*, which has a dimorphic life cycle in which only one of the cell types is flagellated (see Section II.B). In *E. coli* cultures in which cell division was synchronized, transcription of *flhDC* was observed to increase immediately following cell division until it reached a steady-state level in the middle of the cell division cycle (Pruss and Matsumura, 1997). As described in the following sections, *flhDC* is not unique with regard to the multiple environmental signals that mediate its expression through global regulators. Thus, transcription of other master regulator genes is likely also regulated in a cell cycle-dependent fashion similar to *E. coli flhDC*.

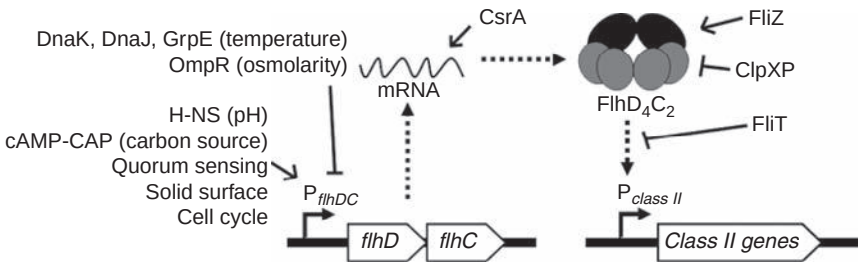

FIGURE 8.3 Regulation of the flagellar master regulator FlhDC. Multiple regulatory proteins influence expression of *flhDC* in response to various environmental factors, and still other regulators influence mRNA stability, protein stability, or activity. Arrowheads indicate a positive effect while blunt-ends indicate a negative effect.

FlhDC activity and levels in the cell are further regulated (Fig. 8.3). FlhDC is regulated at the level of message stability by the global RNA-binding protein CsrA (Wei *et al.*, 2001) and protein stability by the protease ClpXP (Claret and Hughes, 2000b; Tomoyasu *et al.*, 2003). *S. typhimurium* FliZ, a DNA-binding regulatory protein, enhances FlhDC stability probably by down regulating expression of ClpXP or inducing transcription of another factor that directly stabilizes FlhDC (Kutsukake *et al.*, 1999; Lanois *et al.*, 2008; Saini *et al.*, 2008). *X. nematophilus* FliZ has been shown to have a similar function, but instead of acting indirectly, it binds to the *flhDC* promoter region to stimulate transcription (Lanois *et al.*, 2008). A final example of a factor that influences FlhDC activity is FliT, a chaperone that facilitates export of the filament cap. FliT negatively regulates FlhDC activity by sequestering the protein and preventing it from activating transcription (Kutsukake *et al.*, 1999; Yamamoto and Kutsukake, 2006). Since *fliT* is expressed late in the transcriptional hierarchy, the interactions between FliT and FlhDC would function as a negative feedback mechanism to turn off expression of the earlier flagellar genes.

The crystal structure of the FlhDC complex was recently solved (Wang *et al.*, 2006). In contrast to earlier work reporting a $FlhD_2C_2$ structure (Claret and Hughes, 2000a, 2002; Liu and Matsumura, 1996), the crystal structure revealed a $FlhD_4C_2$ hexameric complex (Wang *et al.*, 2006). FlhD and FlhC consist of predominantly alpha-helical structures, and FlhC contains a unique zinc-binding domain (Campos *et al.*, 2001; Wang *et al.*, 2006). FlhDC recognizes sites located approximately 28–88 bp upstream of the transcriptional start site of its target genes, which overlap the −35 promoter elements of these genes (Liu and Matsumura, 1994). The FlhDC binding site contains 17–18 bp imperfect inverted repeats separated by a 10–11 bp spacer (Claret and Hughes, 2002). Upon binding its sites, FlhDC interacts with the C-terminal domain of RNA polymerase α subunit to activate transcription by recruiting σ^{70}-RNA polymerase holoenzyme (σ^{70}-holoenzyme) to the promoter (Liu *et al.*, 1995; Wang *et al.*, 2006).

FlhDC activates transcription of genes whose products include components of the flagellar protein export apparatus, basal body, hook, and the regulatory proteins FliA and FlgM (Claret and Hughes, 2002; Frye *et al.*, 2006; Gillen and Hughes, 1993; Liu and Matsumura, 1994; Pruss *et al.*, 2001). These genes are referred to as class II or middle genes. FlhDC acts as a global transcriptional activator of some genes unrelated to flagella biosynthesis including ones involved in anaerobic respiration using dimethyl sulfoxide, nitrate, or nitrite as a terminal electron acceptor (Pruss *et al.*, 2001, 2003).

One of the flagellar genes under control of FlhDC is *fliA* which encodes the alternative sigma factor σ^{28}. Expression of class III or late genes, including the flagellin FliC, is σ^{28}-dependent (Kutsukake *et al.*, 1990; Ohnishi *et al.*, 1990). FlgM is an anti-sigma factor that interacts with σ^{28}

to prevent transcription of the class III genes (Section IV). Inhibition of class III genes is alleviated upon completion of the basal body-hook structure when the flagellar protein export apparatus switches substrate specificity and transports FlgM out of the cell (Hughes *et al.*, 1993).

From our search of the DOE JGI database, FlhDC appears to be restricted to the β- and γ-Proteobacteria (Table 8.1). FlhDC orthologs were identified in 58 species, all of which are members of the β- or γ-Proteobacteria. Species in these two subgroups make up about 42% of the 303 sequenced species that we predicted to be flagellated. Species that contain FlhDC orthologs constitute 44% of the β- and γ-Proteobacteria that we predicted to be flagellated, suggesting that there is diversity with respect to master regulators even within members of these groups.

B. CtrA

In the α-Proteobacterium *C. crescentus* the complexity of the flagellar gene hierarchy is superseded by the elegant transcriptional regulation of the cell cycle. During the *C. crescentus* cell cycle a flagellated swarmer cell differentiates into a sessile, stalked cell which undergoes asymmetric cell division to give rise to a new swarmer daughter cell. An excellent description of the regulatory network controlling the *C. crescentus* cell cycle can be found in a recent paper by Collier and coworkers and the figures therein (Collier *et al.*, 2007). CtrA is a response regulator that is involved in controlling the cell cycle in *C. crescentus*, as well as serving as the master regulator of the flagellar gene hierarchy (Quon *et al.*, 1996). Flagellar assembly is so intimately connected to the cell cycle that mutations in some flagellar genes can arrest the cell cycle (Shapiro and Maizel, 1973; Yu and Shapiro, 1992)

Expression of *ctrA* is driven from two promoters (Domian *et al.*, 1999). Transcription from the distal promoter is induced by the upstream cell cycle regulator GcrA and is repressed due to methylation of a GANTC motif within the promoter by CcrM, the downstream cell cycle regulator (Holtzendorff *et al.*, 2004; Reisenauer and Shapiro, 2002). Both *ctrA* promoters are subject to autoregulation as CtrA phosphate (CtrA∼P) represses transcription from the distal promoter but induces transcription from the proximal promoter (Domian *et al.*, 1999). CtrA∼P not only acts as a transcriptional regulator but also binds to sites near the origin of replication to inhibit chromosome replication and cell division (Quon *et al.*, 1998). Because of its role in chromosome replication and cell division CtrA∼P must be turned over within the cell to allow continuation of the cell cycle (Domian *et al.*, 1997). Precise modulation of CtrA levels is achieved by localization of CtrA∼P to the stalk-cell pole by RcdA at the

proper time in the cell cycle and colocalization of the protease ClpXP by CpdR (Iniesta *et al.*, 2006; Jenal and Fuchs, 1998; McGrath *et al.*, 2006). CpdR activity is inhibited by phosphorylation (Iniesta *et al.*, 2006). A single phosphorelay involving CckA receives signals from the cell cycle to control both CtrA and CpdR (Biondi *et al.*, 2006; Iniesta *et al.*, 2006; Jacobs *et al.*, 1999).

Flagellar genes make up a major portion of the genes controlled by CtrA (Laub *et al.*, 2002). At class II flagellar gene promoters, CtrA∼P binds TTAA direct repeats (or slight deviations of this motif) that are separated by a critical 7 bp spacer (Ouimet and Marczynski, 2000; Quon *et al.*, 1996) and overlap the −35 promoter element (Reisenauer *et al.*, 1999). An additional CtrA-binding half-site (TTAACCAT) has also been identified (Laub *et al.*, 2000, 2002). Binding of CtrA to sites in the promoter regulator regions of class II genes activates transcription with σ^{73}-RNA polymerase holoenzyme, which is the primary form of RNA polymerase holoenzyme in the bacterium (Reisenauer and Shapiro, 2002; Wu *et al.*, 1998). Class II genes encode components of the basal body and flagellar protein export apparatus, as well as the regulatory proteins RpoN (σ^{54}) and FlbD (Laub *et al.*, 2002; Quon *et al.*, 1996). CtrA binds another set of flagellar gene promoters and is required for transcription of these genes. Unlike traditional class II genes, disruptions in these genes (which encode components of the basal body and chemotaxis proteins—*flgBC*, *fliE*, *flmABCDEFGH*), do not inhibit expression of the subsequent flagellar genes (Boyd and Gober, 2001; Leclerc *et al.*, 1998). The alternative sigma factor σ^{54} is responsible for transcription of the class III and IV flagellar genes (see Section III.A) which encode the remaining basal body, hook, and flagellin proteins (Anderson *et al.*, 1995; Brun and Shapiro, 1992).

Searching the DOE JGI database revealed CtrA orthologs in all of the α-Proteobacteria that are predicted to be flagellated, one *Campylobacter* species, four *Clostridium* species, and one member of the phylum Thermotoga (*Petrotoga mobilis*). In *Hyphomonas neptunium*, which has a dimorphic life cycle similar to that of *C. crescentus*, *ctrA* is in an apparent operon with the flagellar genes *flhF* and *motR*, suggesting a role for CtrA in flagellar gene regulation in this bacterium (Badger *et al.*, 2006) Predicted CtrA-binding sites were identified *in silico* in the promoter regulatory regions of flagellin genes in α-Proteobacteria *Brucella melitensis*, *Mesorhizobium loti*, *S. meliloti*, and *Agrobacterium tumefaciens*, suggesting a role for CtrA in the regulation of flagellar biogenesis in these bacteria (Hallez *et al.*, 2004). If CtrA functions as a regulator of these genes, it probably is not the master regulator since flagellins are required late in flagellar assembly. Moreover, in *S. meliloti* another master regulator as been identified (Section II.C), and CtrA from *Silicibacter* sp. TM1040 does not serve as the master regulator of flagellar genes (Miller and Belas, 2006).

C. VisNR

S. meliloti is a symbiotic, nitrogen-fixing, member of the α-Proteobacteria. The *S. meliloti* flagellar gene hierarchy is controlled by the master regulator VisNR and a second regulator Rem. The *visNR* and *rem* genes are part of a 56-kb island that contains all of the genes required for flagellar assembly, function, and chemotaxis (Rotter *et al.*, 2006; Sourjik *et al.*, 1998, 2000). This region of the chromosome, called the flagellar regulon, contains approximately 50 genes (Rotter *et al.*, 2006; Sourjik *et al.*, 1998). VisNR controls expression of *rem*, and Rem subsequently controls expression of the class II flagellar genes. In this regulatory cascade *visNR* are referred to as class IA genes and *rem* as a class IB gene (Rotter *et al.*, 2006).

Expression of *visNR* is controlled by at least three regulatory networks, Sin-ExpR, ExoS-ChvI, and CbrA. SinR and SinI produce the quorum sensing molecules N-acyl homoserine lactones which are detected by ExpR (Hoang *et al.*, 2004; Marketon *et al.*, 2002). ExpR either directly or indirectly down regulates expression of *visNR* as population density and concentration of *N*-acyl homoserine lactones increase (Hoang *et al.*, 2008). ExoS is a membrane-bound sensor kinase which, together with its cognate response regulator ChvI, regulates extracellular polysaccharide (EPS) production (Cheng and Walker, 1998). ExoR is a periplasmic regulator that also regulates EPS production through ExoS-ChvI (Wells *et al.*, 2007). Transposon insertions in *exoR* or *exoS* result in excess EPS production (Doherty *et al.*, 1988) and a nonmotile, aflagallated phenotype (Yao *et al.*, 2004). Lack of flagella in these mutants is due to a down regulation of *visNR* and is independent of the Sin-ExpR quorum sensing system (Hoang *et al.*, 2008). CbrA, another regulator of EPS production as well as other genes important for symbiosis in the host legume, also affects expression of *visN* (Gibson *et al.*, 2007).

