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# Insights into mechanisms of *Pseudomonas aeruginosa* virulence: Cyanide as a weapon and the complexity of its regulation.

by

Larry Alan Gallagher

A dissertation submitted in partial fulfillment of the requirements for the degree of

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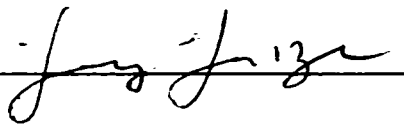
Signature 

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Abstract

Insights into mechanisms of *Pseudomonas aeruginosa* virulence:
Cyanide as a weapon and the complexity of its regulation.

by Larry Alan Gallagher

Chairperson of the Supervisory Committee

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This dissertation presents investigations into virulence mechanisms and virulence-gene regulatory circuitry in the bacterium *Pseudomonas aeruginosa*. *P. aeruginosa* is a medically important human pathogen that causes serious disease in the lungs of cystic fibrosis patients. It also is virulent towards fungi, nematodes, plants and insects. To investigate virulence mechanisms employed by the bacterium, a model pathogenesis system between *P. aeruginosa* PAO1 and *Caenorhabditis elegans* was investigated. PAO1 rapidly paralyzes and kills *C. elegans* by producing a diffusible poison or toxin that requires an intact copy of the *C. elegans* gene *egl-9*. Chapter 2 presents experiments that identify hydrogen cyanide as the sole or primary poison responsible for this killing. A genetic screen for killing-defective mutants, an analysis of the genes identified, and a reconstruction of the killing phenomenon showed that hydrogen cyanide is both necessary and sufficient to effect paralytic killing in an *egl-9*-dependent manner. Many virulence determinants in *P. aeruginosa* are controlled by a complex hierarchy of interacting regulatory factors. Chapter 3 presents a screen for mutants of PAO1 with defects in pyocyanin production. The screen resulted in the identification of four genes responsible for production of a small-molecule regulatory factor, the *Pseudomonas* quinolone signal (PQS). Transcription of quorum-sensing-controlled genes on the chromosome was also examined in mutant strains affected in pyocyanin production. The results revealed new hierarchical interactions between different components of the regulatory circuitry. Chapter 4 presents three additional findings: construction and analysis of a transposon tool (IS*phoA*/hah-Tc) for use in *P. aeruginosa*, analysis of transcription at the hydrogen cyanide biosynthetic locus (*hcnABC*) in mutant strains that don't produce cyanide, and evidence that *egl-9* worms resist cyanide poisoning by enhancing mitochondrial respiration and glycolysis. In conclusion, these studies have elucidated both sides of a virulence/resistance interaction between *P. aeruginosa* and *C. elegans*, and have uncovered new genes and genetic interactions in the virulence-gene regulatory circuitry in *P. aeruginosa*.

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To my father, Alan Charles Gallagher,

and to my mother, Jean Weil Gallagher,

whose brighness and heart were my stronghold,
whose joy is my prayer.

---

Know thou that, according to what thy Lord, the Lord of all men, hath decreed in His Book, the favors vouchsafed by Him unto mankind have been, and will ever remain, limitless in their range. First and foremost among these favors, which the Almighty hath conferred upon man, is the gift of understanding. His purpose in conferring such a gift is none other except to enable His creature to know and recognize the one true God – exalted be His glory. This gift giveth man the power to discern the truth in all things, leadeth him to that which is right, and helpeth him to discover the secrets of creation.

—Bahá'u'lláh

# CHAPTER 1

## Introduction

Bacterial pathogens cause disease using complex, diversified and highly specific virulence mechanisms, the outcomes of co-evolution between pathogen and host. These mechanisms include functions for adherence, functions for internalization and survival within host cells, motility mechanisms, functions for evasion or inhibition of host immunity, the production of scavenging molecules for the acquisition of iron and other scarce metabolites, the targeted secretion of toxic proteins into host cells, and the production of numerous toxic proteins and small-molecule poisons. A hope of modern research is that increased understanding of these mechanisms will lead to better treatments of microbial diseases.

Interestingly, different bacterial pathogens often employ very similar virulence mechanisms against widely divergent hosts or competitors, in part reflective of horizontal gene transfer during evolution (reviewed in Finlay and Falkow, 1997). Although this conservation of virulence mechanisms is not universal, it allows researchers to study bacterial pathogenesis using simple model hosts that are easily manipulated in the

laboratory, and the virulence mechanisms uncovered can often apply to mammalian pathogenesis.

*Pseudomonas aeruginosa* is a medically important pathogen ideally suited for study using model hosts. It causes disease in a wide diversity of species, suggesting that broadly applicable virulence mechanisms are employed. In addition, its broad host range presents a number of organisms that can be exploited as model hosts. Indeed, several virulence determinants important in mammalian pathogenesis by *P. aeruginosa* have been identified and characterized using model hosts such as plants and nematodes. The ease with which *P. aeruginosa* can be studied has also been enhanced by the recent release and annotation of its complete genome sequence (Stover et al., 2000).

My graduate study has focused on a virulence model between *P. aeruginosa* PAO1 (a commonly-used lab strain) and the well-studied nematode *Caenorhabditis elegans*. I have employed genetic approaches to pinpoint a primary weapon that the bacterium uses to kill the nematode. I have also investigated features of the complex regulatory network controlling virulence factor production in *P. aeruginosa*. To introduce the experimental results, this chapter will review *P. aeruginosa* biology, describe some of the bacterium's key virulence determinants, outline the regulatory circuitry with which it controls virulence gene expression, and present background on the nematode model system I have investigated.

**The bacterium *Pseudomonas aeruginosa*.**

*P. aeruginosa* is a Gram-negative bacterium that lives ubiquitously in soil and water. It is known as an opportunistic pathogen because it is clinically dangerous only to individuals who have impaired immunity. Burn wounds and eye lesions are susceptible to serious infections by the bacterium, and HIV-infected individuals and cancer patients undergoing immunosuppressive therapy can contract systemic *P. aeruginosa* infections (Holder, 1993; Lyczak et al., 2000).

*P. aeruginosa* is best known for its role as the primary agent of pulmonary morbidity in cystic fibrosis disease progression. 80-90% of individuals with cystic fibrosis acquire chronic *P. aeruginosa* lung infections that lead to lung tissue breakdown and eventually death (Pier, 1998). These infections are refractory to treatment, in part because the bacterium is naturally resistant to many antibiotics. Drugs that are effective eventually lose their efficacy due to outgrowth of drug-resistant variant strains in individual patients. It is not known why *P. aeruginosa* persists in the cystic fibrosis lung while other bacteria don't. *P. aeruginosa*'s unique makeup of virulence capabilities (see below) undoubtedly facilitates its long-term advantage in the lung, despite the fact that the evolution of the bacterium cannot have taken place in that milieu (before antibiotics, cystic fibrosis patients did not survive early childhood).

In addition to its clinical importance, *P. aeruginosa* has been shown to be virulent towards a wide range of non-mammalian organisms, including bacteria, fungi, plants,

nematodes and insects (Bucher and Stephens, 1957; D'Argenio et al., 2001; Darby et al., 1999; Jarrell and Kropinski, 1982; Plotnikova et al., 2000). It has few nutritional requirements, and can grow on a range of unusual carbon sources. Related species in its genus perform important agricultural biocontrol functions in the rhizosphere, and others find application in biodegredation of toxins and spills.

## *P. aeruginosa* virulence determinants.

The pathogenic versatility of *P. aeruginosa* is reflected in a large arsenal of secreted and surface-associated virulence factors. To convey the diversity of *P. aeruginosa*'s virulence arsenal and to introduce specific virulence factors that will be referred to in later chapters, this section describes in more detail a number of *P. aeruginosa* virulence determinants. The factors described comprise four major classes: adhesins, secreted polysaccharides, protein toxins and small-molecule poisons.

Adhesins are surface-associated structures used by many bacteria to promote attachment to eukaryotic cells (for review see Soto and Hultgren, 1999). A primary *P. aeruginosa* adhesin is the type-4 pilus, which binds specific eukaryotic glycosphingolipids and probably other surface receptors as well (Hahn, 1997). Type-4 pili's importance in pathogenesis is evidenced in mouse models of infection: the $LD_{50}$ for *P. aeruginosa* infecting a susceptible strain of mouse (AB.Y/SnJ) was tenfold higher for

a mutant lacking pili than for its isogenic parent (Hahn, 1997). In addition to pili, other *P. aeruinosa* extracellular factors may have adhesive properties (Arora et al., 2000). The PAO1 genome contains two homologues of the *Bordetella pertussis* filamentous hemagglutinin gene *fhaB*, which in *Bordetella* encodes a large, secreted molecule effecting adherence to epithelial cells (Locht et al., 1993). The *fhaB* homologues in *P. aeruginosa* have not been examined experimentally. Interestingly, we isolated a mutation in one of them in a search for mutants affecting paralytic killing of nematodes (Chapter 2).

The best studied of *P. aeruginosa*'s secreted polysaccharides is alginate, which forms a thick mucous layer encasing the bacteria. Alginate is critical in cystic fibrosis disease progression (for review see Govan and Deretic, 1996). Environmental isolates of *P. aeruginosa* typically do not produce alginate, but once established in the cystic fibrosis lung, the bacterium undergoes a selective adaptation resulting in copious alginate secretion (the "mucoid" phenotype). It is believed that the alginate protects the bacterium against antibiotic attack, opsonization and phagocytic engulfment (Govan and Deretic, 1996). The conversion of infecting strains to the mucoid phenotype correlates with poor disease prognosis for infected patients. Under some growth conditions, polysaccharides secreted from bacteria (including *P. aeruginosa*) form specifically structured matrices in which the bacteria reside. This growth state has been termed biofilm growth (Costerton et al., 1995), and it appears that for *P. aeruginosa,* specific regulatory states are associated with biofilm growth (Singh et al., 2000b). Establishment of biofilms, of which

alginate may form a part, may therefore bring about growth styles and virulence factor production profiles well suited to long-term entrenchment of the infecting bacteria.

The protein toxins produced by *P. aeruginosa* are numerous and diverse. Among the better-studied examples are ADP-ribosylating enzymes, phospholipases and proteases. Exotoxin A and exoenzyme S are ADP-ribosylases which target and inactivate specific proteins in eukaryotic cells. Exotoxin A enters the host cell by endocytosis and kills by the same mechanism as diptheria toxin: it inactivates elongation factor-2 by ADP-ribosylation, thereby stopping cellular protein synthesis (Yates and Merrill, 2001). Exoenzyme S, on the other hand, is delivered directly from the bacterial cytoplasm into the host cell cytoplasm by a sophisticated secretion system (the type-III system) which is activated by bacteria-to-host cellular contact and uses a needle-like apparatus for direct injection (Yahr et al., 1996). Exoenzyme S targets G-proteins, by which it is believed to alter host signal transduction pathways (Frithz-Lindsten et al., 1997). Another protein toxin is phospholipase C, a secreted hemolysin demonstrated to be important for full virulence in infections of both burned mice and *Arabidopsis* plants (Rahme et al., 1995). Several proteases are also elaborated. One is elastase (encoded by the *lasB* gene), which, together with another protease (LasA), proteolyzes elastin, a primary component of lung tissue and blood vessel walls (Galloway, 1991). Another secreted protease is alkaline protease (encoded by *apr*), which has several substrates and may also act with elastase to enhance elastase activity (Galloway, 1991).

A multitude of secreted small-molecule factors constitute a final class of *P. aeruginosa* virulence determinants. Many of these molecules have been classified as secondary metabolites, compounds which are not essential for growth, but which offer ecological or competitive advantages to the bacterium (Vining, 1990). While the *Pseudomonas* secondary metabolites are numerous (the volatile products alone are estimated at over fifty) (Budzikiewicz, 1993; Edwards et al., 1987), three figure prominently in the research described in this dissertation: hydrogen cyanide, pyocyanin and pyoverdine.

Hydrogen cyanide is synthesized in *P. aeruginosa* by the oxidative decarboxylation of glycine by a membrane-bound enzyme complex encoded by the *hcnABC* locus (Blumer and Haas, 2000). Hydrogen cyanide, which inhibits the key respiratory enzyme cytochrome oxidase (and many other metalloenzymes) (Way, 1984), is almost universally poisonous, making it an effective virulence determinant against numerous hosts. *P. aeruginosa* itself is believed to resist the poisonous effects of the cyanide it produces by expressing a cyanide-insensitive cytochrome oxidase (Cunningham et al., 1997). Hydrogen cyanide is also freely diffusible and volatile, with a pK of 9.3 and a boiling point of 26°C.

Pyocyanin is the pigment molecule conferring *P. aeruginosa*'s characteristic blue color. Its virulent effects are ascribed to its ability to undergo redox cycling, producing poisonous reactive oxygen species (Hassan and Fridovich, 1980). Pyocyanin has been shown to inhibit growth of bacteria, fungi and cultured eukaryotic cells (Hassett et al., 1992; Kerr et al., 1999), and has been suggested as an inhibitor of lung ciliary function

(Wilson et al., 1987). Pyocyanin belongs to the class of compounds known as phenazines, which are derivatives of the basic phenazine nucleus, phenazine-1-carboxylate (PCA) (Figure 1). In many phenazine-producing Pseudomonads, PCA is synthesized by the gene products of a highly conserved, seven-gene core biosynthetic locus, the *phzABCDEFG* operon (Delaney et al., 2001; Mavrodi et al., 2001). Additional gene products catalyze the conversion of PCA to other phenazines (Mavrodi et al., 2001). Interestingly, *P. aeruginosa* PAO1 contains two unlinked copies of the seven-gene core operon, designated *phzA1–G1* and *phzA2–G2* (Mavrodi et al., 2001). The two operons are very differently regulated (see below), and although both are capable of supplying PCA in *E. coli* expression systems (Mavrodi et al., 2001), chromosomal mutations in only one of them, the *phzA1-G1* operon, virtually eliminate pyocyanin production under standard laboratory growth conditions (Chapter 3). The conditions under which the

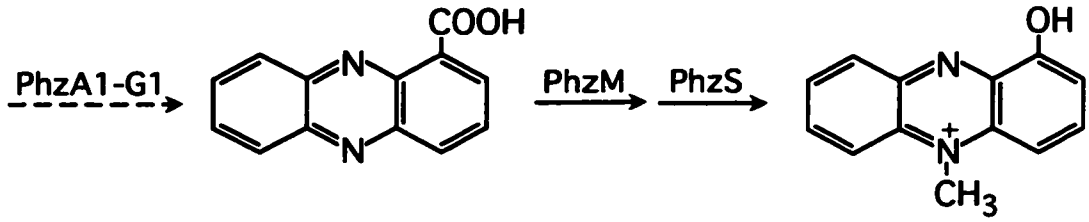


**Figure 1: Phenazine-1-carboxylate (PCA) and pyocyanin.** The common phenazine nucleus PCA is synthesized by the gene products of the core phenazine biosynthetic operon *phzA1B1C1D1E1F1G1*. Pyocyanin is produced from PCA by PhzM and PhzS.

*phzA2–G2* operon is expressed endogenously are unknown. In *P. aeruginosa*, the conversion of PCA to pyocyanin is effected by two genes, *phzM* and *phzS* (Figure 1), both of which are linked to *phzA1-G1* on the chromosome (Mavrodi et al., 2001). Another locus, the *phnAB* operon, is also needed for normal pyocyanin production (Essar et al., 1990), though its role may be regulatory rather than enzymatic. Chapter 3 will address the roles of the *phzA1-G1, phzA2-G2* and *phnAB* loci in pyocyanin production by *P. aeruginosa*.

Pyoverdine is another pigment, yellow-green and fluorescent. Structurally, pyoverdines (numerous variant forms are found naturally) are comprised of a short peptide chain (6 to 12 amino acids residues) attached to a chromophore (Meyer, 2000). Pyoverdines function as siderophores—scavenging molecules for extracellular iron (Meyer, 2000). Thus, they differ drastically in structure and function from the phenazine pigments. Pyoverdine production is examined in Chapter 2 in a study of phenotypic pleiotropy in different strains.

This catalogue of virulence factors is far from exhaustive, but it serves to illustrate the diversity and complexity of virulence capability carried by *P. aeruginosa*. Much remains to be learned. The relative importance of these various factors in human infection is not well understood, and the large proportion of *P. aeruginosa* genes whose functions are described in the annotated genome as 'unknown' suggests that a plethora of virulence factors have yet to be discovered.

**Regulation of virulence factors.**

The actions of specific virulence factors constitute only part of the process of bacterial pathogenesis; equally important is the regulation of expression of these factors. In *P. aeruginosa*, the expression of many virulence determinants is governed by a complex network of interacting regulatory factors. The components of this network coordinately direct expression of numerous virulence factors in response to a multitude of stimuli, such as growth phase, culture density, aerobicity, and metabolite availability. Although many of the regulators in this network have been extensively studied, and some pathways of interaction linking them to virulence gene expression have been elucidated, the understanding of how these various network components respond to stimuli and interact with each other to appropriately express effector genes is still at an early stage.