VisN and VisR have a similar predicted secondary structure and are proposed to form an active heterodimer. Based on the predicted secondary structure, VisN and VisR are LuxR-type transcriptional activators (Sourjik *et al.*, 2000). Members of the LuxR protein family consist of a DNA-binding helix-turn-helix motif and a ligand-binding receptor domain (Fuqua and Winans, 1994). VisNR effector molecules are yet to be identified. Variability in the ligand-binding domains of VisN and VisR suggest these proteins bind distinct effector molecules (Sourjik *et al.*, 2000). Although the VisNR binding site in the *rem* promoter regulatory region has not been identified, VisNR likely activates transcription with σ^{70}-holoenzyme from two different promoters upstream of *rem* (Rotter *et al.*, 2006). Rem is an OmpR-type transcriptional activator that directly controls expression of class II genes by activating transcription with σ^{70}-holoenzyme (Hoang *et al.*, 2008; Rotter *et al.*, 2006). Rem recognizes a 17-bp imperfect tandem repeat that overlaps the −35 element by 3 bp in

its target promoters (Rotter *et al.*, 2006). The close proximity of the Rem-binding site to the promoter is consistent with the transcriptional activation model for OmpR-type regulators (Busby and Ebright, 1994; Rotter *et al.*, 2006).

D. σ^{54}-dependent master regulators

Some master regulators function with σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme). This form of RNA polymerase holoenzyme binds its target promoter to form a closed complex but cannot proceed further in transcription initiation in the absence of an activator (Reitzer *et al.*, 1987; Sasse-Dwight and Gralla, 1988). The activator stimulates the conversion of the closed complex to an open promoter complex in a reaction that requires ATP hydrolysis by the activator (Popham *et al.*, 1989). Hundreds of σ^{54}-dependent activators have been described to date. These activators function with σ^{54}-holoenzyme to transcribe genes involved in a variety of processes, including nitrogen assimilation and fixation, H_2 metabolism, transport of sugars and dicarboxylic acids, degradation of aromatic compounds, response to phage shock, and pilin production, as well as flagellar biosynthesis (Studholme and Dixon, 2003). Most σ^{54}-dependent activators have a three-domain architecture consisting of a N-terminal regulatory domain, a central activation domain, and a C-terminal DNA-binding domain (Schumacher *et al.*, 2006). The activation domain, the most conserved of these domains, is an ATPase that belongs to the AAA+ superfamily, members of which are involved in a variety of cellular functions (Neuwald *et al.*, 1999; Ogura and Wilkinson, 2001). Activators of σ^{54}-holoenzyme generally bind to upstream activation sequences (UAS; sometimes referred to as bacterial enhancers) that are often located 70 bp or more upstream of the target promoter (Reitzer and Magasanik, 1986). After binding the UAS the activator engages σ^{54}-holoenzyme bound at the promoter through DNA looping (Su *et al.*, 1990). DNA looping is often facilitated by an auxiliary DNA-binding protein, such as integration host factor (IHF), which binds to a site between the promoter and the UAS (Hoover *et al.*, 1990).

1. FleQ

The master regulator FleQ from *Pseudomonas aeruginosa* is a σ^{54}-dependent activator (Arora *et al.*, 1997). Several factors have been identified that modulate expression of *P. aeruginosa fleQ*, including the alternative sigma factor AlgT (σ^{E}) which regulates EPS production (Tart *et al.*, 2005). AlgT is required for expression of AmrZ, a regulator that binds to and represses the *fleQ* promoter (Tart *et al.*, 2006). Thus, similar to the situation in *S. meliloti*, when EPS production is turned on flagellar assembly is turned off in *P. aeruginosa*. Interestingly, FleQ appears to bind and repress a set of

promoters for EPS production genes. This repression is relieved by high concentrations of cyclic diguanylate (c-di-GMP), which FleQ appears to bind despite the fact that it does not possess any known c-di-GMP-binding motifs (Hickman and Harwood, 2008). Vfr, a homolog of *E. coli* CAP, also binds the *fleQ* promoter and prevents σ^{70}–holoenzyme from binding at the −10 promoter element (Dasgupta *et al.*, 2002).

FleQ activates transcription of class II genes encoding components of the basal body, motor, and export apparatus as well as the regulator proteins FleN and FleSR. FleN negatively regulates the activity of FleQ through direct interactions to restrict flagellar assembly to a single flagellum (Dasgupta and Ramphal, 2001; Dasgupta *et al.*, 2000). A FleQ-binding site with dyad symmetry was identified 67 bp upstream of the *fleSR* transcriptional start site, and a potential IHF binding site was identified between this site and the *fleSR* promoter (Jyot *et al.*, 2002). A maltose-binding protein-FleQ (MBP-FleQ) fusion protein was also reported to recognize sites immediately downstream of the promoters of three other flagellar genes (*flhA*, *fliE*, and *fliL*) (Jyot *et al.*, 2002). The authors of this study demonstrated by site-directed mutagenesis the importance of the site downstream of the *flhA* promoter for expression. These downstream sites, however, lack homology with the FleQ-binding site at *fleSR* as well as with each other, and appear to be positioned too close to the promoter (located from 5–18 bp from the transcriptional start sites) to allow FleQ to contact σ^{54}-holoenzyme via DNA looping. Thus, it is unclear how FleQ might activate transcription from these downstream sites. It is possible that FleQ contacts σ^{54}-holoenzyme by a different mechanism than DNA looping (e.g., by tracking along the DNA after binding to the downstream sites). Alternatively, a contaminating protein may have been responsible for the reported binding activity since relatively high levels MBP-FleQ (1.0–7.5 μM) were used in the DNA binding assays (Jyot *et al.*, 2002). If this is the case, then the contaminating protein may interact with FleQ and have an important regulatory role.

One of the class II operons activated by FleQ encodes the FleSR two-component system (discussed in Section III.B) which is required for expression of class III genes (Correa *et al.*, 2000). FleR is a σ^{54}-dependent activator that stimulates transcription of the class III genes, which encode the remaining basal body components, hook, and hook associated proteins (Dasgupta *et al.*, 2003). Class IV genes are σ^{28}-dependent and encode the flagellin, chemotaxis proteins, and the regulatory protein FlgM (Dasgupta *et al.*, 2003; Frisk *et al.*, 2002; Starnbach and Lory, 1992).

2. FlrA

Vibrio cholerae FlrA, an ortholog of FleQ, is another example of a σ^{54}-dependent activator that serves as a flagellar master regulator (Klose and Mekalanos, 1998). Similar to the link between motility and EPS

production in *P. aeruginosa* and *S. meliloti*, expression of virulence genes and flagellar genes in *V. cholerae* is mutually exclusive (Gardel and Mekalanos, 1996). ToxR and H–NS have been shown to play a role in this process. ToxR, which is an activator of virulence genes (DiRita, 1992), represses motility while H–NS directly or indirectly represses virulence genes and induces expression of *flrA* (Ghosh *et al.*, 2006).

The regulatory network controlling flagellar gene expression in *V. cholerae* is similar to that of *P. aeruginosa*. Expression of class II genes is initiated by the σ^{54}-dependent activator FlrA. As in *P. aeruginosa*, two of the class II genes encode the regulatory proteins FlrBC which form a two-component system required for expression of the σ^{54}-dependent class III genes. Similarly, the class IV genes are σ^{28}-dependent in *V. cholerae* (Prouty *et al.*, 2001). One significant difference between the two systems is *P. aeruginosa fliA* (σ^{28}) is not regulated by FleQ and σ^{54} (Dasgupta *et al.*, 2003), whereas *fliA* is a class II gene in *V. cholerae* (Prouty *et al.*, 2001). In addition, *V. cholerae* produces multiple flagellin proteins, the majority of which are encoded by class IV genes, but the gene encoding the core flagellin FlaA is a class III gene (Prouty *et al.*, 2001).

3. FlaK and LafK

As mentioned previously, some bacteria possess a polar flagellar system that is utilized for swimming motility and a lateral flagellar (*laf*) system that is expressed for swarming motility. We will only briefly discuss the regulation of such dual flagellar systems and refer the reader to two recent reviews for more information on the subject (McCarter, 2004; Merino *et al.*, 2006). The best characterized dual flagellar systems are in *V. parahaemolyticus* and *Aeromonas hydrophila*. Within each of these bacterial species, the two flagellar systems apparently do not share structural or regulatory genes (McCarter, 2004). In addition, the genes for each system are unlinked. For example, in *V. parahaemolyticus* the genes required for the polar system are distributed among five clusters on chromosome I, while the genes encoding components of the lateral flagella are arranged in two clusters on chromosome II (Kim and McCarter, 2000; Stewart and McCarter, 2003).

The regulatory networks that control the polar and lateral flagellar systems are very similar to those of *V. cholerae* and *P. aeruginosa*. In *V. parahaemolyticus*, the polar flagellum master regulator, FlaK, is dispensable for polar flagellum biosynthesis as the lateral flagella master regulator, LafK, compensates for its loss. FlaK cannot replace LafK, however, in expression of the *laf* system (Kim and McCarter, 2004). Expression of the *V. parahaemolyticus laf* system is induced by iron-limitation and stalling of polar flagellum rotation (McCarter and Silverman, 1989; McCarter *et al.*, 1988). Stalling of polar flagellum rotation occurs when the bacterium

encounters a solid surface or viscous environment. Thus, the polar flagellum functions as a tactile sensor for the cell.

As in *V. cholerae* and *P. aeruginosa*, the class III and class IV genes in both flagellar systems of *V. parahaemolyticus* are dependent on σ^{54} and σ^{28}, respectively, for their expression (McCarter, 2004; Merino *et al.*, 2006). Distinct σ^{54}-dependent activators are used for expression of the class III genes in the two flagellar systems; and likewise distinct σ^{28} and anti-σ^{28} proteins are employed in regulating expression of the class IV genes in the two flagellar systems. Thus, transcriptional control of the polar and lateral flagellar systems in *V. parahaemolyticus* is mediated through parallel regulatory networks.

4. Predicting σ^{54}-dependent master regulators

One of the difficulties in predicting FleQ/FlrA-type master regulators from genome sequences is that σ^{54} is involved in many diverse processes and bacteria can possess several different σ^{54}-dependent activators, each targeting a different set of genes. All σ^{54}-dependent activators share a high degree of homology, particularly within their AAA+ domains, and there does not appear to be any outstanding motifs within FleQ/FlrA-type master regulators that distinguish them from many other σ^{54}-dependent activators. While the presence of a gene encoding a σ^{54}-dependent activator within a cluster of flagellar genes would suggest a role for this activator in flagellar biosynthesis, additional information would be required to predict if the activator served as a master regulator or was involved in later stages of flagellar assembly.

An alternative strategy for predicting whether a given bacterium utilizes a FleQ/FlrA-type master regulator is to search for potential σ^{54}-dependent promoters upstream of class II genes. Promoters recognized by σ^{54}-holoenzyme contain conserved elements located approximate 12 and 24 bp upstream of the transcriptional start site of the gene. Barrios and coworkers identified consensus sequences 5′-TTGCW-3′ and 5′-TGGCACGR-3′ (where W is A or T and R is G or A) for the −12 and −24 elements, respectively, based on a comparison of 186 known or potential promoter sequences (Barrios *et al.*, 1999). The GC and GG dinucleotides (underlined) are the most conserved elements in the consensus. Spacing between these dinucleotides is absolutely critical since insertion or deletion of a single base pair results in loss of promoter function (Buck, 1986). These features facilitate identification of potential σ^{54}-type promoters from genome sequences.

We used the Pattern Locator program (Mrazek and Xie, 2006) (http://www.cmbl.uga.edu/software/patloc.html) to search the intergenic regions of the genomes of several diverse bacterial species for the motif

5′-TGGYAYNNNNN{TT}(1)GCW-3′ (parentheses indicate that only one of the two residues preceding the GC doublet must be a T). This motif was chosen to take into account the variability in σ^{54}-type promoter sequences yet maintain enough stringency to minimize the number of sequences gleaned from the search. Potential σ^{54}-type promoters in the correct orientation were identified upstream of putative class II flagellar genes in several bacterial genomes (Table 8.2). All of these bacteria belonged to the β-, γ-, or δ-Proteobacteria groups, suggesting that FleQ/FlrA-type master regulators are restricted to these groups. A caveat of this approach is that sequences of σ^{54}-type promoters in some bacteria may vary from the consensus. Indeed, the −24 element of σ^{54}-type promoters of flagellar genes in *H. pylori* and other ϵ-Proteobacteria has the consensus 5′-WGGAAC-3′ (Pereira *et al.*, 2006), which would not have been detected in our motif search.

E. Other master regulators

In the Firmicutes *Bacillus subtilis* and *Listeria monocytogenes* two additional early flagellar gene regulators have been identified (Table 8.1). SwrA (also referred to as SwrAA) in *B. subtilis* is required for swimming motility in liquid and swarming motility on solid surfaces (Calvio *et al.*, 2005; Kearns *et al.*, 2004). Binding of SwrA to flagellar promoters has not been demonstrated, but changes in expression of *swrA* affect expression of the 31-gene *fla/che* operon (all genes in this operon are involved in flagellar biosynthesis and chemotaxis) and the overall flagellation of the cell (Kearns and Losick, 2005; Marquez-Magana and Chamberlin, 1994). The promoter of the *fla/che* operon deviates slightly from the σ^{A} consensus, which accounts for its dependence on SwrA (Kearns and Losick, 2005). The *swrAB* gene, which is immediately downstream of *swrA* and is cotranscribed with it, is involved in the increase in flagellation associated with swarming motility. SwrAB is a membrane-bound regulatory protein and appears to be required for proteolysis of SwrA (Calvio *et al.*, 2005). Expression of the *fla/che* operon is also directly regulated by the two-component system DegS-DegU which globally controls changes in gene expression during stationary phase. DegU phosphate (DegU∼P) binds the σ^{A} promoter of the *fla/che* operon and enhances SwrA-dependent transcription (Calvio *et al.*, 2008; Kobayashi, 2007; Tsukahara and Ogura, 2008). In addition, DegU∼P appears to enhance expression of *swrA* from an upstream σ^{A} promoter while a second promoter that is σ^{D}-dependent (Section IV.A) also drives *swrA* expression (Calvio *et al.*, 2008).

SwrA orthologs are found only in three other members of the genus: *Bacillus amyloliquefaciens*, *Bacillus Licheniformis*, and *Bacillus pumilus*.

TABLE 8.2 Potential σ^{54}-dependent promoters upstream of putative class II genes

Group	Bacterium	Gene (Operon)[a]	Locus[b]	Potential promoter[c]	Distance[d]
β-Proteobacteria	*Azoarcus* sp. BH72	*fliE*	Azo2713	GTGGCACGTGGTTTGCT	72
		fliF (+12)	Azo2716	CTGGCACTTTAGCTGCA	120
γ-Proteobacteria	*A. hydrophila* hydrophila ATCC 7966	*fliE* (+25)	Aha_1364	TTGGCACCCTAATTGCT	41
	Pseudoalteromonas haloplanktis TAC125[e]	*fliE* (+27[f])	PSHAa0791	GTGGCACGTTTTGTGCT	84
	Thiomicrospira crunogena XCL-2[e]	*fliE* (+12)	Tcr_1443	TTGGCATATGAATTGCT	80
	Xanthomonas campestris pv. campestris ATCC 33913[e]	*flhF* (+5)	Xcc1908	TTGGCACACCGCATGCA	160
		fliL (+4)	Xcc1920	TTGGCACGCTGATTGCA	33
δ-Proteobacteria	*Geobacter sulfurreducens* PCA[e]	*fliL* (+2)	Gsu0420	CTGGCATTTCGGTTGCA	59
		flhA (+6)	Gsu3056	CTGGCACAACGGTTGCA	51

[a] The numbers in parentheses indicate the number of flagellar or chemotaxis genes that could form an operon with the given gene.
[b] Locus tag for genes with potential σ^{54}-dependent promoters.
[c] Sequence of the potential σ^{54}-dependent promoter with the conserved dinucleotides underlined.
[d] Distance in base pairs between the potential promoter and the predicted translational start of the gene.
[e] Bacterium is also predicted to have potential σ^{54}-dependent promoters upstream of class III genes (Table 8.3).
[f] Divergently transcribed from flaM, which encodes a σ^{54}-dependent activator.

Orthologs of SwrAB are more widespread than SwrA in the phylum Firmicutes. Several Firmicutes that we do not predict to be flagellated contain SwrAB orthologs, while some of the predicted flagellated Firmicutes lack SwrAB orthologs. Thus, SwrAB appears to have other roles outside flagellar biogenesis.

L. monocytogenes MogR is technically not a master regulator since it functions as a repressor of early flagellar genes rather than a transcriptional activator. MogR represses expression of flagellar genes in a nonhierarchal fashion during intracellular growth or growth at 37 °C (Grundling *et al.*, 2004; Shen and Higgins, 2006) by binding TTTT-N_5-AAAA motifs that overlap the −35 promoter element of flagellar gene operons (Shen and Higgins, 2006). MogR activity is controlled by an antagonist, GmaR, the expression of which is subject to temperature regulation through DegU. At lower temperatures GmaR is expressed, sequesters MogR through protein–protein interactions, and allows expression of flagellar genes. Interestingly, GmaR is a functional glycosyltransferase for the flagellin FlaA and is believed to be the first prokaryotic protein identified to control expression of its enzymatic substrate (Shen *et al.*, 2006).

MogR orthologs occur in all of the *Listeria* spp. we predicted to be flagellated and a subset of the predicted flagellated *Bacillus* spp. (see Section IV.A for further discussion about this subset). MogR orthologs were not found in species predicted to be nonflagellated suggesting a conserved function for MogR and its orthologs in regulation of flagellar genes. As expected from its bifunctional nature, GmaR orthologs are found in many diverse species, some of which appear to be flagellated and others which are not. All of the *Listeria* spp. (but not all of the Firmicutes) that contained MogR orthologs also contained GmaR orthologs.

The response regulator FtcR in the α-Proteobacterium *B. melitensis* is a final example of a flagellar master regulator. A cognate sensor kinase to regulate FtcR activity has yet to be identified. Expression of *ftcR* is at least partially controlled by a quorum sensing system (Leonard *et al.*, 2007). FtcR binds to sites located upstream of the open reading frame of *fliF*, which encodes the MS-ring of the basal body, activating transcription of *fliF* and subsequent production of hook and flagellin (Leonard *et al.*, 2007). FtcR orthologs are found in many of the predicted flagellated α-Proteobacteria overlapping with orthologs of both CtrA and VisNR in many of these species including *S. meliloti* and *A. tumefaciens*, but not *C. crescentus*. How or if these multiple regulatory systems are integrated to control flagellar biosynthesis in these bacteria is unknown. Clearly, more work is needed to dissect the complex regulatory networks that initiate the flagellar gene transcriptional hierarchy in these nonparadigm systems.

III. RpoN (σ^{54}) REGULATORS

As alluded to previously, many organisms utilize σ^{54}-holoenzyme for transcription of class III and IV flagellar genes. Utilization of different σ^{54}-dependent activators for regulation of late flagellar genes provides an additional level of temporal control to coordinate gene expression with assembly of gene products (Fig. 8.2). Examples of activators of σ^{54}-holoenzyme that control expression of class III and class IV flagellar genes are discussed in the following sections.

To predict class III genes that are dependent on σ^{54} for their expression in previously uncharacterized systems, we followed the same strategy outlined above (Section II.D.4) using the Pattern Locator program (Mrazek and Xie, 2006). Potential σ^{54}-type promoters were found upstream of class III genes in at least one representative from the α-, β-, γ-, and δ- Proteobacteria but not any other groups of bacteria (Table 8.3). Based the data from Tables 8.2 and 8.3, σ^{54}-type promoters appear to be more prevalent upstream of class III genes indicating that σ^{54}-dependent expression of middle and late flagellar genes may be more common than σ^{54}-dependent master regulators.

A. FlbD

As previously mentioned (Section II.B), *C. crescentus* utilizes σ^{54} and the σ^{54}-dependent activator FlbD to control expression of class III and IV genes (Fig. 8.2, (Brun and Shapiro, 1992; Ramakrishnan and Newton, 1990)). Expression of both *rpoN* and *flbD* is CtrA-dependent (Laub *et al.*, 2002). FlbD is a σ^{54}-dependent activator with three functional domains: a response regulator, AAA+ ATPase and DNA-binding domains (Ramakrishnan and Newton, 1990). Activity of FlbD is both positively and negatively regulated through interactions with FliX, a membrane-bound protein that senses completion of the MS-ring, rotor switch, and export apparatus intermediate (Mohr *et al.*, 1998; Muir and Gober, 2001, 2002, 2004). The *fliX* gene is a class II gene under control of CtrA (Mohr *et al.*, 1998).

FlbD and FliX orthologs are found in several α- Proteobacteria suggesting a conserved function in flagellar gene regulation (Muir and Gober, 2004). Searching the DOE JGI database, we found that all the species that contained FliX orthologs also contained FlbD orthologs. Bacteria that contained FliX orthologs included *Acidiphilium cryptum*, *Bradyrhizobium* spp., *Magnetospirillum magneticum*, *Maricaulis maris*, *Methylobacterium* spp., *Nitrobacter* spp., *Parvibaculum lavamentivorans*, *Rhodopseudomonas palustris*, and *Rhodospirillum rubrum*. We predict potential σ^{54}-type promoters upstream of class III genes in all of these organisms

TABLE 8.3 Potential σ^{54}-dependent promoters upstream of putative class III genes

Group	Bacterium	Gene (Operon)[a]	Locus[b]	Potential promoter[c]	Distance[d]
α-Proteobacteria	*A. cryptum* JF-5	*flgE*	Acry_0110	TGGCACGCTTGCTGCA	94
	Bradyrhizobium japonicum USDA 110	*flgF* (+3)	Blr5838	TTGGCACGGCTTTCGCT	113
		flgI (+3)[e]	Blr5838	GTGGCACAGCACTCGCA	25
	M. maris MCS10	*flgF* (+3)	Mmar10_1946	TGGCACGCCGCGTGCA	65
		flgI (+3)[e]	Mmar10_1952	TGGCACGCCACTGGCA	35
		fliC (+2)	Mmar10_1961	TGGCACCGCGCTTGCT	73
		motA	Mmar10_2419	TGGCACCGCGCTTGCT	72
	M. magneticum AMB-1	*flgE*	Amb1389	TTGGCATCGACCTTGCA	43
		flgI (+4)[e]	Amb3824	TTGGCATGGGTTTCGCA	50
	Methylobacterium extorquens PA1	*flgI* (+3)[e]	Mext_3041	TGGCACGGCCCTGGCA	46
	Nitrobacter winogradskyi Nb-255	*flgI* (+3)[e]	Nwi_1111	TGGCACGGGTCTCGCT	69
		flgF (+3)	Nwi_1122	TGGCACGCATTTCGCT	109
	P. lavamentivorans DS-1	*flgI* (+3)[e]	Plav_2556	TGGCACGGCTTTCGCT	40
		flgF (+3)	Plav_2562	TGGCACAGCCCCTGCA	18
	R. palustris BisA53	*flgI* (+3)[e]	Rpe_1531	TGGCACGGCGCTGGCA	65
		flgF (+3)	Rpe_1538	TGGCACGGCTTTCGCT	110
	R. rubrum ATCC 11170	*flgB* (+4)	Rru_A2842	TGGCACGGGTCATGCA	59
		flgI (+4)[e]	Rru_A2849	TGGCACGACATTAGCA	92

(*continued*)

TABLE 8.3 (*continued*)

Group	Bacterium	Gene (Operon)[a]	Locus[b]	Potential promoter[c]	Distance[d]
β-Proteobacteria	*Nitrosomonas europaea* ATCC 19718	*flgB* (+11)	Ne0302	CTGGCACGATTCCTGCT	34
		fliK ([f])	Ne2088	GTGGCATGAGAATTGCT	43
δ-Proteobacteria	*Desulfovibrio vulgaris* vulgaris Hildenborough	*flgE*	Neu0307	CTGGCACGGCTCGTGCT	42
		MCP[g]	Neu0750	ATGGCACTGCTCTTGCT	184
		fliD (+1)	Neu0863	GTGGCATCTGGATTGCA	54
		fliC ([h])	Neu1441	TTGGGATCGTGTTTGCT	37
		MCP (+2)[g]	Neu1962	TCGGTATCATCTCCGCA	23
	G. sulfurreducens PCA[i]	*flgB* (+12)	Gsu0407	CTGGTACGGCTTTTGCT	35
		flgJ (+4)	Gsu3046	TTGGCACATAACATGCT	60
	Syntrophus aciditrophicus SB	*flgB* (+8)[j]	Syn_01467	GTGGTATGTGCTTTGCT	25
γ-Proteobacteria	*P. haloplanktis* TAC125[i]	*fliC* (+3)	PSHAa0781	TTGGCACAAAACCTGCT	77
		fliS (+1)	PSHAa0786	TTGGCATTATAATTGCT	46
		motY	PSHAa2115	TAGGCATGTAATTTGCT	98
	Shewanella baltica OS155	*cheW* (+18)	Sba1_2949	TTGGCACATGAATTGCT	34

(*continued*)

TABLE 8.3 (*continued*)

Group	Bacterium	Gene (Operon)[a]	Locus[b]	Potential promoter[c]	Distance[d]
	T. crunogena XCL-2[i]	MCP[g]	Tcr_0570	CTGGCATTGGGTTTGCT	41
		cheA (+1)	Tcr_0750	GTGGCACGGTCATTGCT	90
		MCP[g]	Tcr_0759	TTGGCACGGTCATTGCT	39
		fliD (+18)[k]	Tcr_1449	ATGGCATTTTGTATGCT	65
		flgI (+3)	Tcr_1467	ATGGCATACCTAATGCT	51
		flgB (+3)	Tcr_1474	TTGGCATTCGGATTGCT	88
		fliS	Tcr_2133	ATGGCATGCTGTCTGCT	108
	X. campestris pv. campestris ATCC 33913[i]	*flgB* (+4)	Xcc1952	CTGGCACAAACATTGCT	33

[a] The numbers in parentheses indicate the number of flagellar or chemotaxis genes that could form an operon with the given gene.
[b] Locus tag for genes with potential σ^{54}-dependent promoters.
[c] Sequence of the potential σ^{54}-dependent promoter with the conserved dinucleotides underlined.
[d] Distance in base pairs between the potential promoter and the predicted translational start of the gene.
[e] Divergently transcribed from fliX gene encoding a FlbD regulator (Section III.A).
[f] Divergently transcribed from fleSR genes encoding a two-component σ^{54}-dependent regulatory system (Section III.B).
[g] Gene encodes a methyl-accepting chemotaxis protein.
[h] Divergently transcribed from flaG gene encoding a flagellin.
[i] Organism is also predicted to have potential σ^{54}-dependent promoters upstream of class II genes (Table 8.2).
[j] Upstream genes encode a potential two-component σ^{54}-dependent regulatory system.
[k] Putative two-component σ^{54}-dependent regulatory system downstream in operon.