Central players in this network are two "quorum-sensing" systems, the *las* system and the *rhl* system, which activate genes in response to high cell density. Figure 2 illustrates these systems. As shown in the figure, each quorum-sensing system comprises two genes, one encoding a biosynthetic enzyme (the I protein) that produces an acylated homoserine lactone (HSL) signal molecule known as an autoinducer (structures shown in Figure 3), and the other encodes a transcriptional activator (the R protein). As cell density increases, the diffusible autoinducer reaches a threshold concentration at which it binds and activates the transcriptional activator, resulting in induction of target genes in a

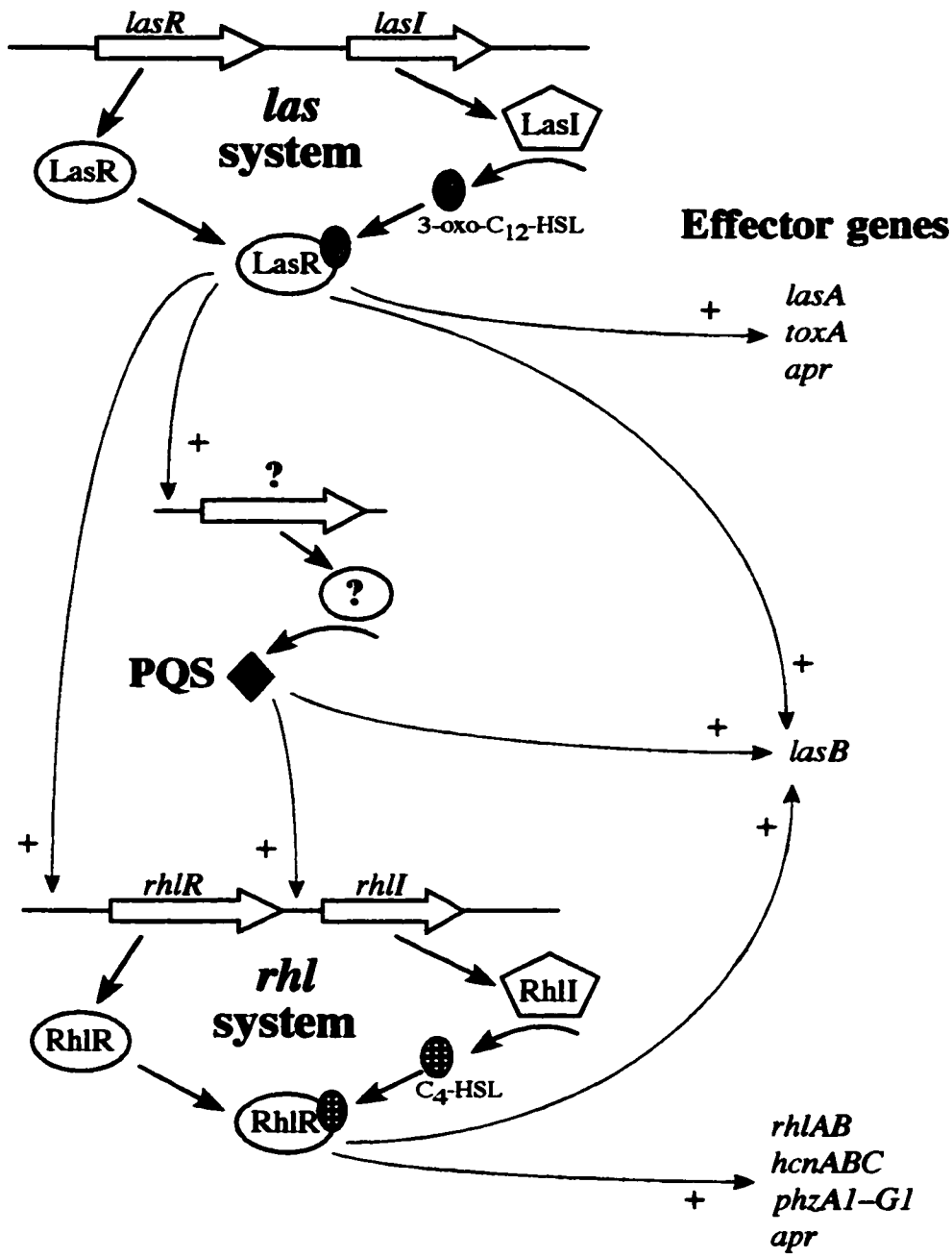


**Figure 2: Model of the quorum-sensing regulatory systems in *P. aeruginosa*.** Blue arrows indicate pathways of transcriptional regulation. For simplicity, only regulatory pathways pertinent to the research described in this dissertation are shown. (Figure modified from McKnight et al., 2000).

cell density-dependent manner (for review see Fuqua et al., 1996). The two quorum-sensing systems utilize distinct autoinducers and control different but overlapping sets of target genes (Figure 2). Together these two systems have been shown to influence expression of over two hundred genes (Whiteley et al., 1999). The *las* system, for example, directs expression of the established virulence factors elastase (the *lasB* gene), the LasA protease (*lasA*), exotoxin A (*toxA*) and alkaline protease (*apr*). The *rlh* system, in contrast, directs expression of rhamnolipid biosynthesis enzymes (*rhlA* and *rhlB*), the phenazine core biosynthetic operon (*phzA1–G1*), hydrogen cyanide synthase (*hcnABC*), *lasB* and *apr*.

The *las* and *rhl* quorum-sensing systems also interact with each other in a hierarchical manner (Pesci and Iglewski, 1997). Specifically, LasR controls transcription of the *rhlR* and *rhlI* genes (Figure 2). *rhl*-regulated genes therefore require activity of the *las* quorum-sensing system for full expression (Pesci et al., 1997; Whiteley et al., 1999). LasR has also been shown to regulate both its own and *lasI*'s expression (Albus et al., 1997; Pesci et al., 1997).

Recently, a third small signal molecule, 2-heptyl-3-hydroxy-4-quinolone, has been identified and shown to participate in the quorum-sensing regulatory network in *P. aeruginosa* (Pesci et al., 1999). Designated the *Pseudomonas* quinolone signal (PQS), this molecule differs significantly in structure from the acyl-HSL autoinducer molecules. Its stucture and the structures of the two acyl-HSL autoinducers are shown in Figure 3. Exogenous PQS strongly induces expression of *lasB* and *rhlI* (Figure 2), and, to a lesser extent, *lasR* and *rhlR* (McKnight et al., 2000; Pesci et al., 1999). In addition, PQS

production depends on *lasR* (Pesci et al., 1999). Together these results place PQS between the *las* and *rhl* quorum-sensing systems in the regulatory cascade, as indicated in Figure 2 (McKnight et al., 2000). The genes directly responsible for PQS synthesis have not been identified, though McKnight et al. hypothesize that they are LasR-regulated (Figure 2). The mechanism by which PQS activates gene expression is also not known.

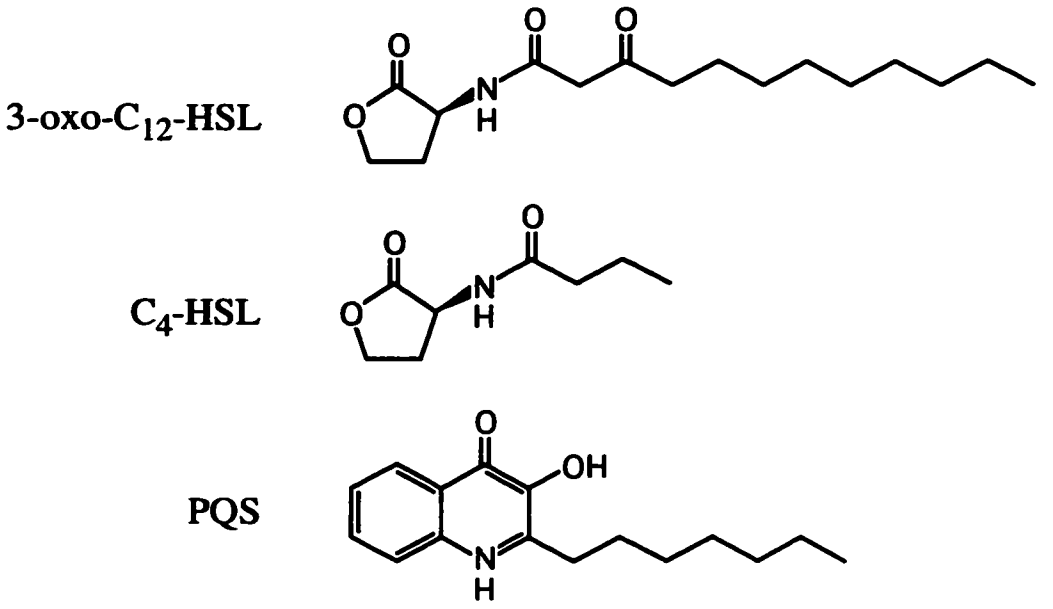


**Figure 3: *P. aeruginosa* signal molecules:** 3-oxo-$C_{12}$-HSL (the *las* system autoinducer, synthesized by LasI), $C_4$-HSL (the *rhl* system autoinduer, synthesized by RhlI) and the *Pseudomonas* quinolone signal (PQS), 2-heptyl-3-hydroxy-4-quinolone.

Numerous other regulatory factors also participate in the network controlling virulence gene expression in *P. aeruginosa*. While these regulators also interact integrally with the quorum-sensing pathways, and clearly play important roles in

virulence gene expression, they are only mentioned here briefly, since only a few enter (and more peripherally so) into the experiments presented in this dissertation. Acting upstream of the *las* quorum-sensing system are two factors, the two-component response regulator GacA (along with its cognate sensor kinase GacS), and Vfr. Mutational analysis has shown that both of these factors mediate *lasR* transcription (Albus et al., 1997; Reimmann et al., 1997). Another element is Anr, which regulates genes (such as *hcnABC*) in response to anaerobiosis (Pessi and Haas, 2000). Yet another regulator is RpoS, the stationary-phase sigma factor, which interacts with the *rhl* quorum-sensing system through pathways that are still controversial (Latifi et al., 1996; Whiteley et al., 2000). Finally, two negative regulators are RsaL, a repressor of *lasI* (de Kievit et al., 1999), and QscR, which represses quorum-sensing-induced gene expression at early growth stages (Chugani et al., 2001). It is clear that although much is known about virulence gene regulation in *P. aeruginosa*, much more remains to be learned. Discovery of new regulatory factors continues, and complexities of the circuitry continue to emerge.

**Paralytic killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*.**

The *P. aeruginosa* virulence model I chose to study was first characterized by Creg Darby (Darby et al., 1999; Darby, 1998): when placed on a lawn of *P. aeruginosa* PAO1 grown for 24 hours on BHI agar, wild-type C. elegans (N2) showed a gradual slowing of their movement, and died within four hours of exposure. This killing was

named "paralytic killing" because the worms showed progressive loss of muscle function, and eventually took on a kinked appearance, seemingly caused by body-wall muscle hypercontraction. Such a phenotype could result from the action of a neurotoxin. It was shown that the toxic substance or substances causing the paralysis were diffusible, and that direct contact between the worms and the bacteria was not necessary for killing.

In addition to characterizing the paralytic killing phenotype, Darby tested several defined mutants of PAO1 for deficiency in paralytic killing of nematodes. Null mutations in either *lasR* or *rhlR*—the two major quorum-sensing regulatory genes—abolished killing, indicating that the responsible factor or factors were under quorum-sensing control.

Darby also undertook a genetic screen to isolate mutant worms resistant to paralytic killing. A single complementation group was identified, and the affected gene, *egl-9*, was cloned. The protein sequence of the gene product contained a putative zinc-finger motif and a section homologous to a cDNA from rat smooth muscle cells. Otherwise, no function was suggested. *egl-9::gfp* reporter strains were constructed, and revealed that the gene was expressed in the nematode in a variety of locations, including body-wall and pharyngeal muscle. The function of the *egl-9* gene, which has remained a mystery until very recently, will be discussed in Chapter 4.

# CHAPTER 2

## *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning

This chapter describes experiments undertaken to identify the bacterial factors mediating paralytic killing of *C. elegans* by *P. aeruginosa* strain PAO1. This chapter, parts of chapter 5 and fragments of chapter 1 have been published together (Gallagher and Manoil, 2001).

## RESULTS

### *P. aeruginosa* mutants with impaired ability to kill *C. elegans*.

To help identify the substance or substances produced by *P. aeruginosa* that are toxic to *C. elegans*, we screened chromosomal mTn5-Tc transposon insertion mutants for reduced nematode killing (see Materials and Methods). Of approximately 3000 mutants

screened, twenty-five strains with significant defects were recovered (Table 1). Slow-growing mutants forming small colonies on nutrient agar were not included in the analysis. The mutants could be grouped into two classes based on the strength and reproducibility of their killing defects. Nine of the mutants (class I) killed ≤13% of the nematodes, whereas 16 of the mutants (class II) killed 27-92% of the nematodes. The class I mutants were quantitatively more reproducible than the class II mutants in terms of the defects in killing observed in different trials. Three of the mutant strains in Table 1 (MP508, MP552 and MP553) showed abundant papillation of secondary colonies upon prolonged incubation (several days) on rich media, suggesting a reduction in viability of the parent strains with an outgrowth of fitter variants (data not shown).

We identified the transposon insertion sites for 24 of the 25 mutants by PCR amplification of genomic sequence flanking each transposon, DNA sequencing and comparison with the PAO1 genome sequence (Materials and Methods). Twenty-one genes were represented in the mutant set. These genes included regulatory genes, genes encoding metabolic enzymes, a gene for a probable metal transporter, and five other genes with known or postulated virulence functions (Table 1). Four of the twenty-one genes interrupted (PA0041, PA0745, PA3946 and PA4725) had not been previously identified except as part of the PAO1 genome sequence.

One mutant (MP507) carried an insertion in the *hcnC* gene, which encodes a subunit of hydrogen cyanide synthase (Pessi and Haas, 2000). This finding suggested that hydrogen cyanide contributed to nematode killing. In addition, we discovered previously that worm killing was inefficient if the petri plate lid was removed during the

## Table 1. Mutants Defective in Paralytic Killing.

| Strain | Insertion Site[a] | Gene[b] | Function | % Killing[c] |
|---|---|---|---|---|
| PAO1 | | | | 99 (±0) |
| **Class I – Strongly avirulent strains** | | | | |
| MP503 | 3,571,648 | ***eda*** | 2-keto-3-deoxy-6-phosphogluconate aldolase; Entner-Deudoroff pathway. | 4 (±2) |
| MP505 | 435,468 | ***proC*** | Δ-1-pyrroline-5-carboxylate reductase; proline biosynthesis. | 4 (±3) |
| MP506 | 435,230 | ***proC*** | Δ-1-pyrroline-5-carboxylate reductase; proline biosynthesis. | 0 (±0) |
| MP504 | 812,969 | PA0745[d] | probably enoyl-CoA hydratase; fatty-acid degradation. | 13 (±10) |
| MP507 | 2,415,450 | ***hcnC*** | Hydrogen cyanide synthase. | 3 (±2) |
| MP508 | 45,846 | PA0041[d] | Homologue of *fhaB*, *Bordetella* filamentous hemagglutinin. | 0 (±0) |
| MP501 | 4,423,808 | PA3946[d] | Homologue of *bvgS*, *Bordetella* two-component sensor kinase virulence gene regulator. | 0 (±0) |
| MP502 | 1,015,249 | *gacS* | Homologue of *P. syringae* two-component sensor kinase controlling disease lesion formation. | 9 (±5) |
| MP511 | Unsequenced[e] | | | 0 (±0) |
| **Class II – Moderately avirulent strains** | | | | |
| MP554 | 3,572,897 | ***zwf*** | Glucose-6-P dehydrogenase; Entner-Deudoroff pathway. | 41 (±19) |
| MP555 | 6,098,814 | *soxA* | Sarcosine oxidase. | 38 (±20) |
| MP556 | 6,100,002 | *soxA* | Sarcosine oxidase. | 39 (±19) |
| MP557 | 1,032,886 | ***purM*** | Phosphoribosylaminoimidazole synthetase; purine biosynthesis. | 92 (±3) |
| MP558 | 4,216,505 | ***purL*** | Phosphoribosylformylglycinamidine synthase; purine biosynthesis. | 90 (±5) |
| MP559 | 874,405 | ***prpB*** | Carboxyphosphonoenolpyruvate phosphonomutase; fatty acid and phospholipid metabolism. | 83 (±15) |
| MP560 | 873,168 | ***prpC*** | Citrate synthase 2. | 72 (±28) |
| MP561 | 1,758,910 | ***gpdA*** | Glycerol-3-P dehydrogenase; specific to fatty acid and phospholipid metabolism. | 57 (±23) |
| MP562 | 2,927,500 | PA2587 | Putative salicylate hydroxylase; quinolone signal synthesis. | 27 (±16) |
| MP571 | 6,193,910 | ***znuB*** | Permease of ABC zinc transporter. | 39 (±20) |
| MP573 | 4,290,026 | ***phpA*** | Aminopeptidase; protein modification, alginate biosynthesis. | 28 (±13) |
| MP574 | 5,993,742 | ***algC*** | Lipopolysaccharide and alginate biosynthesis. | 75 (±15) |
| MP572 | 5,100,129 | ***pilW*** | Type-4 pili. | 56 (±10) |
| MP551 | 1,086,674 | PA1003[d] | Putative transcriptional regulator with LysR family signature. | 31 (±22) |
| MP552 | 5,304,505 | PA4725[d] | Putative amino-acid permease fused to putative two-component sensor histidine kinase. | 42 (±15) |
| MP553 | 5,304,930 | PA4725[d] | Putative amino-acid permease fused to putative two-component sensor histidine kinase. | 76 (±16) |

[a] The transposon insertion site corresponds to the chromosomal location in the PAO1 single contig sequence (www.pseudomonas.com).
[b] Bold letters indicate known *P. aeruginosa* genes; lightface type indicates close homologues of known genes. PA numbers are designations assigned by the web site.
[c] Percentages of killing are averages from at least three independent killing assays for each strain. Numbers in parentheses indicate standard errors of the mean.
[d] Gene not experimentally characterized in studies of pseudomonads.
[e] Repeated attempts to sequence were unsuccessful.

four-hours of the killing assay, suggesting that a volatile factor (such as hydrogen cyanide) contributed to the killing (data not shown).

**Cyanide production strongly correlates with nematode killing.**

To verify that the killing defect in strain MP507 was due to inactivation of the *hcnC* gene rather than to polar effects of the transposon insertion, we complemented the HCN synthase defect by introducing the *hcnABC* gene cluster (lacking downstream open reading frames) in *trans*. The nematode killing phenotype was fully restored in the complemented mutant (Figure 4), implying that paralytic killing truly depends on the *hcn* genes themselves.

To determine whether any of the other killing-defective mutants exhibited reduced cyanide production, we measured the levels of cyanide generated by each strain under growth conditions mimicking those used to assay nematode killing (Materials and Methods). As shown in Table 2, there was an excellent correlation between decreased cyanide production and reduced killing for both class I and class II mutants. These data implicate cyanide as a primary component of PAO1 virulence toward *C. elegans*.

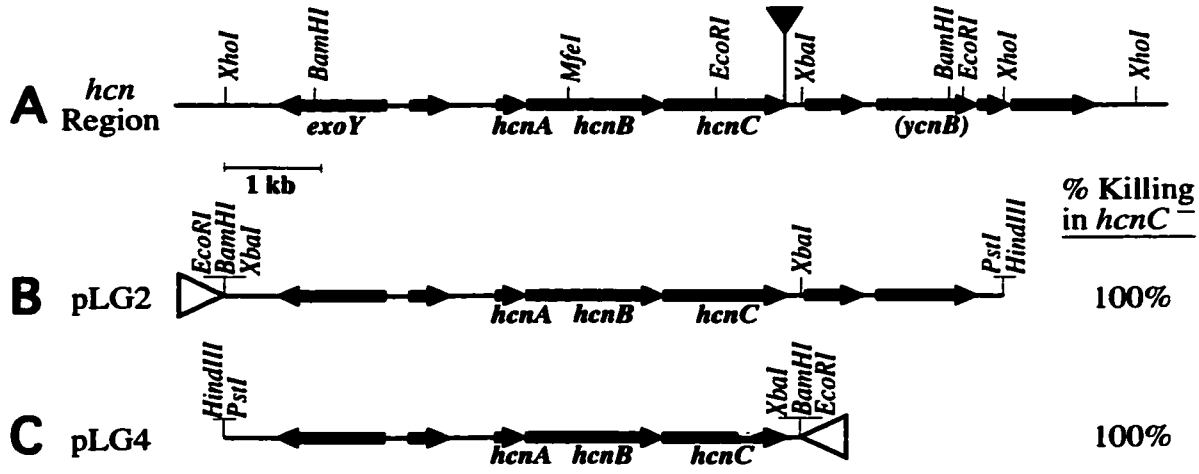


**Figure 4. Complementation of the killing defect in *hcnC* mutant MP507.** (A) Restriction map of the *hcnABC* region, showing the locations and orientations of the known genes *hcnA*, *hcnB*, *hcnC*, and *exoY* and of putative genes (unlabeled arrows), including a homologue of the conserved hypothetical *E. coli* protein *ycnB*. The solid triangle indicates the location of the mTn*5*-Tc transposon insertion in the *hcnC* mutant MP507. (B and C) Maps of the insertion regions in recombinant plasmids carrying the *hcnABC* region. The results of nematode-killing assays of the *hcnC* mutant MP507 carrying these plasmids are also shown. The open triangles indicate the orientation of the Plac promoter in the pUCP18 vector. Killing percentages are averages based on three separate assays. MP507 carrying only the vector plasmid pUCP18 exhibited less than one percent killing.

**Cyanide alone is sufficient to kill *C. elegans*.**

We next examined the response of *C. elegans* to hydrogen cyanide alone at concentrations comparable to those produced by bacteria. When exposed to 1 μmole of cyanide gas (HCN) in a sealed chamber (Materials and Methods), wild-type worms showed a gradual slowing of their movement, with more that 85% becoming fully immobile and unresponsive to touch by five hours after exposure began (Figure 5A). By

**Table 2 – Exoproduct Production by *P. aeruginosa* Mutants.**

| Strain | Mutant gene | % Killing[a] | Cyanide (nmol)[b] | Producion of: Pyocyanin[c] | Pyoverdine[c] | Protease[d] |
|---|---|---|---|---|---|---|
| PAO1 | none | 100 (±1) | 300 (±56) | (1.00) (±.047) | (1.00) (±.079) | ++ |
| PAO-R1 | *lasR* | 0 (±0) | <10 (±0) | .011 (±.000) | 1.54 (±.013) | +/- |
| **Class 1 – Strongly Avirulent** | | | | | | |
| MP503 | *eda* | 7 (±1) | <15 (±8) | .038 (±.015) | .083 (±.099) | + |
| MP505 | *proC* | 9 (±6) | 31 (±3) | ND | ND | ND |
| MP506 | *proC* | 0 | 44 (±34) | .608 (±.057) | .303 (±.006) | ++ |
| MP504 | PA0745 (*fad-1*) | 19 (±21) | <11 (±2) | .036 (±.006) | .379 (±.018) | ++ |
| MP507 | *hcnC* | 13 | <10 (±0) | 2.35 (±.040) | 1.54 (±.009) | ++ |
| MP508 | PA0041 (*fhaB*) | 0 | <10 (±0) | 1.71 (±.059) | .134 (±.035) | +++ |
| MP501 | PA3946 (*bvgS*) | 0 (±0) | <17 (±6) | .521 (±.025) | 1.58 (±.002) | ++ |
| MP502 | *gacS* | 11 | 21 (±12) | .127 (±.002) | .064 (±.070) | ++ |
| MP511 | (unknown) | 0 | 36 (±37) | .034 (±.006) | .033 (±.026) | +/- |
| **Class 2 – Moderately Avirulent** | | | | | | |
| MP554 | *zwf* | 74 (±25) | 200 (±86) | .254 (±.032) | .447 (±.169) | ++ |
| MP555 | *soxA* | 3 | 64 (±7) | 1.00 (±.085) | .353 (±.009) | ++ |
| MP556 | *soxA* | 17 | 55 (±15) | ND | ND | ND |
| MP562 | PA2587 | 0 (±0) | 36 (±31) | .013 (±.006) | 1.03 (±.283) | ++ |
| MP571 | *znuB* | 4 | 96 | .767 (±.042) | .597 (±.476) | ++ |
| MP573 | *phpA* | 14 | 150 | 1.11 (±.021) | .879 (±.046) | ++ |
| MP572 | *pilW* | 36 | 84 | ND | ND | ND |
| MP551 | PA1003 | 0 (±0) | <23 (±23) | .008 (±.000) | 1.54 (±.039) | ++ |

[a] Worm killing was assayed in parallel to two of the three cyanide collection trials by using plates inoculating and incubating exactly like the plates used for the cyanide collection. The numbers in parentheses are standard errors of the mean determined when two assays were conducted.

[b] The values are averages based on one to three independent assays. The numbers in parentheses are standard errors of the mean determined when multiple assays were conducted.

[c] The values are expressed in arbitrary units normalized to the amount in PAO1 and are averages based on two independent assays. The numbers in parentheses are standard errors of the mean. ND, not done.

[d] Total secreted protease production was assessed by relative zones of clearing around equally-sized overnight bacterial patches on skim-milk agar: +/-, no clearing beyond the edge of the patch; +, 1-2 mm clearing; ++, 4-5 mm clearing; +++, >6 mm clearing. ND, not done.

ten hours all of the worms were immobile and unresponsive. In contrast, although mutant *egl-9* worms exhibited a sluggishness similar to that of wild-type worms soon after cyanide exposure began, they recovered completely within a few hours and remained fully viable (Figure 5A). Cyanide gas thus killed *C. elegans* with kinetics and genetic dependency similar to the kinetics and genetic dependency of *P. aeruginosa*-induced paralytic killing, in which complete killing of wild-type but not *egl-9* worms is observed after four hours of exposure to bacteria (Darby et al., 1999). The 1 μmole of HCN used in this protocol approximates the ~300 nmoles recovered from wild-type bacteria grown under standard worm-killing conditions (Table 2).

Exposure to an increased amount of cyanide (4 μmoles) killed both *egl-9* and wild-type worms with indistinguishable kinetics (Figure 5B). This result suggests either that an additional mechanism of killing operates at the higher cyanide level, or that inactivation of *egl-9* simply raises the threshold of sensitivity to cyanide.

**Cyanide as the sole toxic component.**

Although the previous results indicated that the amount of cyanide normally produced by PAO1 should suffice to kill *C. elegans* (Table 2 and Figure 5A), we wondered whether additional factors produced by the bacteria contribute to the killing. To address this possibility, we compared the kinetics of killing by cyanide gas when the worms were placed on either a lawn of *hcnC* mutant bacteria or on agar lacking bacteria.

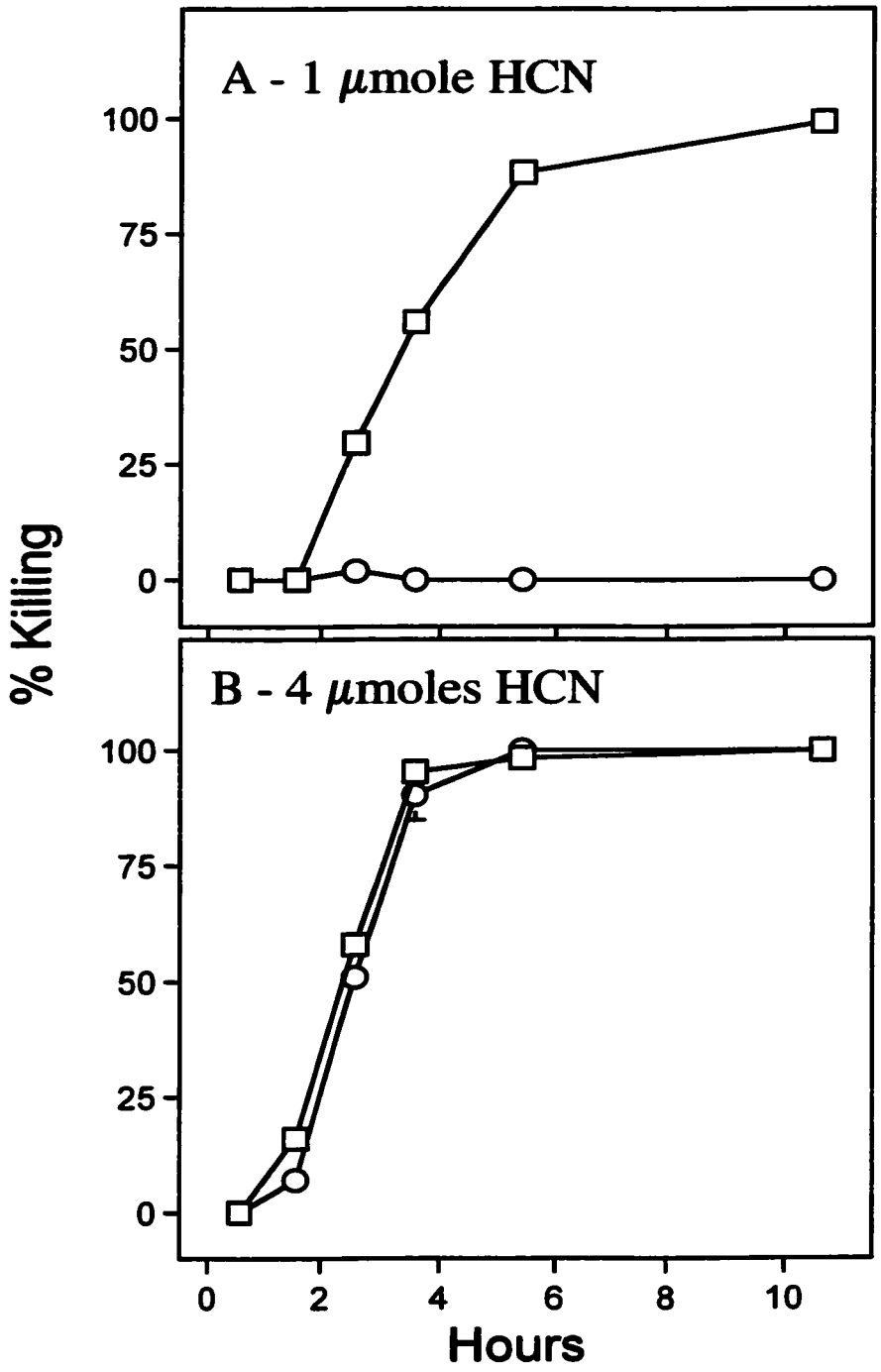


**Figure 5. Direct exposure of *C. elegans* to cyanide gas.** Wild-type (squares) or *egl-9* (circles) nematodes were exposed to 1 μmol (A) or 4 μmol (B) of cyanide gas in sealed 10-cm-diameter petri plates. Worms were considered dead if did not respond detectably when the assay plate was tapped repeatedly against the microscope stage. Each data point represents the average level of killing based on three separate assays.

As shown in Figure 6, the effect of the *hcnC* mutant bacteria on the kinetics of the response to cyanide was negligible for both wild-type and *egl-9* nematodes. The bacteria thus did not augment the toxicity of the added cyanide, suggesting that hydrogen cyanide alone can kill nematodes.

In similar augmentation experiments using the other class I mutants, we discovered that the two *proC* mutants (MP505 and MP506) were unique in that they strongly protected the worms from cyanide-induced killing. Exposure to even eight μmoles of cyanide failed to kill worms placed on a lawn of either mutant (data not shown). We examined whether the *proC* mutant bacteria needed to be in direct contact with the nematodes to provide their protective effect by enclosing worms in a sealed chamber together with a second plate on which bacteria had been grown, then generating cyanide gas in the chamber. As shown in Figure 7, the cyanide gas killed the worms when either no bacteria or *hcnC* mutant bacteria were present on the second plate (athough the *hcnC* bacteria conferred some protection early in the experiment). However, when the *proC* mutant was present on the second plate, the generation of even six μmoles cyanide did not kill the worms. Hence, the *proC* mutant lawn protected the nematodes from the cyanide, even when it was not in contact with them. This protection presumably occurs by inactivation or sequestering of the cyanide by the *proC* mutant bacteria. One possibility is that the *proC* mutant bacteria secrete the proline biosynthetic intermediate glutamic-5-semialdehyde, which reacts with cyanide to form a cyanohydrin.

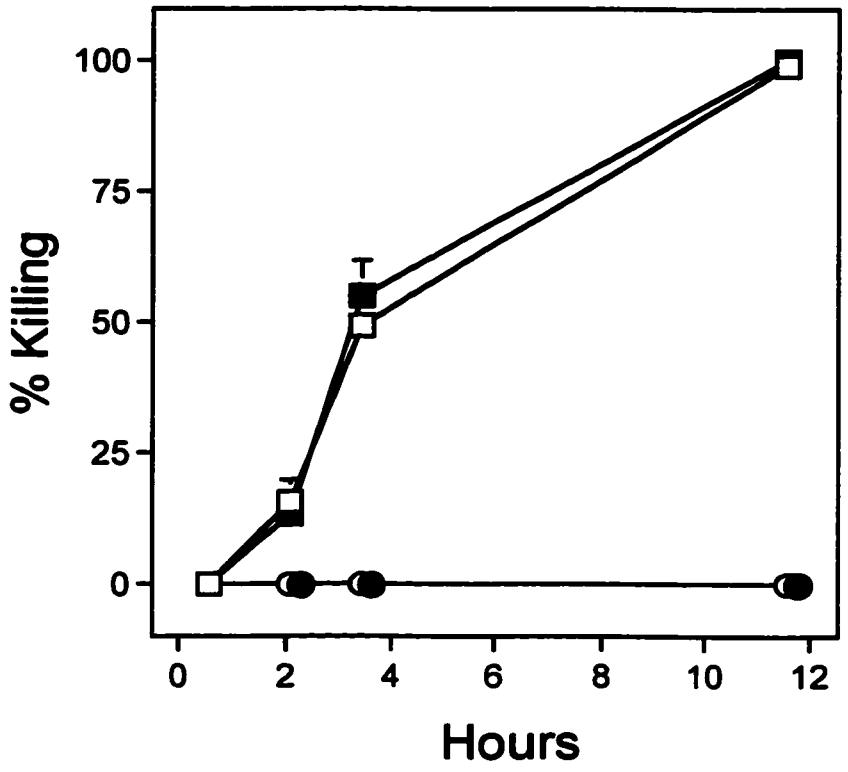


**Figure 6. Killing of *C. elegans* by cyanide gas with and without exposure to bacteria.** Wild-type (squares) or *egl-9* (circles) nematodes placed on either no bacteria (open symbols) or the hcnC mutant MP507 (closed symbols) were exposed to 1 $\mu$mol of hydrogen cyanide in sealed 10-cm-diameter petri plates. Each data point represents the average based on triplicate experiments.

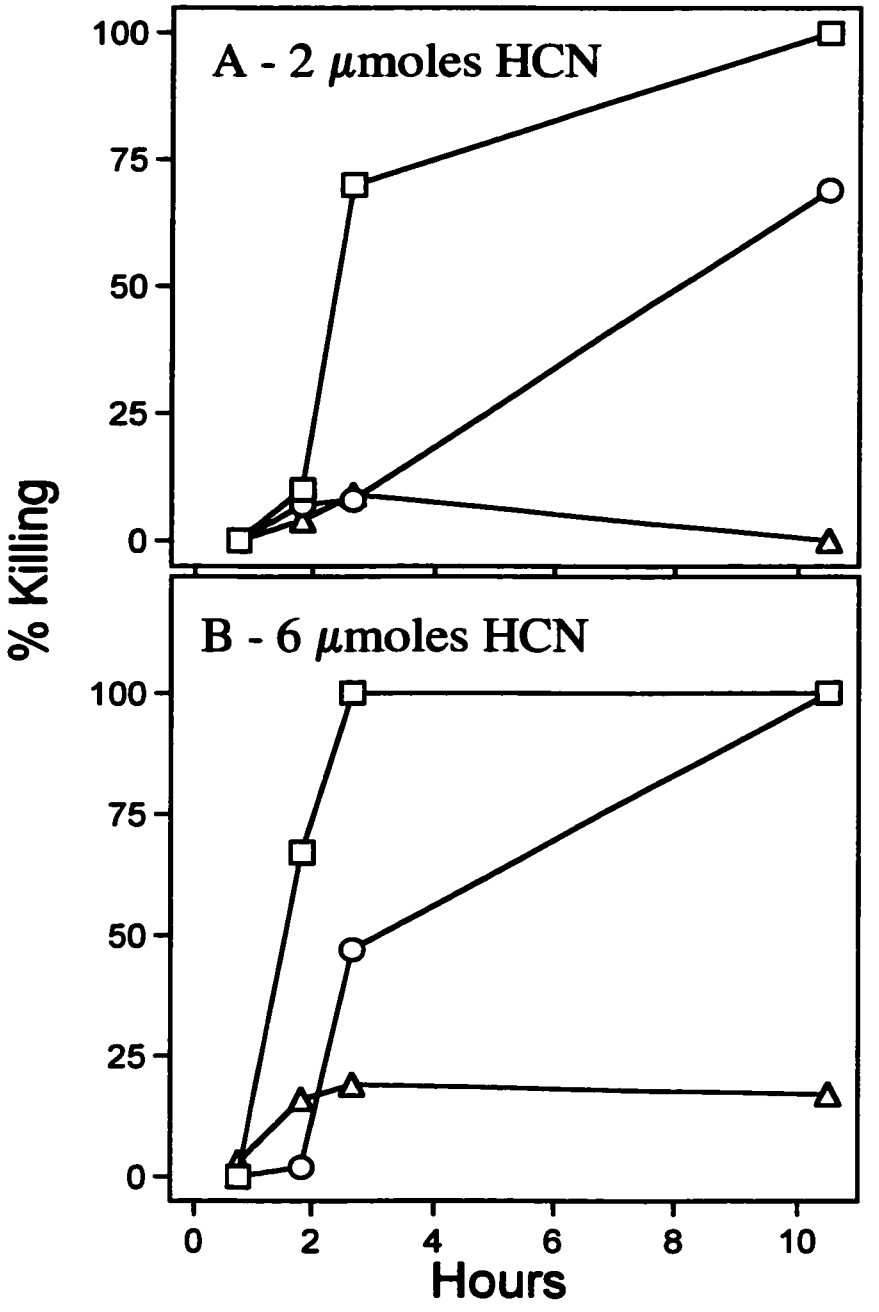


**Figure 7. Killing of *C. elegans* by cyanide gas when bacteria were present but not in contact with the nematodes.** Wild-type nematodes were exposed to 2 μmol (A) or 6 μmol (B) of hydrogen cyanide in sealed 10-cm-diameter petri plates when plates containing either no bacteria (squares), a 24-h lawn of the hcnC mutant MP507 (circles) or a 24-h lawn of the proC mutant MP506 (triangles) were also present in the chamber but were not in contact with the nematodes.