(Table 8.3). In *C. crescentus* and all of the organisms listed above *fliX* is divergently transcribed from *flgI* indicating a possible functional or evolutionary connection between FliX and FlgI.

Basal body assembly, FliX, and FlbD are all required for cell division in *C. crescentus*, (Mohr *et al.*, 1998; Muir and Gober, 2004; Yu and Shapiro, 1992). However, introduction of a variant FlbD or FliX that retain activity independent of basal body assembly restores normal cell division (Muir and Gober, 2001, 2004). Thus, FlbD activity ultimately appears to influence progression of the cell-cycle and is itself subject to cell-cycle control through a FliX-independent mechanism (Muir *et al.*, 2005; Wingrove *et al.*, 1993). FlbE was originally believed to be the cell-cycle dependent regulator of FlbD activity (Wingrove and Gober, 1996) but was later shown to be a structural component of the flagellum probably causing an indirect effect via FliX (Muir and Gober, 2001).

FlbD binds to flagellar transcription regulatory (*ftr*) sites located about 100 bp upstream of class III and IV genes to activate transcription (Benson *et al.*, 1994; Mullin *et al.*, 1994; Ramakrishnan and Newton, 1990; Wingrove *et al.*, 1993). An *ftr* site is also located upstream of the class II gene *fliF* and overlaps the CtrA-dependent promoter (Van Way *et al.*, 1993). FlbD binds this site and acts as a repressor of *fliF* (Benson *et al.*, 1994; Mullin *et al.*, 1994).

B. FleR/FlrC

The two-component systems FleS/FleR in *P. aeruginosa* and FlrB/FlrC in *V. cholorae* are required for transcriptional activation of σ^{54}–dependent class III genes(Dasgupta *et al.*, 2003; Klose and Mekalanos, 1998; Prouty *et al.*, 2001; Ritchings *et al.*, 1995). FleS and FlrB are the sensor kinases in these two-component systems, while FleR and FlrC are the response regulators and activators of σ^{54}-holoenzyme (Klose and Mekalanos, 1998; Ritchings *et al.*, 1995). Phosphorylation of FlrC by FlrB-phosphate is required for flagellar biosynthesis (Correa *et al.*, 2000), but the cellular signals that influence FlrB (or FleS) autophosphorylation have not been identified. Unlike other sensor kinases, FlrB and FleS are predicted to be soluble proteins rather than membrane-bound. In contrast to most activators of σ^{54}-holoenzyme, FlrC binds to sites downstream of its target promoters to activate transcription (Correa and Klose, 2005). FlrC recognizes sites located between 24–95 bp and 11–114 bp downstream of the transcriptional start but still in the noncoding portion of *flaA* (encodes a flagellin) and *flgK* (encodes hook-associated protein 1), respectively. When relocated to a position 295 bp upstream of either the *flaA* or *flgK* promoter, the FlrC-binding site from the *flaA* promoter-regulatory region allowed FlrC to activate transcription indicating that it functions as a bacterial enhancer (Correa and Klose, 2005).

C. FlgR

In the ϵ-Proteobacteria *Camphylobacter jejuni* and *H. pylori* σ^{54} and σ^{28} are used for transcription of flagellar genes similar to the *V. cholerae* and *P. aeruginosa* systems (Fig. 8.2). A master regulator in these ϵ-Proteobacteria has yet to be identified. In *H. pylori* at least three regulons have been defined based on the sigma factor required for their transcription (Niehus *et al.*, 2004). Genes encoding the components of the basal body require the primary sigma factor σ^{80} (Beier *et al.*, 1997; Porwollik *et al.*, 1999; Schmitz *et al.*, 1997); genes encoding the hook, hook-associated proteins and a minor flagellin require σ^{54} (Spohn and Scarlato, 1999; Suerbaum *et al.*, 1993); and genes encoding the filament cap and the major flagellin require σ^{28} (Colland *et al.*, 2001; Kim *et al.*, 1999; Leying *et al.*, 1992). A two-component system composed of the sensor kinase FlgS plus the response regulator and σ^{54}-dependent activator FlgR regulates expression of the RpoN regulon (Beier and Frank, 2000; Spohn and Scarlato, 1999).

H. pylori FlgR lacks the C-terminal DNA-binding domain that is characteristic of most σ^{54}-dependent activators. Instead of binding an UAS, FlgR contacts σ^{54}-holoenzyme directly from solution (Brahmachary *et al.*, 2004). *C. jejuni* FlgR contains a DNA-binding domain, but this domain is dispensable indicating that this FlgR activates transcription by a similar mechanism (Joslin and Hendrixson, 2008). Expression of FlgR in *C. jejuni* is also phase variable with addition or contraction of a nucleotide within any of several homopolymeric tracts resulting in a truncated, nonfunctional protein (Hendrixson, 2006). Like FleS and FlrB, FlgS is predicted to be soluble rather than membrane-bound, and the cellular signals that affect FlgS autophosphorylation are unknown. Disruptions in genes encoding components of the flagellar protein export apparatus in *H. pylori* or *C. jejuni*, however, inhibit expression of the RpoN regulon (Allan *et al.*, 2000; Hendrixson and DiRita, 2003; Niehus *et al.*, 2004; Schmitz *et al.*, 1997). In addition, loss of the hook-length control protein FliK in both of these bacteria stimulates expression of the RpoN regulon (Kamal *et al.*, 2007; Ryan *et al.*, 2005b). These results suggest that similar to *C. crescentus* FliX, the activity of FlgS is controlled by interactions with the flagellar protein export apparatus or basal body structure. Alternatively, the export apparatus may secrete an inhibitor of the RpoN regulon, similar to its role in the export of FlgM to alleviate inhibition of the FliA regulon. In either model, the flagellar protein export apparatus could serve a role in communicating the progression of flagellar assembly to the RpoN regulon.

Flagellar biogenesis in *H. pylori* may also be influenced by regulated proteolysis of σ^{54}. *H. pylori* HP0958, a previously uncharacterized protein, was found to protect σ^{54} from proteolysis and allow expression of the middle and late flagellar genes (Pereira and Hoover, 2005; Ryan *et al.*, 2005a).

HP0958 was originally identified as a protein that interacted with σ^{54} in a high throughput genetic screen that employed the yeast two-hybrid system (Rain *et al.*, 2001). The mechanism by which HP0958 protects σ^{54} from proteolysis is not known. One possibility is that HP0958 acts as a chaperone to assist σ^{54} in binding core RNA polymerase. HP0958 also interacts with the flagellar export apparatus component FliH in the yeast two-hybrid system (Rain *et al.*, 2001), although the significance of these interactions is not known. HP0958 possesses two CxxC motifs near its C-terminus that are predicted to form a zinc ribbon that is possibly involved in binding nucleic acids. Thus, HP0958 may have multiple functions in *H. pylori*. Orthologs of HP0958 are found in a wide variety of bacteria, not all of which are flagellated.

D. FleT

In the α-Proteobacteria *Rhodobacter sphaeroides* a four-tiered flagellar gene hierarchy is proposed similar to that in *V. cholerae* and *P. aeruginosa*. *R. sphaeroides* possesses a *fleQ* ortholog that encodes a master regulator, which controls expression of the *fleT* operon. The *fleT* operon encodes class II structural genes as well as FleT, which is a σ^{54}-dependent activator required for expression of class III genes (Poggio *et al.*, 2005). We address FleT separately here because of its unusual architectural features. FleT consists of only the AAA+ ATPase domain (Poggio *et al.*, 2005). *R. sphaeroides* FleQ is also unusual in that it lacks the N-terminal regulatory domain. FleT and FleQ appear to be constitutively active, suggesting that the activities of these proteins are regulated at the level of expression. Both FleQ and FleT are required for expression of class III genes, which encode components of the basal body, export apparatus, and the hook (Poggio *et al.*, 2005). FleQ binds to a site upstream of *fleT* to activate transcription. FleT inhibits FleQ activity at its own promoter while apparently enhancing FleQ activity at class III gene promoters. Sequences upstream of the *fliO* promoter are not required for transcriptional activation of this class III gene (Poggio *et al.*, 2005), suggesting that FleQ/FleT-mediated transcriptional activation does not require an UAS at target genes. Additional information on unconventional transcriptional activators such as FlgR and FleT can be found in a recent review (Beck *et al.*, 2007).

IV. FliA (σ^{28}) AND FlgM

In many of the systems discussed thus far, expression of the late flagellar genes requires the alternative sigma factor σ^{28}. The flagellar protein export apparatus plays an essential role in regulating σ^{28} activity.

In *S. typhimurium* the flagellar protein export apparatus is made up of the membrane spanning proteins FlhA, FlhB, FliO, FliP, FliQ, and FliR that form a specialized pore at the base of the basal body. In addition, the cytoplasmic proteins FliJ (a chaperone), FliI (an ATPase), and FliH (a regulator of FliI) function as part of the export apparatus (Minamino and Macnab, 1999, 2000b; Minamino *et al.*, 2000). Two of the membrane-bound components, FlhA and FlhB, have large cytoplasmic domains (Kutsukake *et al.*, 1994b; Minamino *et al.*, 1994). The cytoplasmic domain of FlhB is processed by an autocleavage mechanism (Ferris *et al.*, 2005; Minamino and Macnab, 2000a). Processing of FlhB coincides with completion of the mature hook which is signaled to FlhB by interactions with FliK, the hook length control protein (Hirano *et al.*, 1994; Minamino and Macnab, 2000a; Williams *et al.*, 1996). FlhB processing correlates with a switch in substrate specificity of the export apparatus (Fraser *et al.*, 2003; Minamino and Macnab, 2000a). Prior to the switch in substrate specificity the export apparatus secretes rod- and hook-type substrates. Following the switch, the export apparatus displays an increased affinity for filament-type substrates, which includes the filament cap protein and flagellins (Minamino *et al.*, 1999b). The regulatory protein FlgM is an anti-σ^{28} factor (Ohnishi *et al.*, 1992) and is secreted from the cytoplasm via the export apparatus as a filament-type substrate (Hughes *et al.*, 1993; Kutsukake, 1994; Minamino *et al.*, 1999a). Both *fliA* and *flgM* are early genes (Hughes *et al.*, 1993; Kutsukake *et al.*, 1990), but FlgM inhibits σ^{28} activity until the hook-basal body complex is complete and the late gene products are required (Karlinsey *et al.*, 2000). FliA binds to promoters upstream of genes encoding hook associated proteins, filament cap, flagellin, and motor proteins that have a TAAA-N_{15}-GCCGATAA consensus that corresponds to the -35 and -10 elements of σ^{70}-type promoters (Kutsukake *et al.*, 1990). Interestingly, FliA acts as a chaperone to facilitate the secretion of FlgM by the export apparatus (Aldridge *et al.*, 2006). FlgM secretion by the flagellar protein export apparatus has also been demonstrated in *V. cholerae* which produces a polar, sheathed flagellum (Correa *et al.*, 2004).

In the cytoplasm FlgM associates with both free σ^{28}, which prevents it from interacting with core RNA polymerase and also with σ^{28}-RNA polymerase holoenzyme (σ^{28}-holoenzyme), which destabilizes the holoenzyme complex (Chadsey and Hughes, 2001; Chadsey *et al.*, 1998; Ohnishi *et al.*, 1992). FlgM has limited secondary and tertiary structure. The N-terminus of the protein does not form a stable structure and the C-terminus forms nonrigid α-helices (Daughdrill *et al.*, 1998). The C-terminal region, however, forms a rigid helical structure upon binding of FlgM to σ^{28} (Daughdrill *et al.*, 1997). FlgM sequesters σ^{28} through interactions with conserved sigma regions 2.1, 3.1, 4.1, and 4.2(Chadsey and Hughes, 2001; Kutsukake *et al.*, 1994a), but only interacts with region

4 to destabilize σ^{28}-holoenzyme (Chadsey and Hughes, 2001). The crystal structure of *Aquifex aeolicus* σ^{28}/FlgM complex confirmed these observations and showed that FlgM binding causes extensive rearrangement of the conserved sigma domains so that they are tightly packed with FlgM wrapped around the outside (Sorenson *et al.*, 2004). The association of FlgM with σ^{28} in the cytoplasm stabilizes σ^{28} by protecting it from Lon protease (Barembruch and Hengge, 2007).