**Phenazine production does not correlate with nematode killing.**

Recent studies of a form of killing ("fast-killing") of *C. elegans* by a different strain of *P. aeruginosa* (PA14) showed that production of the blue phenazine pigment pyocyanin was compromised in a subset of mutants defective in killing (Mahajan-Miklos et al., 1999). Phenazines are redox-active compounds secreted by Pseudomonads, and pyocyanin is the characteristic phenazine produced by *P. aeruginosa* (Essar et al., 1990; Hassan and Fridovich, 1980; Turner and Messenger, 1986). To examine the potential involvement of phenazines in nematode killing by strain PAO1, we measured the amount of pyocyanin produced by our mutants under the growth conditions used to assay killing. As shown in Table 2, only about one-half of the mutants were defective in the production of pyocyanin. Curiously, the *hcnC* mutant (MP507) produced more than twice as much pyocyanin as its parent. These data show that there is not a strong correlation between reduced pyocyanin production and loss of virulence towards nematodes in the assay described here.

Defects in pyocyanin production in some of the worm non-killing mutants may simply reflect pleiotropic effects of the mutations (Reimmann et al., 1997; Whiteley et al., 1999). To further assess pleiotropy in the killing-defective strains, we assayed production of total secreted protease (Brint and Ohman, 1995) and the secreted siderophore pyoverdine (Cox and Adams, 1985; McMorran et al., 1996). Of fourteen mutants examined, seven showed reduced pyoverdine production and two showed reduced secreted protease production (Table 2). Of the nine class I mutants, the *hcnC*

mutant strain was exceptional in that reduced pyocyanin, pyoverdine or secreted protease production was not evident.

## DISCUSSION

In this report we describe experiments designed to investigate the mechanism by which *P. aeruginosa* PAO1 rapidly paralyzes and kills *C. elegans* (Darby et al., 1999). Our results imply that the poison hydrogen cyanide is the sole or primary bacterial factor responsible for killing of the nematode. That cyanide is necessary for the virulence is implied by findings that seventeen transposon insertion mutants impaired in worm killing, including one mutant in which hydrogen cyanide synthase itself was inactivating, all exhibited reduced cyanide production. That cyanide is sufficient for the nematode killing is implied by results showing that exposure to exogenous cyanide at levels comparable to that produced by the bacteria killed nematodes with kinetics similar to those observed with bacteria. Furthermore, a nematode mutant (*egl-9*) resistant to *P. aeruginosa* killing was also resistant to killing by exogenous HCN.

Hydrogen cyanide is a typical Pseudomonad secondary metabolite, a compound which is not required for growth, energy storage, or primary metabolism but which may offer some ecological advantage to the organism (Vining, 1990). In addition to cyanide,

Pseudomonad secondary metabolites include siderophores such as pyoverdine, redox-active compounds such as phenazines, and polyketide antibiotics (Budzikiewicz, 1993). Cyanide is produced in *Pseudomonas* by oxidative decarboxylation of glycine by the three subunit membrane-bound flavoenzyme encoded by *hcnABC* (Blumer and Haas, 2000). *P. aeruginosa* produces HCN maximally in late exponential and early stationary phase under microaerophilic conditions (Blumer and Haas, 2000), and transcription of the *hcn* genes appears to depend directly on the quorum-sensing regulators LasR and RhlR, as well as the anaerobic regulator Anr (Brint and Ohman, 1995; Pessi and Haas, 2000; Whiteley et al., 1999). Additional components of the complex regulatory circuitry controlling the production of cyanide and other secondary metabolites have been identified (Albus et al., 1997; Chugani et al., 2001; McKnight et al., 2000; Pesci et al., 1999; Whiteley et al., 2000).

The mutations we identified which reduced cyanide production and virulence toward *C. elegans* affect a variety of regulatory and metabolic functions (Table 1). Two of the regulatory mutations affect quorum sensing indirectly: one is in *gacS*, encoding a two-component sensor that influences autoinducer levels (Reimmann et al., 1997), and the other is in a locus (PA2587) needed for synthesis of a quinolone signal (PQS) required for RhlR-RhlI function (Chapter 3 and E. Pesci, personal communication). Mutations in three additional putative regulators were also identified. One of these regulators (PA3946) is homologous to the *Bordetella pertussis* virulence regulator BvgS (Akerley and Miller, 1996), another (PA1003) belongs to the LysR family (Henikoff et

al., 1988), and the third (PA4725) resembles a two-component sensor fused to a membrane permease. A mutation affecting PA1003 was previously identified in a screen for mutations reducing virulence toward *Arabidopsis* (Rahme et al., 1997). The mutations affecting metabolic functions inactivate enzymes that participate in central carbon metabolism, fatty acid breakdown, and proline biosynthesis. With the notable exception of the mutation of the HCN synthase, all of the strongest (class I) non-worm-killing mutations reduced the production of pyocyanin, pyoverdine or secreted protease. This pleiotropy would have made the identification of cyanide as the worm-killing poison difficult if the *hcnC* mutant had not been isolated. An unanticipated benefit of the genetic approach taken is that it appears to have identified several new regulatory and metabolic components of the circuitry controlling the production of secondary metabolites.

Studies of a different strain of *P. aeruginosa* (PA14) showed that about one-half of a collection of transposon insertion mutations that eliminated a fast-killing form of virulence toward *C. elegans* also reduced production of pyocyanin, as did a constructed deletion mutation (Δ*phnAB*) that decreased phenazine biosynthesis (Mahajan-Miklos et al., 1999). The results were interpreted in terms of a model in which phenazines are one component of a multifactorial killing process. Phenazines are toxic to a variety of cell types and are thought to act by generating reactive oxygen species by redox cycling (Sorenson and Joseph, 1993). For strain PAO1, we found no convincing indication that pyocyanin or any other phenazine played a direct role in killing *C. elegans*. Although five of nine strongly avirulent mutants produced significantly less pyocyanin than the

parent, this reduction in production is readily explained by the pleiotropy of the mutations (Table 2). Indeed, two of the mutations affect regulators (LasR and GacS) already known to be required for the expression of multiple genes, and a third affects an enzyme of central carbon catabolism in *Pseudomonas* (Entner-Douderoff aldolase) whose loss might also be expected to be highly pleiotropic (Reimmann et al., 1997; Temple et al., 1998; Whiteley et al., 1999). Furthermore, since exposure of nematodes to HCN in the absence of bacteria reproduced the nematode paralytic killing phenomenon, no additional bacterial substances are required.

The classic cellular target of cyanide inhibition is cytochrome oxidase, although other metalloenzymes are also sensitive to the poison (Solomonson, 1981). Inhibition of mitochondrial respiration can easily account for the rapid and dramatic paralytic killing of nematodes by *P. aeruginosa* PAO1. *Pseudomonas* appears to protect itself from cyanide poisoning by expressing an unusual cyanide-resistant cytochrome oxidase (Cunningham et al., 1997). Studies of human and animal cyanide poisoning indicate that the poison strongly affects neurological tissue (Way, 1984), and it is possible that the nematode killing also reflects a hypersensitivity of neuromuscular tissues to the poison.

It is striking that loss-of-function mutations in a single nematode gene (*egl-9*) confer strong resistance to cyanide poisoning. The mechanism underlying this resistance is mysterious. Since HCN is predominantly uncharged at physiological pH (pK=9.3) and is expected to diffuse freely through membranes, it appears unlikely that a cyanide

transporter is eliminated by the mutations. One possibility is that the *egl-9* mutations constitutively activate an adaptive response to hypoxia (Semenza, 2001), thus conferring some resistance to cytochrome oxidase inhibition by cyanide. Another possibility is that reactive oxygen species generated by cyanide inhibition activate an Egl-9-dependent pathway, such as a stress-dependent MAP (Mitogen Activated Protein) kinase pathway (Tibbles and Woodgett, 1999), leading to paralysis and death. Homologues of *egl-9* exist in humans (Aravind and Koonin, 2001; Dupuy et al., 2000), and may represent potential therapeutic targets for countering the toxic effects of cyanide.

Cyanide is a potent poison expected to be active against most eukaryotic species (Blumer and Haas, 2000; Solomonson, 1981). This compound thus could contribute profoundly to the broad pathogenic host range of *P. aeruginosa* (Boman et al., 1972; Bucher and Stephens, 1957; Jarrell and Kropinski, 1982; Mahajan-Miklos et al., 2000; Patty, 1921). It is thought that cyanide inhibition of fungal growth helps account for the suppression of several plant root and leaf fungal diseases (Haas et al., 2000; Voisard et al., 1989). The activities of cyanide and other small molecule poisons may also contribute to the pathogenesis accompanying the variety of opportunistic infections caused by *P. aeruginosa* (Lyczak et al., 2000). Although the role of cyanide in *Pseudomonas* pathogenesis in humans is largely unexplored, an early study of burn infections detected this poison (Goldfarb and Margraf, 1967). A recent finding that sputa of cystic fibrosis patients contain *P. aeruginosa* in the appropriate quorum-sensing

physiological state to produce cyanide (Singh et al., 2000a) suggest that the poison could also contribute to the tissue destruction that accompanies lung infections in this disease.

# CHAPTER 3

## Identification of genes required for synthesis of the *Pseudomonas* quinolone signal PQS, And analysis of regulatory interactions in the quorum-sensing hierarchy.

The results of the previous chapter stand in contrast to the conclusions of a recent investigation into a killing phenomenon (known as "fast killing") between *C. elegans* and *P. aeruginosa* PA14 (Mahajan-Miklos et al., 1999). Those studies found that mutations in *phzB1* or in the *phnAB* operon—both of which are needed for pyocyanin production (Chapter 1)—prevented fast killing, as did other mutations not affecting pyocyanin production. It was suggested, therefore, that in fast killing, phenazine antibiotics are an important component of a multifactorial virulence mechanism (Mahajan-Miklos et al., 1999). The identity of the other factor(s) involved in the mechanism was not determined. Due to differences in bacterial strains, gene requirements and growth conditions used, fast killing and paralytic killing are probably mechanistically distinct modes of killing

(Chapter 2). It is possible, however, that cyanide represents one of the factors contributing to fast killing. Additionally, phenazines may play a secondary role in paralytic killing. It has been found, for example, that a purified phenazine (phenazine methosulfate) can be used as an electron acceptor for cyanide production *in vitro* (Blumer and Haas, 2000; Castric, 1981).

To address the possibility that phenazines participate in cyanide production *in vivo*, we investigated whether phenazines were required for full virulence in paralytic killing. The results presented in this chapter resolve the question of a metabolic relationship between pyocyanin availability and cyanide production by showing that phenazines are not required for paralytic killing. More importantly, however, these investigations have uncovered new genes involved in virulence gene regulation in *P. aeruginosa*, and have elucidated interactions between several members of the regulatory hierarchy controlling virulence factor production.

## RESULTS

### Isolation of *P. aeruginosa* mutants deficient in production of pyocyanin.

To determine whether phenazines influence cyanide synthesis in *P. aeruginosa* (either through metabolic or regulatory effects), we identified mutants of PAO1 defective

in pyocyanin production and tested them for changes in paralytic nematode killing. Two different growth conditions were used in screening for the mutants: overnight growth on BHI agar, or two-day growth in LPSM detection media, a liquid growth medium producing maximal pyocyanin production (Cox, 1986). In the process of screening, we discovered that different isolates of *P. aeruginosa* PAO1 behaved differently under the different growth conditions. When grown on BHI agar, our standard lab strain of PAO1 (obtained from B. Iglewski), hereby designated "MPAO1", showed much stronger pyocyanin production than did the PAO1 strain whose genome was sequenced (obtained from the laboratory of S. Lory), hereby called "PAO1seq". During LPSM growth, in contrast, PAO1seq produced significantly more pyocyanin than MPAO1 (data not shown). We therefore screened MPAO1 mutants (2,100 IS*phoA*/hah-Tc transposon insertion mutants) for pigmentation defects after overnight growth of lawns on BHI agar, and screened PAO1seq mutants (2,800 IS*phoA*/hah-Tc transposon insertion mutants) for reduced blue pigmentation after two-day growth in LPSM liquid detection media.

Table 3 lists twenty-one mutants from these screens that reproducibly displayed diminished pyocyanin production under standard assay conditions, and that did not show visibly decreased growth rates on nutrient agar. Nine additional pyocyanin-deficient mutants from other studies are also listed (for sources, see Table 3, column 6). We determined the transposon insertion sites for the new mutants by PCR amplification and sequencing of the genomic DNA flanking each transposon insertion (Materials and Methods), followed by BLAST analysis against the completed PAO1 genome sequence.

**TABLE 3: Mutants defective in pyocyanin production.**

| Strain | Parent | Insert site | Gene | Description | Source[a] |
|---|---|---|---|---|---|
| *phzI* cluster | | | | | |
| ME3B56 | MPAO1 | 4,712,812 | *PhzM* | Pyocyanin biosynthesis. | This study |
| L3B111 | PAO1seq | 4,714,312 | *PhzA1* | Core phenazine biosynthetic operon. | This study |
| *LGP105 | MPAO1 | " | " | " | Tfr.f. L3B111 |
| MD5B6 | MPAO1 | 4,715,531 | *phzC1*[b] | " | This study |
| L5C87 | PAO1seq | 4,718,219 | *phzE1*[b] | " | This study |
| *LGP106 | MPAO1 | " | " | " | Tfr.f. L5C87 |
| L5C143 | PAO1seq | 4,720,699 | *phzS* | Pyocyanin biosynthesis. | This study |
| *LGP102 | MPAO1 | " | " | " | Tfr.f. L5C143 |
| *phnAB*-region | | | | | |
| L4E176 | PA01seq | 1,081,114 | PA0998 | Put. β-ketoacyl-acyl protein synth. | This study |
| *LGP103 | MPAO1 | " | " | " | Tfr.f. L4E176 |
| M1B8 | MPAO1 | 1,081,363 | " | " | This study |
| MF4C2 | MPAO1 | 1,081,420 | " | " | This study |
| L5B41 | PAO1seq | 1,082,682 | PA0999 | *fabH1*: β-ketoacyl-acyl protein synth. | This study |
| *LGP104 | MPAO1 | " | " | " | Tfr.f. L5B41 |
| ME2A43 | MPAO1 | 1,082,972 | PA1000 | Hypothetical. | This study |
| LGP110 | MPAO1 | N/D | *phnA* | Anthranilate synthase. | See Chapter 5 |
| MP551 | MPAO1 | 1,086,674 | PA1003 | Putative LysR-like transcriptional regulator. | Chapter 2 |
| PA2587 region | | | | | |
| MP562 | MPAO1 | 2,927,450 | PA2587 | Putative salicylate hydroxylase. | Chapter 2 |
| MF4A9 | MPAO1 | 2,928,247 | PA2587/ PA2588 | (between genes) | This study |
| Known regulators | | | | | |
| LGP101 | MPAO1 | N/D | *lasR* | *lasR* quorum-sensing regulator. | See Chapter 5 |
| MF6C13 | MPAO1 | 3,889,853 | *rhlI/R* | *rhl* quorum-sensing regulatory locus. | This study |
| MF4A18 | MPAO1 | 1,013,291 | *gacS* | Global regulator. | This study |
| MP502 | MPAO1 | 1,015,199 | " | " | Chapter 2 |
| Putative regulators | | | | | |
| MP501 | MPAO1 | 4,423,758 | PA3946 | Homologue of *bvgS*, *B. pertussis* 2-component virulence gene regulator. | Chapter 2 |
| ME1A62 | MPAO1 | 5,302,554 | *dksA* | Regulatory repressor of *rhlI*. | This study |
| MP553 | MPAO1 | 5,304,386 | PA4725 | Putative 2-component sensor kinase. | Chapter 2 |
| MP552 | MPAO1 | 5,304,811 | " | " | Chapter 2 |
| L4F93 | PAO1seq | 5,306,183 | " | " | This study |
| MD3C20 | MPAO1 | 5,482,018 | PA4886 | Putative 2-component sensor kinase. | This study |
| MD7A14 | MPAO1 | 6,192,554 | np20 | 54% similar to zinc-uptake regulator Zur (*E.coli*). | This study |
| Miscellaneous | | | | | |
| ME1A72 | MPAO1 | 447,569 | PA0406 | Hypothetical protein. | This study |
| MC6D13 | MPAO1 | 448,033 | " | " | This study |
| MB5A8 | MPAO1 | 812,005 | PA0744 | Probable enoyl-CoA hydratase/isomerase. | This study |
| MP504 | MPAO1 | 812,919 | PA0745 | Probable enoyl-CoA hydratase/isomerase. | Chapter 2 |
| MD1A8 | MPAO1 | 3,395,202 | PA3031/ PA3032 | (between genes) | This study |

* Strain was derived by transferring into MPAO1 the relevant allele from the stain listed immediately before.

[a] "Tfr.f." indicates "Allelic transfer from."

[b] It is unproven that the insertions in the *phzC1* and *phzE1* mutants are not insertions in *phzC2* and *phzE2*, respectively.

N/D, not determined.

The genomic location of each transposon insertion and the corresponding gene disrupted for each strain are shown in Table 3.

Genes of both known and unknown function were identified in the mutant set. Table 3 organizes the mutants into several groups based on the genes identified. Two major gene clusters were represented: the *phzM/phzA1-G1/phzS* cluster, which is known to encode phenazine biosynthesic enzymes (Chapter 1; Mavrodi et al., 2001), and a cluster surrounding *phnA* and *phnB* (refer to Figure 8). *phnA* and *phnB* encode the subunits of an anthranilate synthase, and have been shown to be required for complete pyocyanin production (Essar et al., 1990; Mavrodi et al., 2001). Numerous known and putative regulatory genes were also identified, including the quorum-sensing regulatory genes *lasR* and *rhlI*, the global regulator *gacS* (Parkins et al., 2001), np20, a homologue of Zur (*E. coli* zinc-uptake regulator) that was found to be induced by respiratory mucus from CF patients (Wang et al., 1996), and three putative two-component regulatory genes—PA3946 (*bvgS*-like), PA4725 and PA4886. Several miscellaneous loci of unknown function were also identified (Table 3).

Since the two supposedly identical strains analyzed (MPAO1 and PAO1seq) behaved differently with respect to pyocyanin production (see above), it was essential for further analysis that the new mutations be characterized in an isogenic strain background. Therefore, the transposon insertion alleles generated in PAO1seq were moved into strain MPAO1 using a technique under development in our laboratory for transferring genomic markers between *Pseudomonas* strains (Materials and Methods). The technique relies on homologous recombination to allow replacement of a locus of interest with a homologous

selectable allele introduced into the recipient strain by transformation of chromosomal DNA bearing the allele. The strains derived by this method have been included in Table 3. Each is marked with an asterisk and is listed immediately below the mutant from which the insertion allele was transferred. Inactivating alleles of *lasR* (from strain PAO-R1, Gambello and Iglewski, 1991) and *phnA* (from strain PECP100, unpublished and provided by E. Pesci) were also transferred into MPAO1, generating strains LGP101 and LGP110, respectively (Table 3).

**Pyocyanin is not required for cyanide production.**

To quantify the pyocyanin-deficiency phenotypes, we measured pyocyanin production for a subset of the mutants, representing most of the genes identified in the mutant set. As shown in Table, fifteen of the twenty mutants examined produced less than 10% of wild-type levels of pyocyanin, and all produced less than 32%.

To assess cyanide production by these strains we measuring paralytic nematode killing. Since cyanide mediates paralytic killing, reductions in killing indicate defects in cyanide production (Chapter 2). As shown in Table 4, many of the strains showed intermediate to severe defects in killing. However, strains ME3B56, LGP105, LGP106 and LGP102, which carry mutations in the genes *phzM*, *phzA1*, *phzE1* and *phzS*, respectively, of the core phenazine biosynthetic locus, did not display significant killing

defects, suggesting that pyocyanin—and probably other phenazines—are not essential for cyanide production in *P. aeruginosa*.

**TABLE 4: Exoproduct and PQS production by *P. aeruginosa* mutants.**

| Strain | Gene | Pyocyanin[a] | | % Killing[a] | | PQS |
|---|---|---|---|---|---|---|
| PAO1 | | (1.00) | | 98 | (±2) | |
| *phz1* cluster | | | | | | |
| ME3B56 | *phzM* | 0.01 | (±0.00) | 100 | (±0) | Yes |
| LGP105 | *phzA1* | 0.04 | (±0.01) | 99 | (±1) | Yes |
| LGP106 | *phzE1* | 0.17 | (±0.02) | 99 | (±1) | Yes |
| LGP102 | *phzS* | 0.02 | (±0.00) | 78 | (±11) | Yes |
| *phnAB*-region | | | | | | |
| LGP103 | PA0998 | 0.04 | (±0.00) | 36 | (±8) | No |
| LGP104 | PA0999 | 0.02 | (±0.01) | 14 | (±2) | No |
| ME2A43 | PA1000 | 0.01 | (±0.01) | 25 | (±8) | Yes |
| LGP110 | *phnA* | 0.04 | (±0.02) | 37 | (±8) | No |
| MP551 | PA1003 | 0.01 | (±0.00) | 12 | (±10) | No |
| PA2587 region | | | | | | |
| MP562 | PA2587 | 0.02 | (±0.01) | 9 | (±4) | No |
| Known regulators | | | | | | |
| LGP101 | *lasR* | 0.01 | (±0.00) | 2 | (±1) | No |
| MF6C13 | *rhlI/R* | 0.02 | (±0.00) | 11 | (±8) | Yes |
| MP502 | *gacS* | 0.10 | (±0.01) | 50 | (±5) | Yes |
| Putative regulators | | | | | | |
| MP501 | PA3946 | 0.31 | (±0.02) | 22 | (±7) | Yes |
| MP552 | PA4725 | 0.19 | (±0.04) | 19 | (±8) | Yes |
| MD3C20 | PA4886 | 0.29 | (±0.03) | 40 | (±6) | Yes |
| MD7A14 | np20 | 0.00 | (±0.00) | 4 | (±3) | No |
| Miscellaneous | | | | | | |
| MC6D13 | PA0406 | 0.07 | (±0.00) | 48 | (±17) | Yes |
| MP504 | PA0745 | 0.03 | (±0.01) | 50 | (±13) | Yes |
| MD1A8 | PA3031/ PA3032 | 0.03 | (±0.01) | 1 | (±1) | Yes |

[a] Values listed are averages from three separate assays. Numbers in parentheses indicate standard errors of the mean.

**PQS production in pyocyanin mutants.**

The recently identified *P. aeruginosa* regulatory molecule PQS (pseudomonas quinolone signal) is believed to modulate phenazine production in *P. aeruginosa* by regulating the *rhl* quorum-sensing system (Figure 2; Mavrodi et al., 2001; McKnight et al., 2000). We decided to examine production of PQS by the mutant strains for two reasons. First, one of the genes identified, PA2587, had already been implicated in PQS production (E. C. Pesci, personal communication). Second, the recent finding that anthranilate is a precursor of PQS (Calfee et al., 2001) suggested that the mutations we isolated in genes adjacent to *phnA* and *phnB* (which encode anthranilate synthase subunits) could inhibit pyocyanin production indirectly by affecting PQS synthesis, rather than by directly disrupting phenezine biosynthetic enzymes. The strains in Table 4 were sent to E. C. Pesci, a collaborator at East Carolina University, who tested them for PQS production. Table 4 summarizes the results of the assays. As expected, the mutation in PA2587 indeed prevented PQS prodution. In addition, mutations in the genes PA0998, PA0999 and PA1003, which surround *phnA* and *phnB* (Figure 8), also prevented PQS production. Surprisingly, however, mutations in *phnA* itself or in PA1000, the gene immediately adjacent to *phnA*, did not prevent PQS production. The genomic context of the region (Figure 8) suggested the presence of a five-gene operon spanning from gene PA0996 to gene PA1000. Although complementation analysis has not yet been carried out to verify the absence of polar effects, we surmise that since mutations in PA0998 and PA0999 prevented PQS production, this operon may play a central role in PQS

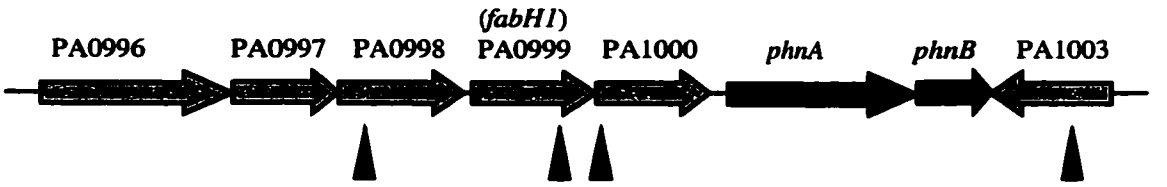


**Figure 8. The *phnAB* genomic region.** Note that all of the genes except PA1003 are transcribed in the same direction. Dark triangles indicate positions of the transposon insertions in strains LGP103, LGP104, ME2A43 and MP551, respectively (reading left to right). An inactivating mutation in *phnA* (strain LGP110, Table 3) is also used. *phnA* and *phnB* are shaded as a visual aid.

biosynthesis. BLAST analysis of the predicted proteins encoded by these genes indicated that some of them could perform functions important for PQS synthesis. Specifically, PQS contains a fatty acid moiety (see Figure 3), putatively derived from β-keto-decanoic acid (Calfee et al., 2001), and both PA0998 and PA0999 (also known as *fabH1*) carry motifs suggesting fatty acid biosynthesis: PA0998 is 47% similar to putative beta-ketoacyl-acyl carrier protein synthase III from Synechocystis sp., and PA0999 (*fabH1*) shows 55% similarity to *E. coli* acetyl-CoA ACP transacetylase. Therefore, these genes may be required for synthesis of the fatty-acid moiety of PQS. We hypothesize that the genes in the *phnAB* region, together with PA2587, constitute a PQS biosynthetic locus.

**Effects of mutations on expression of quorum-sensing-regulated genes.**

A complex and hierarchical network of interacting regulatory factors governs virulence factor production in *P. aeruginosa* (Chapter 1). Central to this network are the

*las* and *rhl* quorum-sensing regulatory systems (Figure 2). Also participating are GacA and GacS, Vfr, QscR, Anr and PQS (Albus et al., 1997; Chugani et al., 2001; McKnight et al., 2000; Pessi and Haas, 2000; Reimmann et al., 1997). Many of these genes control transcription of other regulatory genes in the network (Chapter 1 and Figure 2).

In addition to the genes implicated in PQS synthesis, several new putative regulatory genes were identified in our mutant set (Table 3). These include three (PA3946 (a *bvgS* homologue), PA4725 and PA4886) that appear by homology to be elements of three different two-component regulatory systems. We wished to examine how these regulators, as well as the genes required for PQS synthesis, fit into the emerging hierarchy of regulatory factors identified in *P. aeruginosa*. To do this, we examined transcription in the various mutants of a number of genes shown to be quorum-sensing-regulated. For transcriptional reporters, we used *lacZ* reporter fusions isolated by Whiteley et al. (Whiteley et al., 1999).

Whiteley and colleagues have constructed a library of chromosomal *lacZ* reporter fusions to quorum-sensing-controlled (qsc) genes (Whiteley et al., 1999). They described 47 such fusion strains, and classified them according to their patterns of qsc gene induction in response to exogenous addition of the two acyl-homoserine lactone (HSL) autoinducers in a strain background that does not produce either autoinducer. Four classes were described: class I genes, which responded early (before stationary phase) to the addition of 3-oxo-$C_{12}$-HSL (the *las* autoinducer, Figure 3), class II genes, which responded late (after the onset of stationary phase) to the addition of 3-oxo-$C_{12}$-HSL, class III genes, which responded early to the addition of both 3-oxo-$C_{12}$-HSL and $C_4$-HSL

(the *rhl* autoinducer, Figure 3) and class IV genes, which responded late to the addition of both 3-oxo-$C_{12}$-HSL and $C_4$-HSL. We chose six of these strains, representing three of the four classes, and, using the chromosomal allele replacement technique described above, transferred these six reporter alleles into the chromosomes of MPAO1 and most of the mutant strains listed in Table 4 (Materials and Methods). The six qsc-gene reporter alleles chosen were qsc101 (class I), which is the gene *phzD2* of the *phzA2-G2* operon (see Chapter 1); qsc105 (class I), which is the gene PA2587 itself, one of the genes we found to be required for PQS synthesis (Table 4); qsc118 (class III), which is *rhlI*, encoding the $C_4$-HSL autoinducer synthase; qsc128 (class III), which is *hcnB* of the hydrogen cyanide biosynthetic operon *hcnABC*; qsc131 (class III), which is *phzC1* of the *phzA1-G1* operon, known to be required for full pyocyanin production (see Chapter 1); and qsc135 (class IV), which is *cytC*, encoding a cytochrome C precursor.

To determine the optimal growth phase at which to compare the qsc gene expression levels in the various mutants, we monitored expression of each of the six fusion alleles in the wild-type (MPAO1) background during growth in liquid culture. Figure 9 shows representative plots of culture density ($OD_{600}$) and β-galactosidase levels for all six strains over fifteen hours of growth, starting from innocula at an $OD_{600}$ of 0.02. The shapes of all six qsc–*lacZ* expression curves were similar, though the scales varied considerably (~200 β-gal units for the *phzD2–lacZ* reporter, and more than 5000 β-gal units for the *rhlI–lacZ* reporter). Note that the growth phase at which β-galactosidase activity was first detected varied somewhat between reporter strains, ranging from mid-

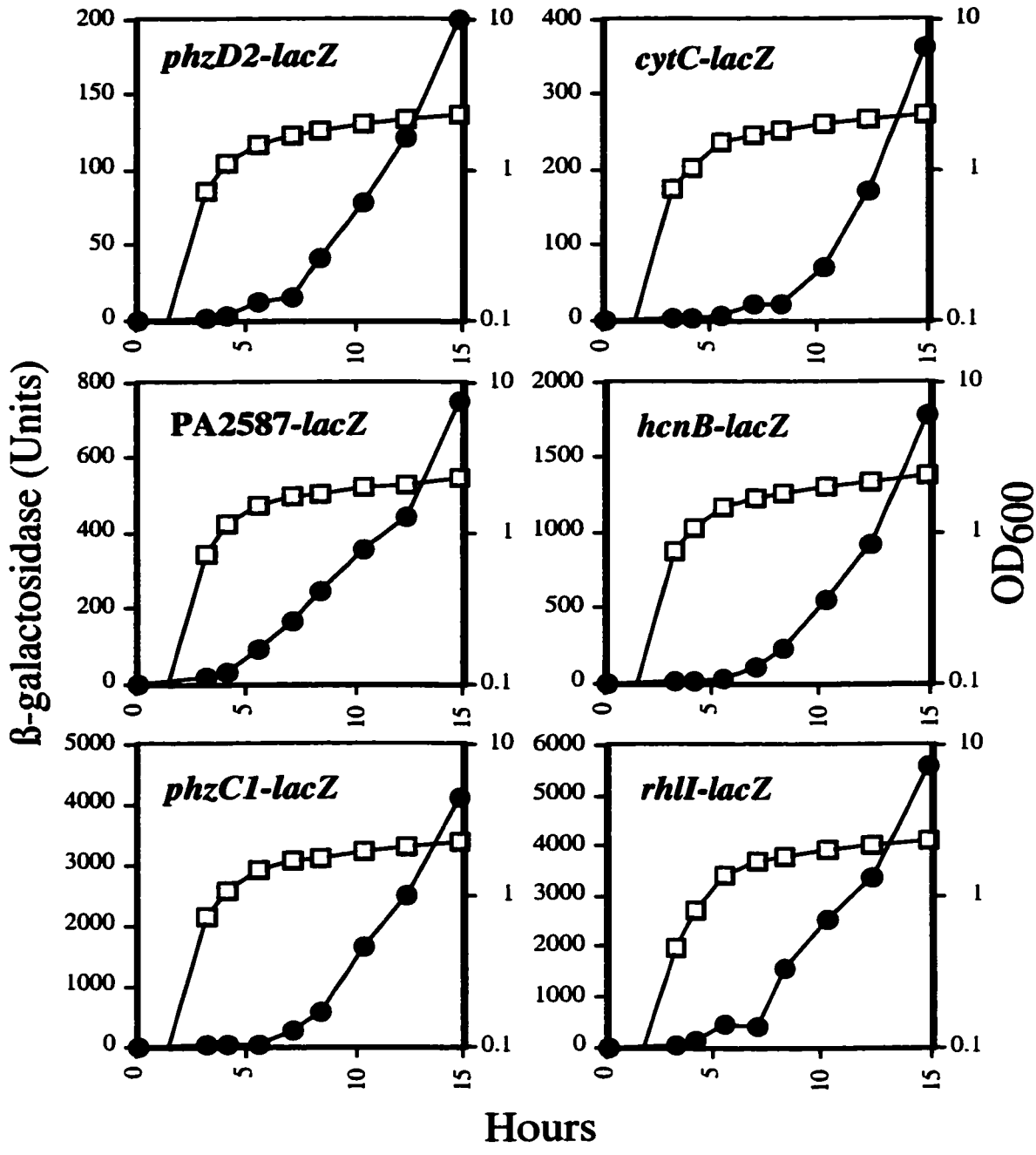


**Figure 9. β-galactosidase activity (filled circles) and culture density (open squares) measured over time for chromosomal *lacZ* reporter fusions to quorum-sensing-controlled (qsc) genes.** All fusion constructs were in wild-type MPAO1 background. Starting innoculum was at $OD_{600}$ of ~0.02 for all cultures. Similar results were obtained in multiple assays for all six strains.

log to early stationary phase (Figure 9). For all six strains, however, the β-galactosidase levels reached a linear rate of increase by mid-stationary phase, and the slopes showed no signs of leveling off, even after fifteen hours of incubation (Figure 9). The results were reproducible in multiple trials. Since β-galactosidase levels were well established and reproducible by thirteen hours of growth for all strains, we chose the thirteen-hour time point as a comparison point for assaying expression levels in the different mutant backgrounds.