A. SigD and other systems

The flagellar transcription factor σ^{28} was originally characterized in *B. subtilis* where it is referred to as σ^{D} or SigD (Helmann *et al.*, 1988). SigD controls both late flagellar gene expression and genes required for separation of daughter cells after cell division (Kearns and Losick, 2005; Margot *et al.*, 1999). SigD promoters are upstream of several flagellar gene operons that are distant from the *fla/che* operon and encode the hook associated proteins, filament cap, and flagellin as well as FlgM (Serizawa *et al.*, 2004). FlgM functions with SigD in a similar manner as FliA in *S. typhimurium* (Caramori *et al.*, 1996; Fredrick and Helmann, 1996). A weak SigD-dependent promoter is located upstream of the *fla/che* operon (Estacio *et al.*, 1998), but transcription of this operon is mainly driven from the SwrA-dependent σ^{A} promoter as described previously (Section II.E). The *sigD* gene is the next to last gene in the *fla/che* operon, and the gene immediately downstream designated *swrB* encodes a SigD activator that is required for swarming motility (Estacio *et al.*, 1998; Kearns *et al.*, 2004; Werhane *et al.*, 2004).

Functional homologs of FlgM can be difficult to predict from genome sequences due to the lack of well conserved primary structure. Many FlgM orthologs can be found by searching for orthologous group COG2747 or PFAM group PF04316. Outside of *S. typhimurium,* functional FlgM homologs have been identified in *V. cholerae* (Correa *et al.*, 2004), *P. aeruginosa* (Frisk *et al.*, 2002), and *H. pylori* (Colland *et al.*, 2001). *C. jejuni* has a FlgM homolog, but this protein appears to play only a minor role in regulation of flagellin genes (Hendrixson and DiRita, 2003). Recently, three FlgM clades were defined by comparing a group of 77 FlgM sequences from diverse bacteria (Pons *et al.*, 2006).

When the DEO JGI database was searched FliA/SigD orthologs were found in 552 genomes representing 300 species of which 359 genomes representing 230 species were predicted to be flagellated. These species make up 76% of the species we predicted to be flagellated. Not all FliA/SigD orthologs are involved in flagellar biogenesis. For example, the alternative sigma factor WhiG from *Streptomyces* spp. is a FliA/SigD ortholog, but this protein is involved in sporulation and glycogen biosynthesis rather than flagellar biogenesis. One pattern that emerged from the

search for FliA/SigD orthologs was that the majority (83%) of species that lack FliA/SigD orthologs belong to the α-Proteobacteria group. Indeed, the only members of the α-Proteobacteria that contained FliA orthologs are *Erythrobacter* spp., *Loktanella vestfoldensis*, *Novosphingobium aromaticivorans*, *R. sphaeroides*, *Roseovarius* spp., *Sphingomonas* spp., *Sphingopyxis alaskensis*, and *Zymomonas mobilis*. With the exception of *N. aromaticivorans*, all of these bacteria also possess FlgM orthologs. All other predicted flagellated α-Proteobacteria presumably utilize σ^{54} for transcription of late flagellar genes as in *C. crescentus*. Temporal regulation of the late flagellar genes in *C. crescentus* is achieved through post-transcriptional regulation by FlbT which binds to the 5′ noncoding portion of the flagellin transcript and prevents its translation until completion of the hook-basal body complex (Anderson and Gober, 2000). Orthologs of FlbT are found in many α-Proteobacteria, including *R. sphaeroides* and *Rosovarius* spp. suggesting that temporal control of late flagellar genes in *R. sphaeroides* and *Roseovarius* spp. could be mediated by both FlgM and FlbT.

A second pattern that emerged is that a subset of *Bacillus* spp. lack SigD orthologs. These species include *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus weihenstephanensis* and are the same species that were found to have orthologs of the flagellar gene repressor MogR. In addition, disruption of the export apparatus component FlhA in *B. thuringiensis* did not inhibit expression of the flagellin gene as would be predicted if FlgM-SigD regulation was operating in this bacterium (Ghelardi *et al.*, 2002). These findings suggest that during the course of evolution these *Bacillus* species acquired a flagellar gene regulatory network that differs from that found in many other members of the genus.

Another interesting observation from the search for FliA/SigD orthologs is seen in the Spirochetes. *Borrelia* spp. lack FliA/SigD orthologs as well as σ^{28}-type promoter consensus sequences upstream of their flagellar genes. Rather, the flagellar genes of *Borrelia* spp. are predicted to have σ^{70}-type promoters (Ge *et al.*, 1997; Sal *et al.*, 2008). Two recent studies have shown that in *Borrelia burgdorferi* regulates expression of flagellins at a post-transcriptional level, and this regulation is dependent on formation of the hook-basal body complex (Motaleb *et al.*, 2004; Sal *et al.*, 2008). While other Spirochetes including *Leptospira* spp. and *Treponema* spp. have FliA/SigD orthologs, at least for *Treponema denticola* a similar post-transcriptional regulation appears to control expression of flagellins (Limberger *et al.*, 1999).

A final interesting twist on the search for FliA/SigD orthologs is found in *Buchnera aphidicola* which is the only predicted flagellated γ-Proteobacteria that lacks a FliA ortholog. This endosymbiotic bacterium is nonmotile due to the absence of the genes encoding the flagellin, but produces hundreds of basal body-hook structures that cover its cell surface (Maezawa *et al.*, 2006). These structures are predicted to function in

B. aphidicola symbiosis, and given the metabolic expense required to produce so many of these structures this prediction seems likely.

V. CONCLUSIONS

Tremendous diversity exists within the transcriptional regulatory networks that bacteria employ to coordinate flagellar gene expression with assembly of the flagellum. These regulatory systems are highly sophisticated and are often responsive to a variety of environmental signals and cellular cues. From a few paradigms, we have learned much about how bacteria are able to assemble complex cellular structures like the flagellum with precision and efficiency. Still, we have much to learn about the molecular mechanisms that control flagellar gene expression in these paradigms. Moreover, we are only beginning to understand the multiformity of regulatory systems that control flagellar biogenesis in other bacteria. The ready availability of proteomic and genomic experimental tools coupled with the wealth of accessible genomic sequence data will undoubtedly lead to new findings that will expand our appreciation of the variety of mechanisms used by bacteria to regulate transcription of flagellar gene hierarchies. As we begin to elucidate these mechanisms in more detail, it is likely that we will discover common threads that unite these diverse regulatory networks.

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CHAPTER 9

Genetic Tools to Study Gene Expression During Bacterial Pathogen Infection

Ansel Hsiao and **Jun Zhu**

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Abstract

The study of bacterial pathogenesis is in many ways the study of the regulatory mechanisms at work in the microbe during infection. The astonishing flexibility and adaptability of the bacterial cell has enabled many pathogenic species to freely transition between dramatically different environmental conditions. The transcriptional changes that underlie this ability can determine the success of the pathogen in the host. Many techniques have been devised to

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examine the transcriptional repertoire of bacteria *in vivo* during infection. Here, we review a class of technologies known as *in vivo* expression technology (IVET), which use promoter-trapping with a variety of different reporter constructs to allow researchers to probe the transcriptional changes taking place in bacteria under various environmental conditions. Using IVET techniques, researchers have been able to catalogue a wide variety of virulence factors in the host for several important human pathogens, as well as examining the timing of virulence gene regulation. Most recently, IVET techniques have also been used to identify transcriptional repression events *in vivo*, such as the suppression of anti-colonization factors deleterious to infection. As the array of IVET reporters and promoter-trapping strategies grow, researchers are increasingly able to illuminate the myriad transcriptional activities that allow bacteria to survive and cause disease in the host.

I. INTRODUCTION

Microorganisms, especially pathogens, which might exist in both environmental reservoirs and host populations, are rarely in a static environmental context. These bacteria are thus necessarily protean in nature; they must adapt, retooling their transcriptional and translational repertoires to suit the varied environments they encounter. This network of transcriptional, posttranscriptional, and posttranslational regulatory events affects a wide variety of proteins and bacterial structures, ranging from metabolic pathways necessary to shift between different nutrient sources, to cell wall and glycoprotein modification to withstand physical and chemical stress, to motility and cell–cell communication. In addition, toxins, host-specific adhesins, invasion factors, and others, loosely classified under the designation ''virulence genes,'' are also regulated in a context-specific manner.

Dissecting these pathways has proved to be fertile ground for researchers wishing to understand not only the inner workings of bacterial metabolism and regulation, but also how microorganisms establish themselves in hostile host environments and mediate disease. Traditional approaches, using forward genetics and saturation transposon or chemical mutagenesis, have been extremely useful in determining the order and requirements of transcriptional control in these bacterial environmental transitions under *in vitro* conditions. However, *in vivo*, such approaches can lead to a bottleneck in identifying important genes for survival in the host, as the survival and virulence potential of mutants often need to be validated individually against the wild-type strain. The inaccessibility of bacteria in their natural niche in the host is also a vexing issue that researchers must grapple with.

II. *IN VIVO* EXPRESSION TECHNOLOGY

Though bacterial transcription can be examined directly by isolation whole RNA (LaRocque *et al.*, 2005), the difficulties of isolating high quality bacterial RNA from infected host tissues, coupled with the cost of microarray or quantitative reverse-transcriptase PCR reactions, limit the applicability of direct measures of transcriptional activity *in vivo*. Several groups, including our own, have attempted to design genetic systems using reporter fusions as a surrogate for the transcriptional state of bacteria to probe the complex weave of regulation employed by pathogens within an infected host. The combination of a reporter system used in conjunction with a promoter trap to analyze changes in bacterial transcription during infection has been dubbed *in vivo* expression technology (IVET) (Fig. 9.1).

Ideally, such systems would conform to several criteria. Isolation of promoter–reporter fusions under differing growth regimes should involve some form of selection to cull any nonproductive or inactive

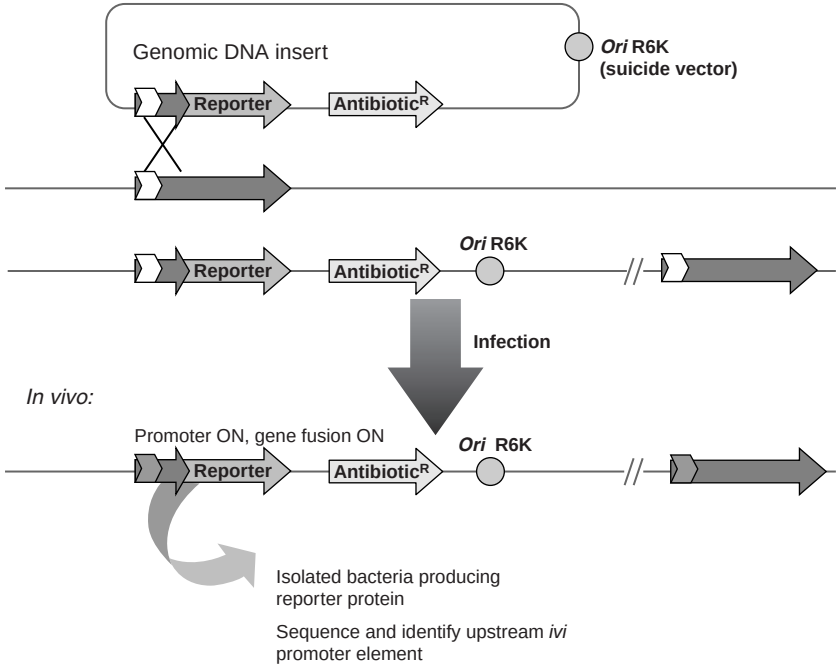

FIGURE 9.1 IVET promoter-trapping approaches. A promoter–reporter fusion library is generated as a merodiploid by single-crossover of a genomic DNA-reporter library to the chromosome. The original gene is maintained, and a single copy of the reporter fusion is introduced to the organism under study. Under varying environmental conditions, the activation of the promoter can be measured through the activity of the reporter protein.

fusions. This process should be convenient and rapid. The reporter construct used must also be expressed robustly, with an unambiguous phenotype conferred on bacterial cells by expression of the promoter–reporter fusion. This system must also be maintained under differing growth conditions, ideally without the need for supplemental growth or maturation factors. Plasmid borne-reporters are problematic *in vivo* in the host because of difficulties in maintaining selection, coupled with variability introduced by different copy numbers of reporter plasmids per cell.

Here we will review several case studies in using reporter fusion promoter-traps techniques to examine bacterial gene regulation during infection. As such tools are developed and refined, researchers in the field of bacterial pathogenesis are slowly lifting the veil over the complexities of gene regulation *in vivo*.