Table 5 shows β-galactosidase levels measured at the thirteen-hour time point in 114 bacterial strains. These 114 strains represent the six qsc-gene–*lacZ* reporter alleles transferred individually into each of the mutant and wild-type (MPAO1) strain backgrounds. To make the table easier to read, β-galactosidase levels for the reporter alleles in the wild-type background are given in absolute β-gal units, and, for each reporter allele, β-galactosidase levels in the mutant backgrounds are shown as percentages relative to the average level in the wild-type background (Table 5). Multiple independent β-galactosidase assays were carried out for each strain, and good reproducibility was observed (standard errors are included in the table). For many of the mutation/reporter categories, two different isolates of the relevant strain were constructed and tested, and both strains are reported in the table. In all such cases but one (the *phzC1–lacZ* reporter in the PA3946 mutant background; see below for discussion), the two different isolates behaved comparably (Table 5). Note that two of the qsc genes whose expression was measured, *rhlI* and PA2587, are also represented in the set of

**Table 5: ß-galactosidase activity in mutant strains with chromosomal *lacZ* reporter fusions to quorum-sensing-controlled genes.**

| | *phzD2-lacZ* | | | *rhlI-lacZ* | | | *hcnB-lacZ* | | | PA2587-*lacZ* | | | *phzC1-lacZ* | | | *cytC-lacZ* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mutation | strain | n | ß-gal Units | strain | n | ß-gal Units | strain | n | ß-gal Units | strain | n | ß-gal Units | strain | n | ß-gal Units | strain | n | ß-gal Units |
| w-t | LGP201.1 | 5 | 169 (±15) | LGP221.1 | 3 | 4,227 (±174) | LGP241.1 | 6 | 999 (±32) | LGP261.1 | 4 | 605 (±23) | LGP281.1 | 6 | 1,904 (±181) | LGP301.1 | 4 | 175 (±16) |
| | LGP201.2 | 3 | 194 (±18) | LGP221.2 | 2 | 4,255 (±404) | LGP241.2 | 2 | 973 (±24) | LGP261.2 | 2 | 537 (±19) | LGP281.2 | 3 | 1,298 (±75) | LGP301.2 | 2 | 185 (±19) |
| | | | (178=100%) | | | (4,238=100%) | | | (993=100%) | | | (562=100%) | | | (1,702=100%) | | | (179=100%) |
| PA0998 | LGP203.1 | 2 | 111% (±16%) | LGP223.1 | 2 | 94% (±4%) | LGP242.1 | 3 | 51% (±4%) | LGP262.1 | 2 | 86% (±4%) | LGP282.1 | 3 | 17% (±2%) | LGP302.3 | 2 | 82% (±16%) |
| PA0999 | LGP204.3 | 2 | 133% (±9%) | LGP224.1 | 2 | 87% (±7%) | LGP243.1 | 2 | 35% (±5%) | LGP263.1 | 2 | 84% (±1%) | LGP283.1 | 3 | 4% (±0%) | LGP303.1 | 2 | 77% (±12%) |
| PA1000 | LGP212.1 | 2 | 116% (±10%) | LGP232.1 | 2 | 90% (±5%) | LGP248.1 | 2 | 36% (±3%) | LGP268.1 | 2 | 86% (±2%) | LGP284.1 | 3 | 3% (±0%) | LGP304.1 | 2 | 86% (±14%) |
| | | | | LGP232.3 | 2 | 90% (±8%) | | | | | | | | | | | | |
| *phnA* | LGP217.2 | 2 | 102% (±19%) | LGP220.1 | 2 | 92% (±6%) | LGP254.1 | 2 | 45% (±4%) | LGP274.1 | 2 | 84% (±5%) | LGP294.1 | 3 | 15% (±2%) | LGP316.1 | 2 | 88% (±21%) |
| PA1003 | LGP210.4 | 2 | 129% (±21%) | LGP230.1 | 2 | 87% (±7%) | LGP247.1 | 3 | 32% (±1%) | LGP267.1 | 3 | 83% (±2%) | LGP285.1 | 5 | 6% (±1%) | LGP305.1 | 3 | 89% (±11%) |
| | LGP210.3 | 2 | 154% (±17%) | LGP230.2 | 3 | 91% (±5%) | LGP247.3 | 2 | 32% (±1%) | LGP267.3 | 2 | 78% (±3%) | LGP285.2 | 3 | 4% (±1%) | | | |
| PA2587 | LGP207.1 | 3 | 134% (±15%) | LGP227.1 | 2 | 87% (±9%) | LGP245.1 | 3 | 38% (±2%) | X | | | LGP286.1 | 5 | 8% (±2%) | LGP306.1 | 3 | 83% (±9%) |
| | LGP207.2 | 2 | 102% (±20%) | LGP227.2 | 2 | 88% (±7%) | | | | | | | | | | LGP306.2 | 2 | 86% (±15%) |
| *lasR* | LGP218.1 | 2 | 6% (±0%) | LGP236.3 | 2 | 5% (±0%) | LGP253.1 | 2 | 18% (±0%) | LGP273.1 | 3 | 9% (±2%) | LGP292.1 | 3 | 2% (±0%) | LGP312.1 | 2 | 51% (±3%) |
| | LGP218.3 | 2 | 4% (±0%) | LGP236.4 | 2 | 5% (±1%) | LGP253.2 | 2 | 7% (±5%) | | | | | | | LGP312.3 | 2 | 64% (±6%) |
| *rhlI/R* | LGP214.2 | 3 | 118% (±6%) | X | | | LGP250.1 | 3 | 109% (±15%) | LGP270.2 | 2 | 87% (±4%) | LGP290.1 | 3 | 53% (±5%) | LGP310.1 | 3 | 85% (±9%) |
| | LGP214.4 | 2 | 96% (±12%) | | | | LGP250.3 | 2 | 103% (±16%) | LGP270.3 | 2 | 88% (±0%) | | | | | | |
| *gacS* | LGP208.2 | 2 | 69% (±10%) | LGP228.1 | 3 | 67% (±1%) | LGP252.1 | 2 | 100% (±26%) | LGP272.1 | 3 | 145% (±7%) | LGP291.1 | 3 | 50% (±16%) | LGP311.2 | 2 | 53% (±7%) |
| | LGP208.3 | 2 | 26% (±2%) | LGP228.2 | 2 | 71% (±4%) | | | | | | | LGP291.3 | 3 | 52% (±17%) | LGP311.3 | 2 | 49% (±5%) |
| PA3946 | LGP209.3 | 2 | 939% (±175%) | LGP229.2 | 2 | 58% (±0%) | LGP246.1 | 3 | 24% (±1%) | LGP266.1 | 3 | 46% (±0%) | LGP289.1 | 3 | 41% (±15%) | LGP309.1 | 3 | 74% (±6%) |
| | LGP209.1 | 2 | 1016% (±141%) | LGP229.1 | 2 | 60% (±0%) | LGP246.2 | 2 | 21% (±1%) | LGP266.2 | 1 | 51% | LGP289.2 | 3 | 139% (±11%) | | | |
| PA4725 | LGP206.1 | 3 | 80% (±3%) | LGP226.1 | 3 | 110% (±3%) | LGP244.1 | 3 | 175% (±26%) | LGP264.1 | 2 | 132% (±3%) | LGP287.1 | 3 | 68% (±12%) | LGP307.1 | 2 | 105% (±22%) |
| | LGP206.2 | 2 | 38% (±3%) | | | | LGP244.3 | 2 | 163% (±16%) | LGP264.2 | 2 | 134% (±6%) | LGP295.1 | 3 | 63% (±6%) | LGP307.3 | 2 | 97% (±13%) |
| PA4886 | LGP213.1 | 3 | 94% (±6%) | LGP233.1 | 2 | 93% (±7%) | LGP249.1 | 2 | 107% (±15%) | LGP269.1 | 2 | 91% (±4%) | LGP288.1 | 3 | 61% (±7%) | LGP308.1 | 2 | 86% (±10%) |
| | | | | LGP233.2 | 2 | 97% (±9%) | | | | LGP269.3 | 2 | 95% (±10%) | LGP288.3 | 3 | 76% (±11%) | | | |

Each of the six thick-bordered boxes shows ß-galactosidase levels measured for strains carrying the chromosomal reporter fusion indicated at the top of the box; refer to the far left column of the table for the mutant backgrounds of the strains listed.

For each of the six reporter fusions, ß-galactosidase values in all mutant strains carrying that reporter are normalized to the average of the measurements for the reporter in the wild-type background. Standard errors of the mean are listed paranthetically. n, number of assays performed. X, no strains constructed.

mutant strains. For these two reporter alleles, no reporter strains were constructed in the *rhlI* and PA2587 mutant backgrounds, respectively, since the reporter allele would have merely replaced the mutant allele and therefore been uninformative (marked as 'X' in table 5).

The data in Table 5 present a wealth of findings bearing on our understanding of regulatory interactions in *P. aeruginosa*. Even if we confine ourselves to mutant strains showing greater than twofold effects in qsc-gene–*lacZ* transcription relative to the wild-type background, numerous interesting results emerge (Table 5). Most striking are the values reporting transcription of the *phzD2–lacZ* reporter in the PA3946 mutant background. The β-galactosidase levels measured were almost tenfold higher than the levels from the same reporter in the wild-type background, suggesting that under these growth conditions PA3946 (a homologue of *bvgS*, the global virulence gene regulator in *B. pertussis*) reppresses transcription of the *phzA2-G2* operon. Interestingly, the same mutation reduced (with smaller effects) transcription of three of the other reporter alleles, the *rhlI–lacZ* reporter (transcription was reduced to ~60% of wild-type levels), the *hcnB–lacZ* reporter (~23%) and the PA2587–*lacZ* reporter (~50%). Together these data suggest that PA3946 negatively regulates at least one gene while positively regulating others. This finding will be addressed further in the discussion. For one of the reporter alleles (*phzC1–lacZ*) in the PA3946 mutant background, the results from the two isolates tested were inconsistent (44% vs. 148%, Table 5). We cannot rule out the possibility that this result is due to an error made in construction of one of the strains.

Also striking are the transcription levels exhibited in mutant backgrounds of genes which we have shown affect PQS production (Table 4: genes PA0998, PA0999, PA1003 and PA2587). Two reporter alleles—*hcnB–lacZ* and *phzC1–lacZ*—showed significantly lower β-galactosidase levels in these mutant backgrounds compared to the wild-type background (30–50% and 4–18%, respectively). These results are consistent with the reported role of PQS as a regulator of *rhl* quorum-sensing (McKnight et al., 2000), which in turn controls *hcnB* and *phzC1* transcription (Mavrodi et al., 2001; Pessi and Haas, 2000) (see Figure 2). It is interesting that the nematode killing data and the pyocyanin production data for the corresponding mutant strains were consistent with the transcription data: percent killing and *hcnB–lacZ* transcription were both at intermediate levels, and pyocyanin production and *phzC1–lacZ* transcription were both at very low levels (Tables 4 and 5). These results suggest that effects at the transcriptional level can account for the observed effects on both killing and pyocyanin production in these mutants.

Curiously, for both the *hcnB–lacZ* and the *phzC1–lacZ* reporters, transcription was not only affected by mutations in the genes PA0998, PA0999, PA1003 and PA2587, which were shown to be important for PQS production, it was also affected, and equally so, by mutations in PA1000 or *phnA*—genes apparently not essential for PQS production (Tables 4 and 5). These results cause us to wonder whether PA1000 and *phnA* do in fact play a role in PQS synthesis. Perhaps other genes perform functions redundant with PA1000 and *phnA*, so defects in PA1000 or *phnA* do not eliminate PQS synthesis, but

still reduce it enough to affect transcription of *hcnB* and *phzC1*. *trpE* and *trpG*, for example, also encode an anthranilate synthase (Essar et al., 1990).

In the *lasR* mutant background, transcription of all the reporter alleles was decreased, and in most cases by more than a factor of ten (Table 5). Given that *lasR* has been shown to act upstream of most other components in the quorum-sensing hierarchy (Figure 2; Pesci and Iglewski, 1997), this result is not surprising. The dependence on *lasR* seen for PA2587–*lacZ* transcription is notable, however, since it provides an explanation for a previous observation that PQS is not produced in *lasR* mutant bacteria (Pesci et al., 1999). Recently it has also been shown that inhibiting PQS production by supplying an anthranilate analogue (anthranilate is a precursor of PQS) reduces production of elastase, a *las*-regulated gene product (Calfee et al., 2001). Therefore, some of the regulatory effects ascribed to *lasR* may in part act via PQS regulation, due to *lasR*'s transcriptional control over PA2587.

Unlike in the *lasR* mutant background, however, qsc gene expression in the *rhlR/I* mutant backgound (the transposon insertion is between the *rhlR* and *rhlI* genes) was not significantly affected for any of the six reporters, despite the clear reduction in both paralytic killing and pyocyanin production observed in the *rhlR/I* mutant (Tables 4 and 5). This result is very surprising since both *hcnB* and *phzC1* have been shown to be under *rhl* quorum-sensing control (Mavrodi et al., 2001; Whiteley et al., 1999). One possible explanation is that since the transcription assays were carried out at an earlier time point and under different growth conditions than the pyocyanin and killing assays, the *rhl* system may not have yet significantly contributed to gene regulation. Another

possibility is that the insertion, which is between the *rhlR* and *rhlI* genes, is not a null allele of *rhlI*; instead it may only partially reduce *rhlI* transcription, so that *rhl* target genes are still induced, but other processes important for production of cyanide and pyocyanin are adversely affected.

Mutations in the two other putative regulators investigated, PA4725 and PA4886, both of which encode putative two-component sensors, did not cause significant effects on transcription of the tested reported fusions. The observed effects on pyocyanin production and paralytic killing seen for these mutants (Table 4) may result from metabolic disturbances affecting availability of pyocyanin and cyanide precursors. Alternatively, their effects may be at a post-transcriptional level, or may come into play at later growth phases.

Finally, we note that in Table 5, mutations in any of the genes implicated in PQS production (PA0998–PA1003, PA2587) did not reduce transcription of *rhlI*. This finding appears to contradict results of McKnight, et. al, who showed that exogenously supplied PQS greatly enhanced *rhlI* transcription from a multi-copy plasmid in a *lasR* mutant background (McKnight et al., 2000). It may be that McKnight and colleagues' results were skewed by the pleiotropic effects of the *lasR* mutation and the artificiality of the assay conditions used. Alternatively, the two results may reflect important differences in *rhlI* transcription at different growth stages. These possibilities are discussed below.

# DISCUSSION

The experiments presented in this chapter reveal several significant characteristics of the regulatory circuitry controlling virulence gene expression in *P.aeruginosa*. Specifically, we have uncovered the identity of several genes needed for synthesis of the diffusible signaling molecule PQS, we have identified new regulatory genes affecting virulence gene expression, and we have elucidated pathways and mechanisms of interaction among the regulatory components and downstream effector genes in the regulatory hierarchy.

We initially set out to determine whether phenazine antibiotics, which had been associated with a form of nematode killing by *P. aeruginosa* strain PA14 ("fast killing") (Mahajan-Miklos et al., 1999), were needed for full virulence in the cyanide-mediated nematode killing by strain MPAO1 ("paralytic killing") that was described in Chapter 2. Mutants showing reduced producion of pyocyanin were isolated and tested for paralytic killing defects. Several mutants with severe defects in pyocyanin production showed normal killing (Table 4), indicating that pyocyanin is not required for paralytic killing by strain MPAO1. Turning to the genes affected in the mutant set, we noticed several that we suspected might play a role in synthesis of the *Pseudomonas* quinolone signal (PQS), the recently identified small regulatory molecule (Pesci et al., 1999). Testing for the presence of PQS (Table 4) revealed that mutations in four different genes, three adjacent to *phnAB* (PA0998, PA0999 and PA1003) and one unlinked (PA2587), eliminated PQS

production, indicating that these genes are needed for PQS biosynthesis. To determine how these and the other genes identified in the mutant set fit into the emerging virulence-gene regulatory network in *P. aeruginosa*, we examined in the various mutant backgrounds transcription of both downstream effector genes and several of the regulatory genes themselves (Table 5). The results revealed several important regulatory interactions, suggesting both additions and potential revisions to the accepted model of the quorum-sensing hierarchy.

The important findings in this final experiment are highlighted in Figure 10, which is a revised model of the regulatory hierarchy depicted in Figure 2. First, transcription of PA2587, one of the genes required for PQS synthesis, was severely reduced in a *lasR* mutant background (Table 5). Given that PQS is not produced in *lasR* mutant strains (McKnight et al., 2000), this finding suggests that PA2587 fits into the quorum-sensing hierarchy as the proposed link between the *las* and the *rhl* quorum-sensing systems (McKnight et al., 2000). This proposed role for PA2587 is shown in Figure 10. We do not know whether the other three genes implicated in PQS synthesis, PA0998, PA0999 and PA1003, are also regulated by *lasR*, but their importance in PQS production is also indicated in Figure 10. Incidentally, PA1003, which is transcribed from the opposite strand to PA0998, PA0999, PA1000 and *phnAB* (Figure 8), is presumably a regulatory gene (encoding a member of the LysR family of transcriptional regulators) (for review see Schell, 1993). Due to its genomic location and its imporance

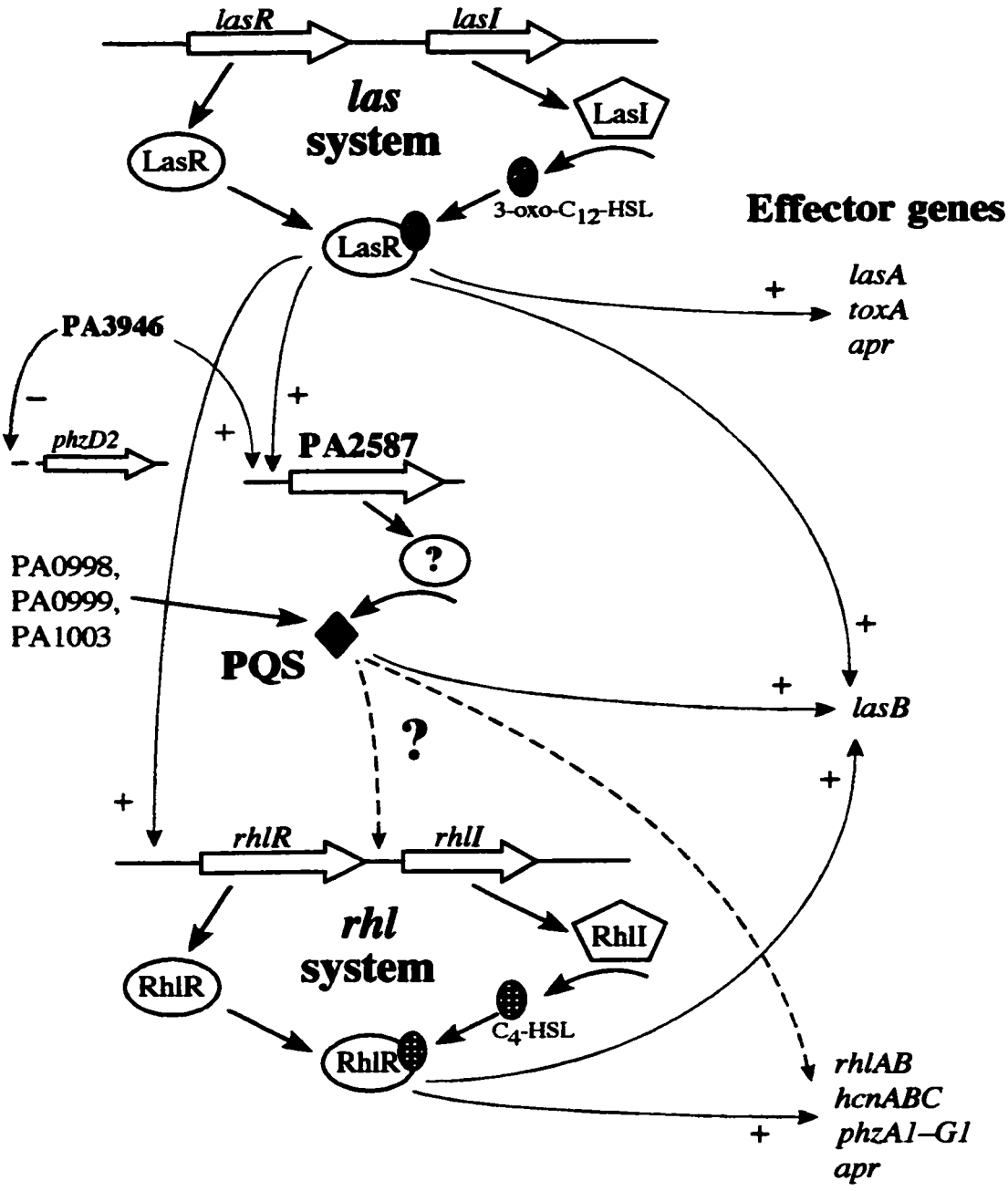


**Figure 10: Revised model of the quorum-sensing regulatory systems in *P. aeruginosa*.** Revisions and additions to the previous model (Figure 2) are indicated in red. See text for explanations of the changes.

in PQS production, this gene merits further investigation as a potential regulator of the other genes responsible for PQS synthesis.

Second, the putative two-component regulator PA3946 (a homologue of the *Bordetella pertussis* virulence-gene regulator *bvgS*) negatively controls transcription of some genes and positively controls transcription of others (Table 5). Since it appears to positively control PA2587, the predicted deficiency in PQS synthesis in the mutant could account for the effects observed on transcription of *hcnB*. PA3946's proposed influence on PA2587 transcription is shown in Figure 10. Unexpectedly, PA3946 appears to exert strong negative transcriptional control on *phzD2* (Table 5 and Figure 10). Precedent for its role as a repressor is found in its homologue in *B. pertussis*, where *bvgS* and its cognate response-regulator *bvgA* activate some genes and repress others (Beattie et al., 1992). Given the results in Table 5 and the close homology between PA3946 and *bvgS*, we surmise that PA3946 represents a novel virulence-gene regulatory factor that exerts both positive and negative regulatory effects in *P. aeruginosa*.

Third, mutations in genes required for PQS synthesis caused significantly reduced transcription of both *hcnB* and *phzC1*. Since *hcnB* and *phzC1* are both under *rhl* quorum-sensing control (Whiteley et al., 1999), these results were consistent with previous findings showing that PQS positively regulates *rhl* quorum-sensing through transcriptional effects on *rhlI* (McKnight et al., 2000). Surprisingly, however, transcription of *rhlI* itself, which is also under *rhl* quorum-sensing control (Whiteley et al., 1999), was not significantly affected by the mutations eliminating PQS production (Table 5). This result raises the possibility that, contrary to the previous findings, PQS

may not transcriptionally regulate *rhl* quorum-sensing. If this is so, then PQS may bypass the *rhl* system to regulate *hcnB* and *phzC1*. This possibility and the possibility that PQS does not affect *rhlI* transcription are indicated in Figure 10 by dashed lines with a question mark. It should be noted that the studies implicating PQS as a regulator of *rhlI* were based on a complicated expression analysis of a plasmid-born *rhlI–lacZ* reporter construct in a *lasR* mutant background, to which exogenous PQS was added (McKnight et al., 2000). The pleiotropies associated with *lasR* mutations and the artificial effects possible from the exogenous PQS addition in those studies may have caused amplified effects not seen under normal growth conditions. The same pleiotropies and possible artificial effects also make a straightforward comparison of the differing results difficult. A possible explanation for the disparity is that PQS exerts transcriptional control over *rhlI,* but only at later stages of growth. Our finding that *cytC*—a gene characterized as a late responder to *rhl* quorum-sensing control (Whiteley et al., 1999)—was also not transcriptionally affected by the PQS-abolishing mutations (Table 5) supports this hypothesis; perhaps at later growth stages the transcription of *cytC* and *rhlI* would be more significantly affected by the mutations eliminating PQS synthesis. Curiously, however, *rhlI*, unlike *cytC*, was characterized as an early responder to the homoserine-lactone autoinducers (Whiteley et al., 1999), yet it did not show a response to the absence of PQS. We conclude from these data that, at least at the growth stage examined, PQS does not significantly control *rhlI* transcription.

Thus, this systematic pair-wise analysis of mutant effects on quorum-sensing-regulated gene expression has produced a profusion of valuable findings, many of which deserve significant follow-up study. Several potential directions of further investigation are apparent: to investigate the importance of timing in the hierarchical interactions, a number of the mutant/reporter strains assayed in Table 5 could be examined again at later growth stages; to better understand the relationships between the various regulatory factors, additional fusion constructs, such as *lasR–lacZ, lasI–lacZ, rhlR–lacZ, lasB–lacZ* and PA0998–*lacZ,* could be studied; finally, it would be useful to examine effector-gene transcription in the presence of mutations in other implicated regulatory factors—such as *qscR* (a negative regulator of virulence genes), *rpoS* (stationary-phase sigma factor) and *anr* (anaerobiosis regulator).

The results presented in this chapter not only offer several novel findings, they also represent a proof-of-principle of the utility of this kind of pair-wise analysis. It should be noted that the prodigious strain construction needed for this analysis would have been far more cumbersome without the chromosomal allelic replacement technique described above. Nicole Benkers, Jeannie Bailey and other lab members developed the technique.

Regarding the genes implicated in PQS synthesis, two loci were identified: the *lasR*-regulated gene PA2587, and three genes surrounding *phnA* and *phnB* (Figure 8). Some suggestions for the functions of these genes come from the primary sequences of the encoded gene products. PA2587 encodes a protein 43% similar to salicylate hydroxylase from *Sphingomonas* sp. Its structural features suggest a probable FAD-

dependent monooxygenase, indicating that it may directly participate in synthesis of PQS, which carries an aromatic hydroxyl group (see Figure 3). The sequences of PA0998 and PA0999 show similarity to fatty-acid biosynthesis enzymes, also suggesting a possible connection to PQS synthesis (see results). PA1003, the LysR-family homologue, is most likely a regulator. Regulators in the LysR family activate target genes in response to the binding of a coinducer metabolite, and often control expression of adjacent genes that are divergently transcribed (Rhee et al., 1999). Therefore, it is possible that the PA1003 gene product controls expression of the operon containing PA0998 and PA0999. The coinducer molecule that binds the PA1003 regulator has not been identified. Except for *phnA* and *phnB*, none of the genes we have described at this genomic locus have received significant experimental attention.

*phnA* and *phnB*, however, present an interesting history of an unproven and questionable early hypothesis assumed to be true in later work. The genes were first characterized by Essar, et al. (Essar et al., 1990), who identified them as homologues of *trpE* and *trpG*, which are tryptophan biosynthesis pathway genes encoding anthranilate synthase subunits. Mutations in *phnA* and *phnB* (or *trpE* and *trpG*) did not create tryptophan auxotrophy, but *phnA*, *trpE* double mutants did. In addition, *phnA* and *phnB* could complement the tryptophan auxotrophy of *E. coli trpE* mutants. Together these results implied that *phnA* and *phnB* encode an anthranilate synthase. Since *phnA* and *phnB* were highly expressed in stationary phase rather than log phase, since they were not feedback inhibited by tryptophan, and since mutations in *phnA* resulted in a four- to five-fold decrease in pyocyanin production, the genes were ascribed a role in phenazine

biosynthesis rather than tryptophan biosynthesis (and the genes were therefore named *phnA* and *phnB*) (Essar et al., 1990). Essar and colleagues further proposed that since *phnA* and *phnB* encode an anthranilate synthase, anthranilate is likely an intermediate in phenazine biosynthesis (Essar et al., 1990). In support of this hypothesis the researchers offered the finding that the residual pyocyanin produced by the *phnA* mutant was eliminated in a *trpE, phnA* double mutant grown on supplemental tryptophan. "Apparently," the authors wrote, "the tryptophan-specific anthranilate synthase [*trpEG*] is capable of providing some anthranilate for pyocyanin production." (Essar et al., 1990). To explain a previous finding (Turner and Messenger, 1986) that radiolabeled anthranilate was not incorporated into pyocyanin, however, Essar et al. hypothesized that the *phnAB* gene products form part of multi-enzyme complex, such that the anthranilate produced is enzymatically channeled to the next active site, so exogenous anthranilate cannot enter the pathway (Essar et al., 1990). These last two points may be contradictory, unless anthranilate from the tryptopahan-specific anthranilate synthase is only able to enter the pyocyanin biosynthesis pathway in the absence of the *phnA* and *phnB* gene products. In any case, researchers have since referenced the results of Essar and colleagues in attributing phenazine biosynthesis functions to *phnA* and *phnB*. Mahajan-Miklos, et al., for example, noted that a transposon insertion affecting fast killing in strain PA14 was "immediately downstream of the phenazine biosynthetic genes *phnA* and *phnB*, as identified in *P. aeruginosa* strain PAO1" and referenced the work of Essar, et al. (Mahajan-Miklos et al., 1999).

However, the *phnA* and *phnB* gene products may play no direct role in phenazine biosynthesis. Given (i) that anthranilate has recently been identified as a PQS precursor (Calfee et al., 2001), (ii) that PQS levels may influence *rhl* quorum-sensing activity, thereby affecting expression of the phenazine biosynthetic locus *phzA1-G1* (see above), and (iii) this chapter's findings that mutations in several genes surrounding *phnAB* eliminate PQS production, the pyocyanin-deficiency phenotype observed in *phnA* mutants could be accounted for solely by a decrease in PQS synthesis. Additionally, recent biochemical analyses have suggested that anthranilate is probably not an intermediate in phenazine biosynthesis (Mavrodi et al., 2001). Thus, given their genetic context, *phnA* and *phnB* seem more likely to produce anthranilate primarily for the purpose of PQS biosynthesis. If this proves to be true, then *phnA*, *phnB* and the surrounding genes PA0998, PA0999 and PA1003 (as well as PA2587) might be more aptly named *pqs* genes.

In summary, while the regulation of virulence gene expression in *P. aeruginosa* is extraordinarily complex, involving dozens of interacting regulatory genes and signaling molecules, we have presented data uncovering several significant features of the network. Four genes required for the production of the small signaling molecule PQS have been pinpointed, several new regulators participating in the network have been identified, and a number of interactions between factors in the hierarchy have been elucidated.

# CHAPTER 4

## Additional Findings

In addition to the work presented in the previous two chapters, my graduate work included three smaller projects that deserve mention. They are described here.

### Construction and characterization of the transposon IS*phoA*/hah-Tc.

The transposable element used for much of the random mutagenesis of P. aeruginosa described in this dissertation was IS*phoA*/hah-Tc. It was constructed by replacing the chloramphenicol-resistance gene of its parent transposon, IS*phoA*/hah-Cm, with a tetracycline-resistance cassette (for construction, see Materials and Methods). This replacement was necessary for effective transposon mutagenesis in *P. aeruginosa* MPAO1, which is naturally chloramphenicol-resistant (see Kohler et al., 2001).

IS*phoA*/hah-Cm, a Tn*5*-based transposable element (de Lorenzo et al., 1990) conceived by Colin Manoil and constructed by Jeannie Bailey, carries a number of

sequence features that serve as powerful tools for geneticists and molecular biologists (Figure 11). First, like its predecessor IS*phoA* (Manoil and Beckwith, 1985), it carries at one end the coding region for a version of the *phoA* gene (alkaline phosphatase, AP) missing the signal sequence required for export of AP from the cytoplasm. Insertions of the transposon in the proper orientation and reading frame into expressed genes therefore result in translational fusions to *phoA*. If the expressed gene encodes an exported protein, then the AP portion of the protein fusion is also exported. Since AP is only active extracellularly and can be detected easily using substrates that produce color upon catalysis, the transposon can therefore be used to identify genes for exported proteins. Second, IS*phoA*/hah-Cm contains *loxP* recombination sites at both ends. As shown in Figure 11, if *cre* recombinase—whose substrates are the *loxP* sites (Van Duyne, 2001)—is transiently introduced into a cell carrying an IS*phoA*/hah-Cm insertion, then the bulk of the transposon is excised from the chromosome, leaving a 189-base-pair (63 codon) insertion. For in-frame transposon insertions, these 189 base pairs do not interrupt transcription or translation, and translation of the target gene then results in the insertion of 63 additional amino acids into the middle of the protein. The 63 amino-acid domain contains a hemagglutinin epitope and a hexahistidine moiety that can be used for protein purification. While the transposon was used solely as a mutagenic agent in the research presented in this dissertation, it was chosen over simpler transposons for the tools it offered to potential future analyses of the mutants. For example, *phoA* fusions can be used as reporters of gene induction, and purification of fusion proteins using the hemagglutinin epitope or hexahistidine features of the 63-amino-acid-insert form of the

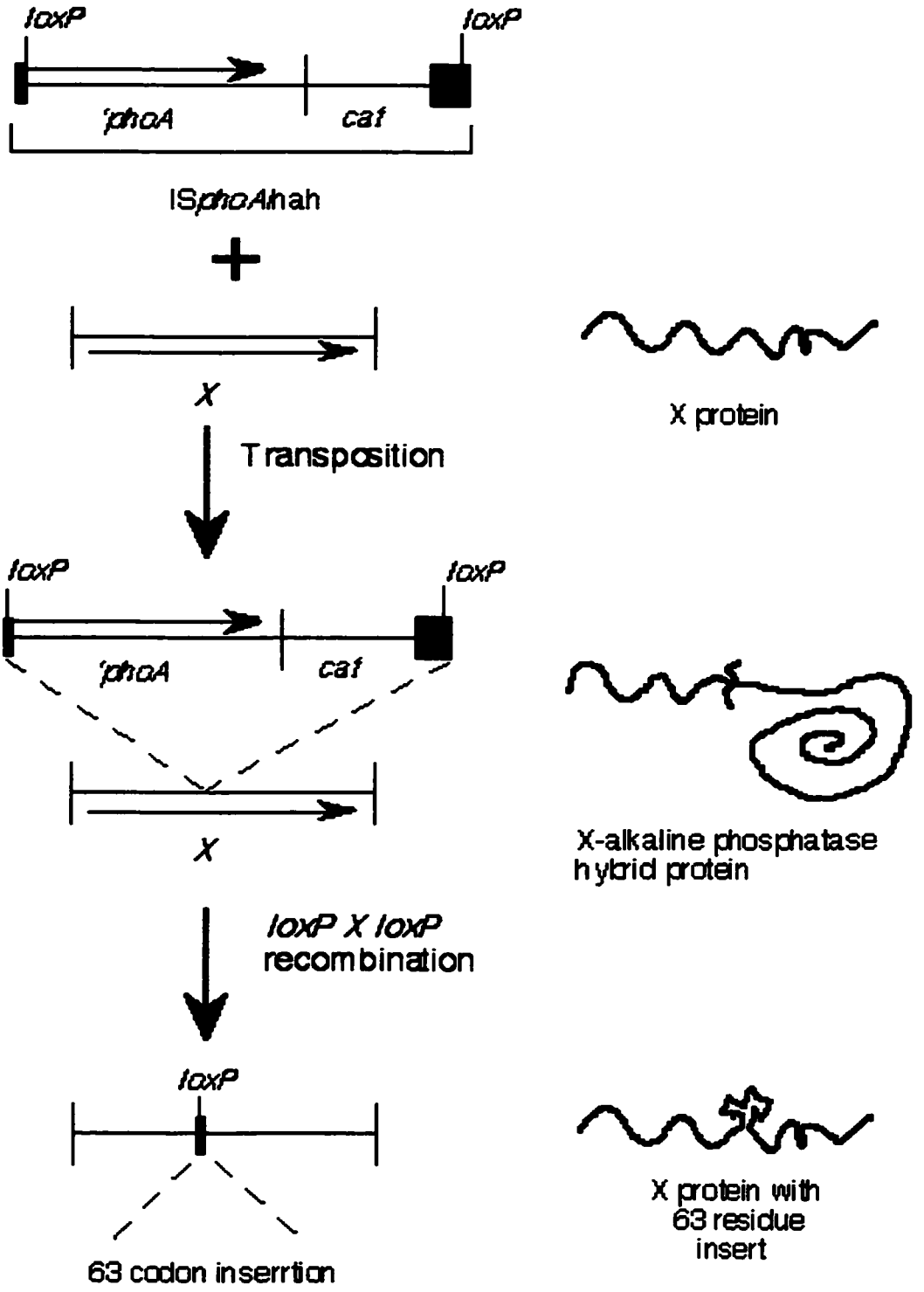


**Figure 11. Features of transposon IS*phoA*/hah-Cm.** In-frame insertions create alkaline-phosphatase fusion proteins. *loxP* X *loxP* recombination excises the bulk of the transposon, leaving a 189-base-pair insertion that codes for a 63-amino-acid insert in the protein. Figure provided by C. Manoil.

transposon could be useful for studies of protein function. In addition, *cre*-mediated excision of the bulk of the transposon restores tetracycline sensitivity, thus allowing further transposon mutagenesis or maintenance of $Tc^R$ plasmids in mutant strains.