A. Selection by selection for complementation of auxotrophy in *Salmonella*

Reporter assays for gene expression *in vivo* are much more complicated due to problems with access to bacterial cells during infection, the stability of reporter proteins, and the difficulty of separating *in vivo* expression patterns from responses induced by *in vitro* expansion of host-passaged cells.

The Gram-negative bacteria of the genus *Salmonella* comprises several important human and animal pathogens that have long been studied as a model system for bacterial gene regulation and pathogenesis. *Salmonella* serotypes are the etiologic agents for several human diseases including bacteremia, typhoid fever, and enterocolitis; indeed, *S. typhimurium* is one of the most common causes of infectious diarrhea (Zhang *et al.*, 2003). To select for gene expression events during infection of *Salmonella*, Mahan and colleagues in the laboratory of John Mekalanos developed a promoter trap system using complementation of auxotrophs, which they dubbed IVET. The promoter trap consisted of a genomic DNA library of *S. typhimurium* placed upstream of a transcriptional fusion of the purine biosynthesis gene *purA* and the β-galactosidase reporter genes *lacZY* on an oriR6K plasmid. This plasmid library was then mobilized into a *purA* strain of *S. typhimurium*, which also lacked λ*pir* required for maintaining oriR6K plasmid replication, and single-crossover insertion mutants isolated. The resulting merodiploid bacteria contained the target chromosomal fragment upstream of a promoter-less *purA–lacZY*, and the cloned fragment upstream of the wild-type gene on the chromosome. Thus, potential promoter–reporter fusions were integrated while maintaining the original wild-type gene, and the reporter cassette was expressed in single-copy, obviating any problems with copy number and plasmid maintenance. The parental *purA S. typhimurium*, being deficient for purine biosynthesis, had previously been shown to be avirulent in mouse models of infection (McFarland and Stocker, 1987).

The library was plated on selective agar containing lactose indicator to test for Lac phenotype and also minimal media to examine whether *purA* expression was sufficient to complement the parental purine auxotrophy. The authors found that the levels of purine complementation roughly corresponded to the expression of *lacZY*; strongly Lac^+ bacteria were also Pur^+ on minimal medium, while Lac^- bacteria were Pur^- when grown on minimal medium. Only 33% of the selected library strains tested expressed enough *purA* and *lacZY* to complement purine biosynthesis and exhibit a Lac^+ phenotype *in vitro*.

This library was then injected intraperitoneally to BALB/c mice, and infection allowed to proceed for 3 days, after which bacteria were isolated from the spleens of infected animals. In theory, only those members of the library with functional promoter-*purA* fusions active *in vivo* would be able to complement the auxotrophy of the parent strain and multiply in the host. When the bacteria collected after infection were examined, the percentage of Lac^+ Pur^+ isolates had risen 86%, suggesting that infection had selected for transcriptionally active fusions between *S. typhimurium* genomic DNA and the *purA–lacZY* reporter cassette. Approximately 5% of these strains were $LacZ^-$ *in vitro*, and thus were chosen by the authors for further examination as containing potential *in vivo*-induced (*ivi*) genes. When used to infect mice, the bacteria isolated from the spleens of infected animals all showed an increase in β-galactosidase activity per CFU ranging from 40 to 1,000-fold over the activity exhibited by growth in rich media *in vitro*. In comparison, a strain carrying a transposon insertion from the initial library that was strongly Lac^+ in rich medium displayed no change between β-galactosidase/CFU activity *in vivo* and *in vitro*.

When several of the genes with the promoters identified in the screen, including *carAB* involved in arginine and pyrimidine biosynthesis and the *pheST–himA* operon coding for phenylalanyl-tRNA synthetase (*pheST*) and host integration factor (*himA*), were individually deleted and the resulting mutants tested for virulence in the mouse model of infection, they displayed between a 10^2 and 10^4-fold increase in LD_{50}, suggesting that expression of these genes during infection is important for *S. typhimurium* pathogenesis.

B. Antibiotic-based IVET selection for *ivi* genes

The initial IVET studies in *S. typhimurium* relied on the previously known dependency of this pathogen on *purA in vivo*. Since the metabolic requirements of other bacteria under different environmental pressures may not be known, Mahan and colleagues decided to expand their IVET design to replace *purA* with a chloramphenicol acetyl-transferase (*cat*) resistance gene, thus allowing for selection of active promoter-*cat-lacZY* fusions

in vivo or *in vitro* under different conditions through the addition of antibiotic (Mahan *et al.*, 1995).

Using an integrated library of genomic-DNA-*cat-lacZY* fusions generated as in the previous *purA* IVET, the authors were able to examine using growth on MacConkey-lactose agar the ratio of Lac^+ and Lac^- strains both *in vitro* and after passage through the mouse model of infection. For the *in vivo* assays, BALB/c mice were again infected intraperitoneally with the *S. typhimurium* promoter-*cat-lacZY* library. After infection, chloramphenicol was added both by twice-daily intraperitoneal injection as well as in the drinking water. After a period of incubation, the percentage of Lac^+ bacteria were then quantified from bacteria recovered in spleens by plating on MacConkey-lactose agar. The preinfection ratio of Lac^+/Lac^- bacteria was 21/71%, respectively, but after two rounds of infection of BALB/c mice, 95% of the recovered bacteria were Lac^+. Though growth outside of the animal host may introduce some altered expression patterns, this indicated that cells containing active promoter-*cat-lacZY* fusions were being selected for in the host due to administration of chloramphenicol. The 5% of Lac^- strains on MacConkey-lactose agar after mouse passage were likely to be expressed at levels sufficient to confer resistance to chloramphenicol *in vivo*, but not necessary for growth *in vitro*, and were selected for future study.

This method was then used to examine transcriptional changes during intracellular growth in macrophages, where *S. tymphimurium* are thought to multiply during infection (Fields *et al.*, 1986). For this assay, the bacterial library was incubated with cultures of RAW264.7 murine macrophages grown in culture. Gentamycin, an antibiotic that cannot penetrate the macrophage membrane, and chloramphenicol, which can, were then added to kill any extracellular bacteria and select for active promoter–reporter fusions. When several of the 5% of Lac^- mouse-passed strains were used in this assay, they exhibited a 500–1,000-fold increase in bacterial survival compared to isogenic Lac^- strains picked randomly from the initial library before passage in mice. These data demonstrated that antibiotic-reporter selection can be used as a reporter for IVET promoter trapping. This form of IVET can be performed without prior knowledge of the metabolic requirements of the microorganism to be examined, other than their sensitivities to a set of antibiotics.

C. Recombinase-mediated IVET and analysis of *Vibrio cholerae* gene regulation

While auxotrophy and antibiotic-based IVET have proved potent tools for studying gene regulation during infection in many pathogens, there remained a need for a general method that could be applied across many bacterial species, and be easily selectable outside of the host. A modified form of IVET analysis that met some of these goals was

developed for the study of *Vibrio cholerae*, a Gram-negative bacterium that is the causative agent of cholera, a devastating acute dehydrating diarrhea that affects millions of people in the world each year (Faruque *et al.*, 1998; Sack *et al.*, 2004). In between epidemics in human populations, *V. cholerae* are normally found in association with plankton in coastal and estuarine surface water (Chiavelli *et al.*, 2001; Huq *et al.*, 1983). Persistence in these aquatic reservoirs and survival in the context of human infection pose very different sets of challenges for *V. cholerae*. *Vibrio*s outside of the host must express factors that allow bacteria to attach to and use nutritive substrates, and survive temperature, osmolaric, and nutrient stress (Reidl and Klose, 2002). This necessary set of transcriptional shifts and its genetic tractability has caused *V. cholerae* to be intensely studied as a model for the regulation of pathogenesis.

Camilli and colleagues decided to use a modified IVET technique to probe the transcriptional changes occurring in this organism under varying growth conditions (Lee *et al.*, 1999). These studies took advantage of recombinase-mediated *in vivo* expression technology (RIVET), where in place of an antibiotic or metabolic gene reporter, a site-specific recombinase (resolvase) was used to excise cassettes from the chromosome of *V. cholerae*, generating irreversible markers of promoter activation (Fig. 9.2). The resolvase used was *tnpR* from the γδ transposable element, which resolves the transposition cointegrate intermediate of two repeated γδ elements formed by donor and recipient DNA each containing copies of the original transposable element (Reed and Grindley, 1981). Resolution occurs between two *res* sites within the cointegrate recognized by the resolvase.

As a proof of principle, Camilli and co-workers placed a promoter-less *tnpR* under the control of an inducible and well-characterized promoter, that of *irgA*, which is iron-sensitive, being highly induced at low levels of iron and repressed at high concentrations of iron (Goldberg *et al.*, 1990, 1991). To measure the activity of the resolvase, a cassette containing a tetracycline-resistance gene flanked by *res1* resolvase-target sites was inserted into the *V. cholerae* chromosome; activation of *irgA–tnpR* excises the antibiotic selection marker, and irreversibly generates Tc^S bacteria. As many as 99.5% of bacterial colonies were Tc^S after 11 generations of growth in low-iron, *irgA–tnpR*-inducing, conditions, indicating successful resolution of the *res*-flanked chromosomal cassette. Over 90% of bacteria had lost tetracycline resistance within 60 min of shifting growth to *irgA*-inducing conditions (Camilli *et al.*, 1994).

The sensitivity of RIVET can also be adjusted by several modifications for the specificity and activity of the resolvase used. In the study using *irgA–tnpR*, a high level of background transposition was observed during normal growth at the conditions initially used. Accordingly, the sequence of the *res* sites was modified to create *res1* sites, at which resolution occurred at ~10% of efficiency of that seen at wild-type *res* flanking sequences.

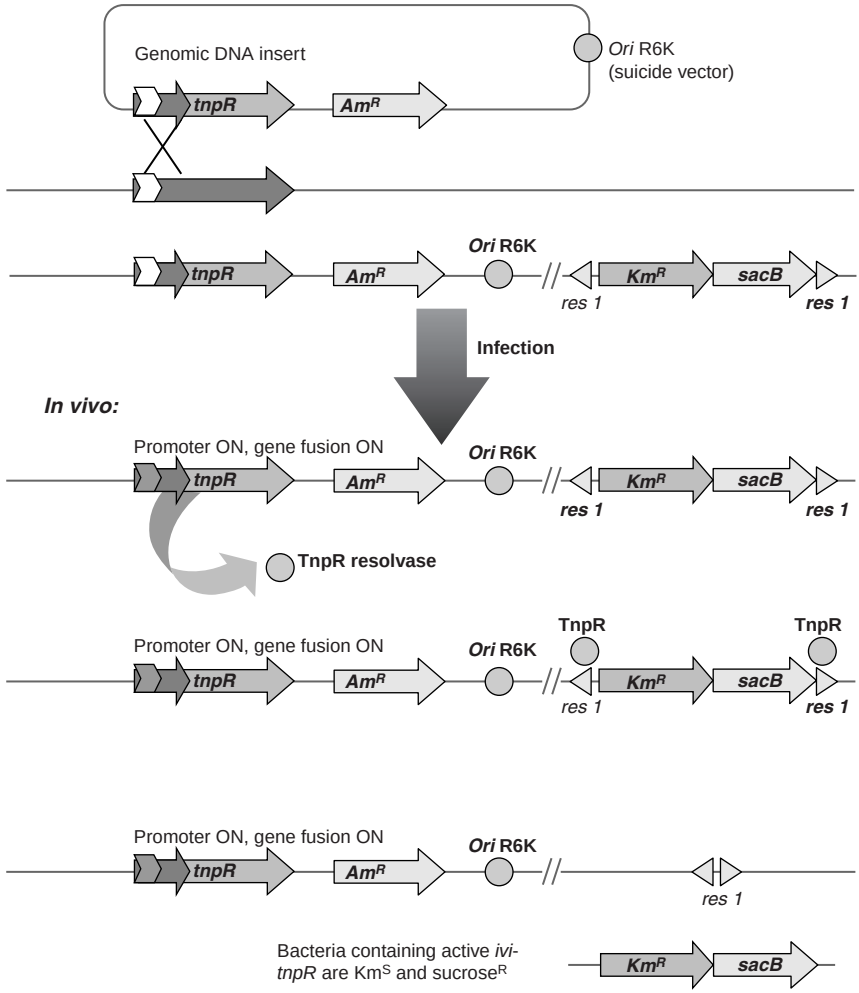

FIGURE 9.2 Recombinase-mediated IVET (RIVET). A merodiploid promoter–reporter library is generated through single-crossover of a genomic DNA-reporter fusion. Activation of promoters causes production of the resolvase enzyme. A cassette containing antibiotic resistance or sucrose sensitivity markers flanked by *res1* resolvase target sites is integrated into the chromosome of the parental bacterial strain. The resolvase excises this cassette, causing the cell to be irreversibly marked by the resulting sensitivity to antibiotics or ability to grow in sucrose.

This system was then adapted to examine the timing of virulence gene activation in *V. cholerae in vivo*. Virulence gene expression in this pathogen is tightly controlled by a series of transcriptional regulatory cascades. In the host intestine, the ToxRS and TcpPH regulatory protein complexes

respond to a set of ill-defined host environmental signals that result in the production of the AraC family transcription factor ToxT (Heath *et al.*, 1999; Lee *et al.*, 1999). Among the critical genes upregulated by ToxT at the end of this process are those encoding the cholera toxin (*ctxA, ctxB*) responsible for the diarrhea associated with cholera (Faruque *et al.*, 1998), and those genes of the toxin-coregulated pilus (TCP) operon, a Type IV pilus which is necessary for colonization of the small bowel (Herrington *et al.*, 1988; Shaw and Taylor, 1990). Using forward genetic transposon screens *in vitro*, many elegant studies had been used to characterize much of the regulatory cascade that culminates in the transcription of *toxT*, and ToxT- and ToxR-mediated activation of TCP biogenesis and CT genes (DiRita and Mekalanos, 1991; DiRita *et al.*, 1991; Hase and Mekalanos, 1998; Miller and Mekalanos, 1985; Miller *et al.*, 1987, 1989; Taylor *et al.*, 1987). However, little was known about the timing of this cascade in the host.

By using *tnpR* fused to a variety of virulence-associated promoters, and again excising a *res1*-flanked tetracycline resistance cassette, a profile of activation of these promoters *in vivo* can be assembled by assaying for the number of tetracycline sensitive cells over the course of an infection (Lee *et al.*, 1999). Using this method, the Camilli group determined that *tcpA* was expressed by almost all *V. cholerae* within 5 h of infection, and that, in common with *in vitro* studies, this activation was dependent on ToxT. Interestingly, these studies also revealed that the activation of key virulence genes *in vivo*, including *tcpA*, and *ctxAB*, do not mirror their activity in *in vitro* inducing conditions. For example, *tcpA* transcription could be activated in either *toxRS* or *tcpPH* mutant backgrounds, but not a double mutant background, whereas *in vitro*, *tcpA* activation requires the presence of both of these regulatory complexes. *ctxAB* activation *in vivo* was similarly at odds with *in vitro* data; during infection, these genes were only activated after *tcpA* (Lee *et al.*, 2001), and again was not dependent on *tcpPH*. These studies confirmed the importance of analyzing virulence in the natural setting of infection, rather than *in vitro*, and further highlighted the advantages in this regard provided by IVET techniques.

A second-generation RIVET screen has also been developed in the Camilli lab to probe for multiple gene activation events *in vivo* by *V. cholerae* (Osorio *et al.*, 2005). A genomic DNA library was cloned upstream of *tnpR* on an oriR6K suicide vector for introduction into *V. cholerae*. In developing this system, several modifications were made to address some of the key shortcomings of the original RIVET scheme. The rate of TnpR production, which can lead to high background levels of resolution outside the host, was modulated by modifying the ribosomal binding site to produce *tnpR* alleles of varying translational efficiency. This also allows selection for genes mildly induced outside the host, and different *tnpR* alleles can be selected to adjust the sensitivity of the

resulting screen. In addition, the resolvable cassette was modified to include a *neo* kanamycin-resistance gene and a counter-selectable *sacB* gene that causes inhibited growth in media containing sucrose (Reyrat *et al.*, 1998). The presence of *sacB* thus allows for a positive selection for resolution, rather than selection based on analysis of antibiotic sensitivity. A final improvement was the introduction of a screening step prior to preparation of the RIVET library; by treating the library with kanamycin before infection, any prematurely resolved promoters are thus removed.

Recently, RIVET in *V. cholerae* has also been expanded to screen for genes only induced late in infection (Schild *et al.*, 2007). Schild and colleagues employed a modified the *tnpR* RIVET system described above (Osorio *et al.*, 2005). The library of promoter-*tnpR* fusions generated previously (Osorio *et al.*, 2005) and described above was used again for these studies. Again, activation of *tnpR*, instead of conferring tetracycline sensitivity, confers sensitivity to kanamycin and resistance to sucrose. However, in these studies the library of promoters can be culled at any given point during infection by the oral administration of kanamycin to infected animals, which kills those cells that have already expressed *tnpR* and resolved the *neo-sacB* cassette. Interestingly, many of the genes found to have been induced in later stages of infection (>7 h post-infection) were involved in bacterial metabolism, indicating that *V. cholerae* initiates a transcriptional program to prepare for life outside the host. In addition to the upregulation of metabolic genes late in infection, it has been demonstrated that *Vibrio*s shed from human cholera patients are actually hyper-infectious (Merrell *et al.*, 2002). Transcriptome-wide analysis of these shed bacteria indicates that chemotaxis genes are downregulated, in concordance with findings from previous work that indicate that loss of chemotaxis actually improves *V. cholerae* fitness during infection, though the mechanism behind this phenotype is unclear (Lee *et al.*, 2001). In addition, biogenesis genes required for the environmental adhesin, MSHA pili (see below), were also upregulated, further suggesting that *V. cholerae* are transcriptionally committing to life outside of the host during exit.

III. DFI: *S. TYPHIMURIUM* GENES INDUCED BY LOW-pH AND INTRACELLULAR GROWTH CONDITIONS *IN VITRO* AND *IN VIVO*

The irreversibility of resolution is at the once a key advantage and limitation of RIVET in examining changes in bacterial gene activation. Background activity of resolvase, low levels of promoter activation outside of the environmental conditions to be used in the screen can

limit the cohort of genes identifiable by this method. In addition, once the promoter fused to *tnpR* has been activated, subsequent analysis of changes in the promoter's transcriptional state is not possible. Another limitation of RIVET is the need to examine the rate of resolution individually for each mutant, which involves potentially laborious serial dilution and measurement by antibiotic resistance.

Valdivia and Falkow developed an IVET scheme to examine the transcriptional activation profile of *S. typhimurium* within macrophages using the green fluorescent protein gene (*gfp*) as a reporter (Valdivia *et al.*, 1996). They named this method differential fluorescence induction (DFI), as the state of activation of a promoter-*gfp* reporter can be used to rapidly identify by fluorescence the activity of the reporter under different environmental conditions. GFP as a reporter has the advantage of being easily assayed by fluorimeter or flow cytometry measurements, but the ability of fluorescence activated cell sorting (FACS) flow cytometers to rapidly isolate GFP^+ cells also opens up the possibility of extremely high-throughput isolation of active promoter-*gfp* reporter fusions from culture or host tissues. As *S. typhimuirum* is known to be able to survive in the phagolysosome of host macrophages after uptake (Fields *et al.*, 1986; Prost and Miller, 2008), a compartment that is acidified after formation within the phagocyte (Rathman *et al.*, 1996), Valdivia and colleagues sought to first test their new IVET system to screen for acid-induced genes in this pathogen (Valdivia and Falkow, 1996).

As in previous IVET studies, a genomic DNA library was inserted upstream of a promoter, in this case, *gfpmut*, an FACS-optimized GFP variant (Cormack *et al.*, 1996). The plasmid libraries so generated were introduced into *S. typhimurium* and allowed to insert into chromosomal DNA, generating merodiploid strains with an intact target gene and promoters fused to *gfp*. These libraries were then subjected to acid shock by shifting growth pH to 4.5, and sorted using FACS to isolate the GFP^+ cells, as defined against the fluorescent properties of isogenic *S. tymphimurium* without *gfp*. To identify acid-induced promoters, this collection was then grown at pH 7, and then the lowest 10% of bacteria by fluorescence sorted out to identify those cells with promoter-*gfp* fusions inactive under normal pH but active at low pH. This collection of bacteria was finally subjected to a last round of growth at pH 4.5, and the top 10% of bacterial cells by fluorescence isolated by FACS sorting for future analysis. A number of these promoters were then sequenced out, and individually confirmed as being activated within RAW264.7 macrophage-like cells by fluorescence microscopy (Valdivia and Falkow, 1996).

These studies were then expanded by using FACS sorting to identify bacteria with active promoters driving *gfp* expression within macrophages themselves (Valdivia and Falkow, 1997). RAW264.7 cells were again used as hosts for infection with a *gfp S. typhimurium* library.

After a 6 h infection, infected macrophages were isolated using FACS sorting for GFP^+ cells, and the bacteria contained within them recovered by lysis of the phagocyte with mild detergent. These bacteria were then grown in the same medium used to culture macrophages for infection, and the lowest 15% of bacteria by fluorescence, i.e., those containing promoters mostly induced during intracellular growth in macrophages, were isolated and expanded by growth on selective agar. These bacteria were then used to infect a second population of RAW264.7 macrophages. In this infection, a much stronger fluorescence signal was detected within the infected macrophages, suggesting that the first round of sorting did in fact enrich for *S. typhimurium* containing promoters fused to *gfp* that were active during intracellular growth, i.e., macrophage induced genes (*mig*). As before, a selection of the promoters isolated by this method were analyzed independently, confirming their activation profile in macrophages using fluorescence microscopy. In addition, *mig–gfp* fusions were also shown to be active within the spleen of infected animals, suggesting that the phenotype seen in macrophage culture was representative of the expression profile seen during actual infection.

IV. REPRESSION OF AN ANTI-COLONIZATION FACTOR IN *V. CHOLERAE*

In addition to the known transcriptional cascades that sense the presence of the host and activate the expression of virulence gene hierarchies in *V. cholerae*, our laboratory reasoned that this microorganism may also repress a set of genes in order to efficiently transition from aquatic reservoir to host and back again. While previous IVET studies have identified a large contingent of metabolic genes activated during infection to deal with differences in nutrient availability, a set of genes may encode for products superfluous, or even actively deleterious, in the face of pressures in the environment of the mammalian host. Not least of the challenges facing this pathogen are the effects of the host immune system, which employs many factors to exclude pathogens from the epithelium, especially secreted immunoglobulins and a thick glycocalyx of mucins (Jenny M Woof, 2006).

Our laboratory set out to examine the set of genes that might be transcriptionally repressed during infection, which had the potential to provide new insights into the biology of *V. cholerae* survival, virulence, and dissemination within the host. However, this goal came with the limitations of identifying negative events in the complex milieu of the host intestine, i.e., the cohort of *in vivo* repressed genes (*ivr*).

To address issues of speed of isolation of bacterial samples, as well as the need for a positively identifiable marker for gene repression, we

devised a modified DFI promoter-trapping approach, which we have dubbed differential fluorescence repression (DFR). Our promoter trap consisted of a promoterless *tetR* gene and a *neo* kanamycin-resistance cassette on a transposable element flanked by inverted repeats recognized by a Mariner family transposase (Fig. 9.3). TetR is the repressor protein that negatively regulates the tetracycline resistance system of Tn10 in the absence of tetracycline (Hillen and Berens, 1994; Ramos *et al.*, 2005).

In the presence of tetracycline, TetR is released from then its operator sites in the P_{tet} promoter, allowing for the expression of TetA. For our

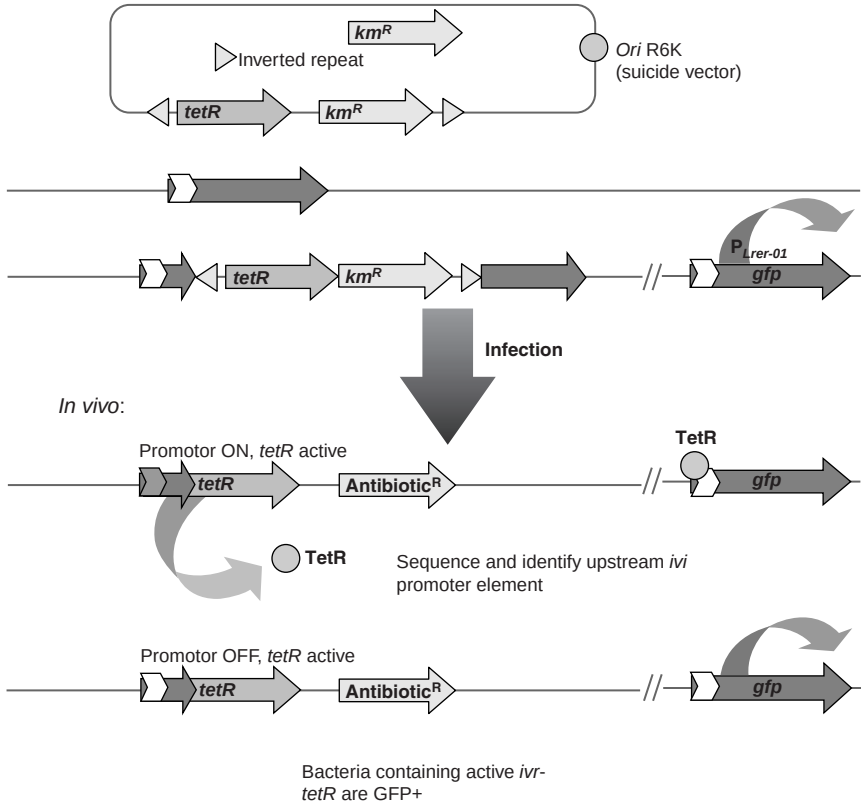

FIGURE 9.3 The differential fluorescence repression technique for examining *in vivo* repressed promoters. A library of promoter-*tetR* fusions is generated by transposon mutagenesis with a promoter-less *tetR* at the upstream end of the transposable element. A $P_{L\text{-}tet\text{-}O1}$-*gfp* reporter is integrated into the chromosome of the parental bacterial strain. In the presence of an active promoter-*tetR* fusion, the TetR repressor tightly inhibits production of GFP. However, repression of the library promoter de-represses *gfp*, allowing for rapid selection of GFP+ bacteria containing promoters repressed under the tested environmental conditions.

reporter, we used a $P_{Ltet\text{-}O1}$-*gfp* construct integrated within the *lacZ* locus of *V. cholerae*. The novel $P_{LtetO\text{-}1}$ promoter consists of the P_L lambda phage promoter with the CI repressor sites replaced by the *tetR* operator sites of P_{tet}. This promoter is strongly active in the bacterial cell in the absence of the TetR repressor protein, as measured by a $P_{LtetO\text{-}1}$-luciferase fusion; in the presence of the inducer anhydrotetracycline, an up to 5,000-fold induction of this construct was observed when placed on a low-copy number pSC101 backbone plasmid vector (Lutz and Bujard, 1997). Thus, activity of any promoter-*tetR* fusions corresponds to the tight repression of $P_{Ltet\text{-}O1}$-*gfp* and bacterial cell fluorescence, which could be tracked *in vitro* by either fluorescence microscopy, fluorimeter measurements or flow cytometry.

We confirmed using flow cytometry that bacteria with $P_{Ltet\text{-}O1}$-*gfp* but lacking TetR expressed were approximately 100-fold brighter for GFP than isogenic *V. cholerae* without the reporter. However, no GFP+ cells were seen when *tetR* was constitutively expressed in this reporter strain (Hsiao *et al.*, unpublished). This system thus allows for positive identification of repressed promoters by coupling promoter repression to repression of *tetR*, and subsequent de-repression of the $P_{Ltet\text{-}O1}$-*gfp* reporter.

We chose to generate our promoter trap library using a Mariner/Himar1 family transposase, which have been used extensively in previous mutagenesis studies in a variety of bacteria (Akerley *et al.*, 1998; Hendrixson *et al.*, 2001; Lampe *et al.*, 1999; Liberati *et al.*, 2006; Liu *et al.*, 2007; Rubin *et al.*, 1999; Wong and Akerley, 2008; Wu *et al.*, 2006). This family of transposases has the advantage of not requiring cellular co-factors for transposition, and will randomly insert the cognate transposable element without significant site specificity, though it does prefer TA sequences (Lampe *et al.*, 1996). We thus generated a library of *tetR* insertions by introducing via conjugation to *V. cholerae* of an oriR6K suicide shuttle vector containing the Mariner transposase and a promoter-less *tetR* at the upstream end of the transposable element. The effects of gene disruptions in this system are mitigated by the fact that we are specifically targeting repressed genes during infection; thus, the potential for dysregulation of normal bacterial functions in the host is minimized. However, this system can readily be adapted to generate a merodiploid library through ligation of a genomic-DNA library upstream of a promoter-less *tetR* and insertion via recombination to a suicide shuttle vector in a manner similar to that used in other RIVET studies in *V. cholerae* (see above).

Since a fluorescent reporter was used, this system allowed for the rapid isolation of cells containing different states of *tetR*, and thus $P_{Ltet\text{-}O1}$-*gfp*, activity by FACS. Since a transposon-based approach to library generation may produce many nonproductive *tetR* insertions, we initially isolated any cells containing functional genomic DNA-*tetR* fusions *in vitro* by two rounds of FACS for GFP^- bacteria.

We inoculated infant mice with the library we generated and culled, and allowed infection to proceed for 18 h. At the conclusion of this time, mice were sacrificed, and bacteria isolated from by homogenization of intestinal tissues, and the final suspension was strained to remove any remaining large tissues to prepare the sample for FACS. The sorted population of GFP^+ cells derived from infected intestines was then plated on selective agar, and arbitrary PCR used to identify the locations of *tetR* transposon insertion. Using this modified promoter-trap strategy, we demonstrated that *V. cholerae* expresses the genes encoding for the biogenesis of a Type IV mannose-sensitive hemagglutinin (MSHA) during growth *in vitro*, but not during early colonization of the infant mouse model (Hsiao *et al.*, 2006). Expression of a P_{msh}-*tetR* fusion in the $P_{Ltet\text{-}O1}$-*gfp* reporter strain during infection led to the appearance of a large population of GFP^+ cells in homogenized infected intestinal tissues. Conversely, no GFP^+ bacteria were observed from tissues of mice infected with a $P_{Ltet\text{-}O1}$-*gfp V. cholerae* expressing a constitutive P_{lac}-*tetR* fusion (Hsiao *et al.*, 2006).

This scheme retains room for improvement and refinement. Depending on the optics available for the flow cytometer to be used, a second, constitutive fluorophore can be used to isolate live bacteria from bacteria-sized debris. Autofluorescence in the GFP channel has also proved to be an issue when used with flow cytometry with host cells (Thöne *et al.*, 2007), which may be mitigated in the future using red, orange, or yellow fluorescent protein variants (Shaner *et al.*, 2004). Furthermore, GFP^+ bacteria isolated from intestinal tissues are approximately one log less bright than the same bacteria passage in broth culture (Hsiao *et al..*, unpublished data). The fluorescent signal *in vivo* may in the future be improved using recently reported anaerobically active fluorophores (Drepper *et al.*, 2007).

V. CONCLUSION AND FUTURE PROSPECTS

Ever since the start of molecular techniques to examine how bacteria transcriptionally regulate how they respond in the many environments they are faced with, there has existed the possibility for studying how microbes respond in the way that arguably influences human populations most directly: pathogenesis. The development of IVET technologies, which can directly probe the transcriptional regulation underpinning bacterial functions during infections, has dramatically expanded our understanding of how microbes adapt in, subvert, and modulate their hosts. As the array of available reporter technologies expand, researchers are able to sample bacterial transcriptional regulons under many more different conditions. These improvements, coupled to the advent of

high-throughput techniques that have reduced the difficulty in processing and identifying bacterial populations from infected tissues and animals, promise to open a larger window into how the microbial population manages its interactions with the host population in the cat-and-mouse game of infection.

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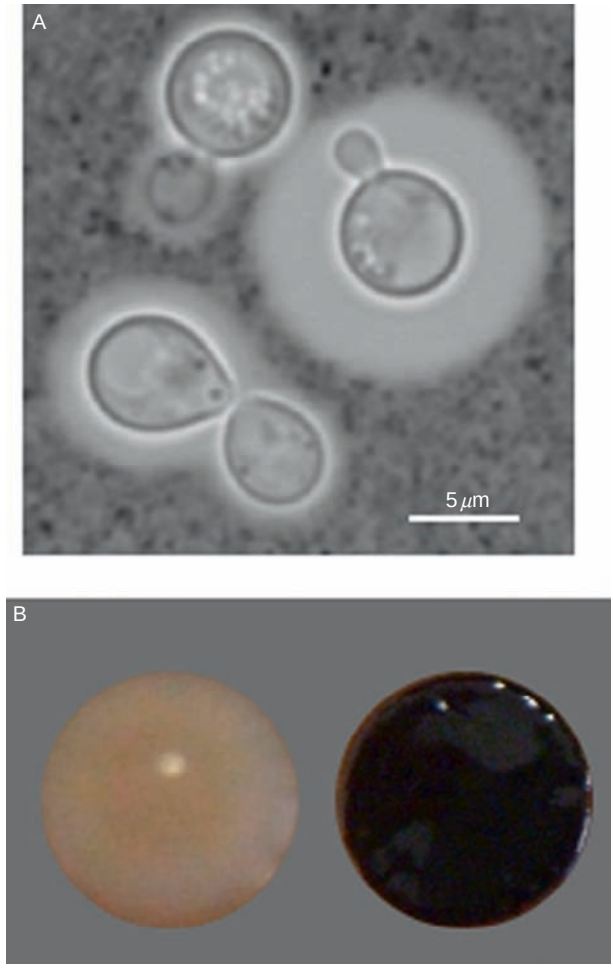

FIGURE 5.1 (A) India ink staining reveals the capsule (of various sizes) around budding *C. neoformans* cells; (B) Melanin and nonmelanin forming colonies of *C. gattii* serotype B on L-DOPA medium after 7 days at 25 °C.

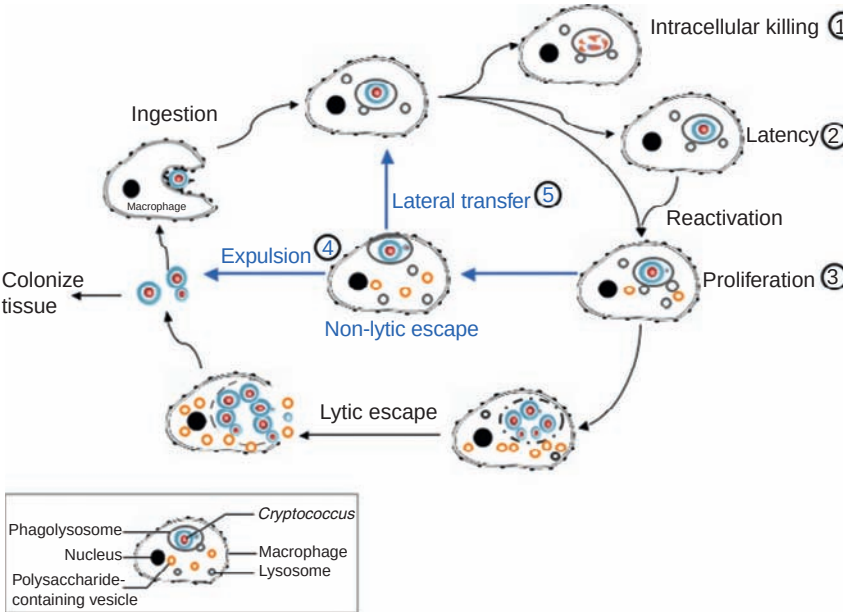

FIGURE 5.3 Macrophage parasitism by *C. neoformans*. Following phagocytosis, the internalized cryptococci can be killed by macrophages (1) or remain latent (2). When the host becomes immunocompromised, some of the cryptococci or latent population can reactivate and proliferate intracellularly (3), followed by the lytic burst of the host cells and release of the intracellular yeast cells into the extracellular environment. The released yeast cells can then carry on infecting more macrophages or establish extracellular dominance. Nonlytic escape pathways were also observed for *C. neoformans*, during which the yeast cells are expelled by macrophages without causing death of either party (4) or the intracellular yeast cells are directly delivered to a neighboring macrophage via lateral transfer (5).

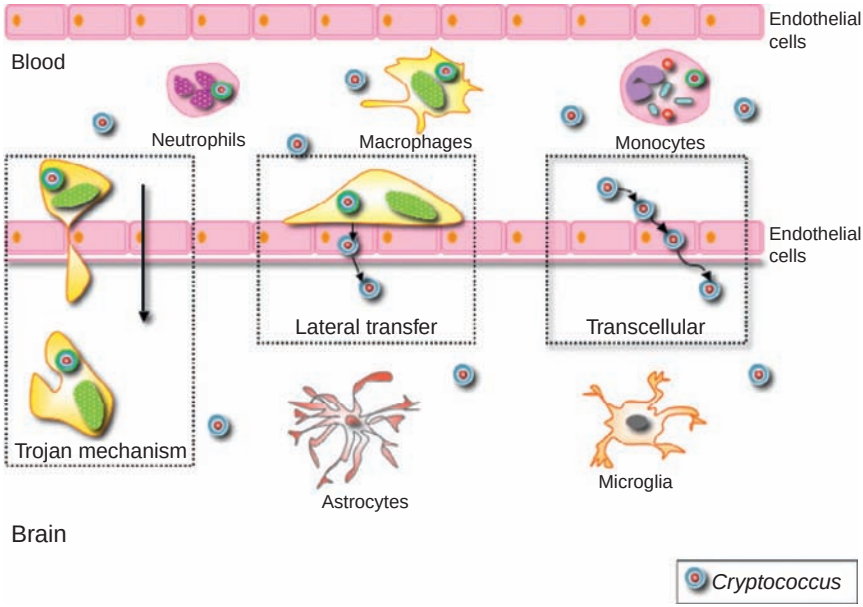

FIGURE 5.5 Possible routes for cryptococci to cross the blood–brain barrier: (1) Trojan horse mechanism; (2) Lateral transfer; and (3) Transcellular crossing.