To test the use of IS*phoA*/hah-Tc as a tool for identifying exported proteins in *P. aeruginosa*, insertion mutagenesis was performed in our standard lab strain MPAO1 (Materials and Methods). Transposants were selected on tetracycline agar containing XP for detection of alkaline phosphatase activity (Materials and Methods). Colonies showing blue color were picked and scored for the intensity of the color (light, medium or dark). In one test, 11 out of 364 colonies (~3%) were blue. This percentage may be high compared to the true proportion of genes for exported proteins in the genome, since a number of the blue colonies turned out to contain insertions in genes not encoding exported proteins (see below). In collaboration with Tran Nguyen and Jeannie Bailey, sixteen blue colonies were picked, and the transposon insertion sites were determined by PCR amplification and sequencing of the genomic DNA flanking the insertion, followed by comparison to the PAO1 genome sequence (Materials and Methods). Table 6 lists these insertion mutants.

**Table 6: IS*phoA*/Hah-Tc insertion strains.**

| Strain | Color | Insertion Point | Gene | In-frame Insertion? | Function | Exported? |
|---|---|---|---|---|---|---|
| MP1010 | Light blue | 1,162,218 | *braC* | Yes | Amino acid transport. | Yes |
| MP1007 | Light blue | 1,162,248 | *braC* | Yes | Amino acid transport. | Yes |
| MP1008 | Light blue | 2,809,533 | *mexE* | Yes | Multidrug efflux. | Yes |
| MP1002 | Light blue | 3,480,562 | *xcpT* | Yes | General secretion. | Yes |
| MP1018 | Medium blue | 22,522 | PA0020 | Yes | Unknown. | Unknown |
| MP1019 | Medium blue | 626,694 | PA0572 | Yes | Unknown. | Unknown |
| MP1036 | Medium blue | 4,260,589 | PA3801 | Yes | Unknown. | (Predicted TM domain) |
| MP1020 | Medium blue | 4,263,980 | PA3804 | Yes | Unknown. | Unknown |
| MP1021 | Medium blue | 4,898,840 | PA4370 | Yes | outer membrane metalloproteinase. | Yes |
| MP1012 | Medium blue | 5,678,213 | *pilO* | Yes | type 4 fimbria. | Yes |
| MP1035 | Medium blue | 5,678,608 | *pilP* | Yes | type 4 fimbria. | Yes |
| MP1004 | Dark blue | N/A | PA5369 | No | *pst* operon. | Unknown |
| MP1005 | Dark blue | N/A | *pstC* | No | *pst* operon. | (Predicted TM domain) |
| MP1022 | Dark blue | N/A | *pstA* | No | *pst* operon. | (Predicted TM domain) |
| MP1009 | Dark blue | N/A | *pstB* | No | *pst* operon. | Unknown |
| MP1001 | Dark blue | N/A | *pstB* | No | *pst* operon. | Unknown |

N/A – Not available.

Of the sixteen blue transposants sequenced, eleven were in-frame insertions (Table 6). Of the ten genes targeted in these eleven insertions, seven encode proteins known or predicted (by the presence of predicted transmembrane domains in their coding sequence) to be exported from the cytoplasm (Table 6). The other three genes with in-frame insertions are of unknown function. These results indicate that IS*phoA*/hah-Tc can be used to identify genes encoding exported proteins. Remarkably, however, five of the transposants (those forming the darkest blue colonies) did not contain in-frame insertions (Table 6). As shown in Table 6, all five of these strains carried insertions in genes of the *pst* operon, encoding functions for the transport of inorganic phosphate. We believe that these insertions result in constitutive expression of *P. aeruginosa*'s endogenous alkaline phosphatase(s). In *E. coli*, an intact Pst transport system is required for the normal

repression of *phoA* (and other *phoB*-regulated genes) when inorganic phosphate is plentiful (Wanner, 1996). Similarly, in *P. aeruginosa,* inactivating mutations in *phoU*, the last gene in the *pst* operon, cause constitutive alkaline phosphatase activity (Kato et al., 1994). Since the insertions we isolated should inactivate the Pst transport system and/or could be polar on *phoU*, loss of repression of endogenous *phoA* could explain the high levels of phosphatase activity seen in these mutants. To investigate this possibility, we selected two of the *pst* mutant strains (with insertions in *pstB* and *pstC*, respectively) for further analysis. The internal sequences of the transposon insertions in these strains were excised using the *loxP* recombination feature (Figure 11 and Materials and Methods), leaving 189-bp insertions that still caused the dark blue phenotype. We remutagenized these strains using the transposon mTn5-Tc (Materials and Methods) and picked colonies showing loss of the blue colony phenotype on XP agar. Two strains were picked, one from each of the original mutants. The transposon insertions in these two suppressor strains were found to be in the genes *phoB* and *phoR*, respectively, the two genes encoding the two-component regulatory system controlling the *pho* regulon (Anba et al., 1990). Therefore, the high levels of AP activity observed in the *pst* mutants depended on *phoB* and *phoR*, suggesting that, like in *E. coli*, disruption of the Pst transport system causes constitutive expression of the *pho* regulon (which includes *phoA*) (Anba et al., 1990). We note that this phenomenon will make large-scale screening for exported proteins using IS*phoA*/hah-Tc somewhat less efficient than hoped: highly expressed genes for exported proteins will be difficult to distinguish on plates from the very common *pst* mutants (all five of the dark blue colonies we isolated were *pst*

mutants). A possible solution would be to screen in a strain lacking endogenous alkaline phosphatases.

**Examination of *hcnABC* gene transcription in mutants defective in cyanide production.**

A broad variety of genes were identified in the screen for *P. aeruginosa* mutants unable to paralytically kill nematodes (Chapter 2 and Table 1). These genes included regulatory genes, genes for secreted proteins, and genes affecting metabolic pathways. We sought to examine which of the mutations prevented cyanide production by affecting *hcnABC* transcription, and which affected cyanide production by other means, such as by metabolic effects or via post-transcriptional regulation. Most of this work was carried out as a rotation project by Jesse Goldmark.

A plasmid (pJG1) carrying a transcriptional *hcnABC–lacZ* reporter construct was transformed into twelve of the mutants described in Chapter 2. The transformants were grown on BHI agar as for the worm-killing assay (Materials and Methods), and β-galactosidase levels were assayed at the 24-hour time point (when worm killing would normally be tested). The results are shown in Table 7.

**Table 7. ß-galactosidase activity after plate growth of mutants with plasmid pJG1.**

| Strain | Gene | ß-Gal Units | (SEM) |
|---|---|---|---|
| MPAO1 | **(MPAO1)** | **2,449 (100%)** | (±5%) |
| PAOR1 | **lasR** | **2%** | (±0%) |
| MP501 | **PA3946** | **5%** | (±1%) |
| MP504 | **fad-1** | **11%** | (±1%) |
| MP502 | **gacS** | **13%** | (±1%) |
| MP562 | **PA2587** | **16%** | (±2%) |
| MP551 | **PA1003** | **40%** | (±1%) |
| MP503 | **eda** | **108%** | (±10%) |
| PAOR3 | **qscR** | **117%** | (±3%) |
| MP506 | **proC** | **149%** | (±7%) |
| 64-15 | **PA4856** | **178%** | (±12%) |
| MP552 | **PA4725** | **184%** | (±3%) |
| MP508 | **PA0041** | **207%** | (±22%) |

It is clear from the data shown that the mutants tested fall neatly into two classes: those that express wild-type (or greater) levels of β-galactosidase activity, and those that express severely reduced levels (<16%). Only one mutant, the PA1003 mutant, displayed an intermediate level of expression (40%). The genes mutated in the six strains that showed wild-type or greater β-galactosidase levels were *eda* (Entner-Doudoroff aldolase, a central carbon metabolism enzyme), *qscR* (a negative regulator that interacts with the *las* quorum-sensing system), *proC* (proline biosynthesis), PA4856 (an uncharacterized two-component regulator), PA4725 (a two-component regulator described in Chapters 2 and 3) and PA0041 (homologue of *B. parapertussis* filamentous hemagglutinin). Four of these six genes—*eda*, *proC*, PA4725 and *fhaB*—had been implicated in paralytic killing (Chapter 2), implying that they are required for full cyanide production. Since the data in Table 7 indicate that *hcnABC* transcription is not diminished in these four mutant strains, we hypothesize that the cyanide deficiencies observed are due to post-transcriptional or

metabolic effects. Consistent with this hypothesis, *eda* and *proC* are known metabolic enzymes, suggesting that the mutations in these genes alter metabolite availability, such as by diminishing pools of glycine (the immediate precursor of cyanide). The effects of the mutations in PA4725 or *fhaB* are less clear. These mutations may influence post-transcriptional regulation of hydrogen cyanide synthase, since it has been shown that *hcnABC* gene-expression is regulated both transcriptionally and post-transcriptionally in *P. aeruginosa* (Pessi and Haas, 2001).

The genes mutated in the six strains that showed reduced β-galactosidase levels were *lasR* (the quorum-sensing regulator), PA3946 (the *bvgS*-like two-component regulator), *fad-1* (fatty-acid degradation), *gacS* (global two-component regulator that mediates *lasR* transcription), PA2587 (required for PQS biosynthesis; Chapter 3) and PA1003 (LysR-like regulator also needed for PQS production; Chapter 3). The transcriptional effects observed are easily explainable for most of these mutants: *lasR* and *gacS* have previously been implicated in regulating *hcn* transcription (see Chapter 1); PA1003 and PA2587 are both required for production of PQS, which regulates *hcnABC* transcription (Chater 3); and PA3946 is a regulatory protein that interacts with the quorum-sensing hierarchy (Chapter 3). The only transcriptional effect that is hard to explain is that of the *fad-1* mutant. However, since *fad-1* participates in fatty-acid degradation, it may therefore influence the levels of fatty-acids available for autoinducer synthesis.

It is interesting to compare Table 7 to Table 5 (Chapter 3). Both tables report *hcnABC* transcription in various mutant backgrounds, but the conditions under which the

two experiments were carried out differed slightly: transcription was measured from the chromosome in Table 5 and from a multi-copy plasmid in Table 7, and the assays were done at different growth phases and under different growth conditions in the two experiments. Nonetheless, there is generally good agreement between the two tables. In both cases, significant transcriptional defects were seen in *lasR*, PA3946, PA2587 and PA1003 mutants, and wild-type or better transcription was seen in the PA4275 mutant. The only conspicuous discrepancy seen was for the *gacS* mutant: wild-type levels of *hcn* expression were seen for the chromosomal reporter (Table 5), but only 13% of wild-type expression was observed for the plasmid-born reporter (Table 7). This difference may be due to differences in growth phase or differences in reporter construct copy number. In either case, the experiment suggests that *Pseudomonas* researchers need to be careful when comparing results obtained using different experimental designs. In no case is this more true than for studies of the regulatory hierarchy in *P. aeruginosa*, where experiments from which important conclusions are drawn have varied drastically in the methodologies and strategies employed.

**Nematode response to Methyl Viologen.**

The finding that hydrogen cyanide poisoning underlies paralytic nematode killing by *P. aeruginosa* PAO1 prompted us to examine the mechanism by which *egl-9* mutant worms resist this killing. Since cyanide inhibits a number of enzymes that participate in

oxygen metabolism, including cytochrome oxidase, catalase, peroxidase and superoxide dismutase (Blumer and Haas, 2000; Solomonson, 1981; Way, 1984), we examined the sensitivity of the mutant worms to agents affecting oxygen metabolism. Methyl viologen (also known as paraquat) is a redox active substance that increases mitochondrial superoxide production and slows development of wild-type *C. elegans* (Hartman et al., 1995; Ishii et al., 1990). Indeed, when exposed to 0.2 mM methyl viologen (Materials and Methods), L1 N2 larvae reached adulthood approximately one day later than in the absence of the compound (Figure 12). In contrast, *egl-9* worms showed a surprising sensitivity to methyl viologen (Figure 12): at 0.2 mM methyl viologen, L1 *egl-9* larvae failed to reach adulthood even three days after animals that had not been exposed reached adulthood. Indeed, many of the exposed animals appeared arrested at the L3 to L4 stages (data not shown). Thus, *egl-9* worms are hypersensitive to methyl viologen. No strong differences were observed between wild-type and *egl-9* worms in their sensitivity to several other agents affecting oxygen metabolism, including hydrogen peroxide, azide, cadmium and pure oxygen (data not shown).

Recently it has been discovered that EGL-9 acts as an oxygen sensor in *C. elegans* (Epstein et al., 2001). According to those studies, EGL-9 utilizes molecular oxygen to hydroxylate proline residues on the transcription factor HIF-1 (hypoxia-inducible factor). This proline-hydroxylation targets HIF-1 for proteolytic degradation, thus preventing it from forming a transcription complex and activating target genes. Under hypoxia, the model states that EGL-9 no longer hydroxylates HIF-1, and HIF-1 can then activate transcription of hypoxia-inducible genes (Epstein et al., 2001). These

findings help explain the observation that *egl-9* worms are hypersensitive to methyl-viologen, since in the absence of EGL-9 function, HIF-1 is likely to constitutively activate hypoxia-inducible genes such as mitochondrial respiratory-complex genes. Excessive mitochondrial respiration should lead to enhanced mitochondrial superoxide production in response to methyl viologen exposure, thus slowing worm development excessively.

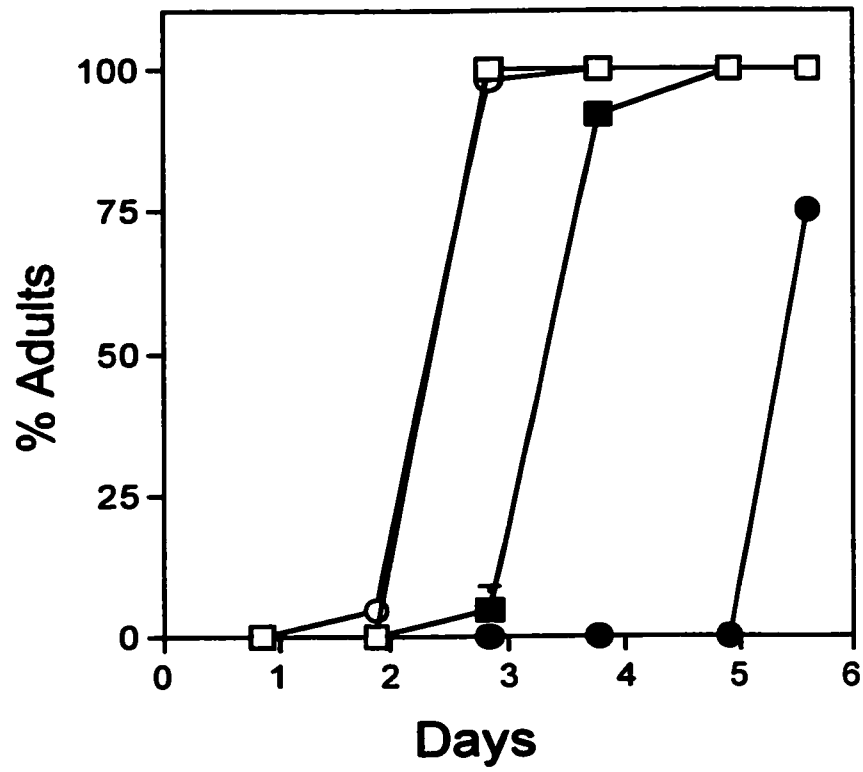


**Figure 12. C. elegans growth in the presence of methyl viologen.** Freshly-laid wild-type (squares) or *egl-9* (circles) embryos were grown on seeded NGM agar (open symbols) or seeded NGM agar containing 0.2 $\mu$M methyl viologen (closed symbols). Each data point represents the average from duplicate experiments.

EGL-9's role as an oxygen sensor also brings to light the resistance *egl-9* worms display against paralytic-killing by *P. aeruginosa*. The hypoxia-inducible genes regulated by HIF-1 include both mitochondrial respiratory-complex genes and genes encoding glycolytic enzymes. Therefore, increased ATP production from the enhanced respiration and glycolysis in *egl-9* worms may provide a buffer against the effects of cyanide-caused inhibition of respiration, thus making the worms more tolerant to the poisonous effects of cyanide at the concentrations produced by *P. aeruginosa*.

The findings presented here therefore provide some closure to the story of an interesting virulence interaction discovered between *P. aeruginosa* and *C. elegans* (Darby et al., 1999; Darby, 1998). Both sides of the interaction have been elucidated: the poison used by the bacterium to kill the worms has been identified, and the mechanism of the resistance displayed by *egl-9* worms against this poisoning has been explained.

# CHAPTER 5

## Materials and Methods

**Strains, plasmids, growth media, and culture conditions.**

The *P. aeruginosa* strains used were PAO1 (2 isolates: PAO1 from the laboratory of B. Iglewski (which we also call MPAO1), and the isolate whose genome was sequenced (which we call PAO1seq)) (Holloway et al., 1979), PAO-R1, a *lasR* mutant of MPAO1 (Gambello and Iglewski, 1991), two *pvd* strains carrying transposon insertions in the PA2401 and PA2424 genes (provided by D. D'Argenio), the mTn*5*-Tc and IS*phoA*/hah-Tc (de Lorenzo et al., 1990) insertion mutants listed in Tables 1 and 3, and the constructed strains listed in Table 5. The *E. coli* strains used were DH5α (Sambrook et al., 1989) for plasmid construction and SM10λpir (Silhavy et al., 1984) for conjugal suicide plasmid delivery. The growth media used were brain heart infusion (BHI) agar (Difco), L agar (Sambrook et al., 1989), skim-milk agar (Sokol et al., 1979), King's B (King et al., 1954), low-phosphate succinate-minimal liquid media (LPSM) (Cox, 1986) and L broth. Plasmids were maintained in *P. aeruginosa* in media supplemented with

100 or 200 μg carbenicillin per ml and in *E. coli* in media supplemented with 100 μg ampicillin or 40 μg tetracycline per ml.

To construct plasmids used in *hcn* complementation, an 8,968 bp *XhoI* fragment carrying the *P. aeruginosa hcnABC* operon was gel purified from an *XhoI, BglII, ScaI* digest of Cosmid 011 from the PAO1 genome sequencing project (supplied by Matt Wolfgang and S. Lory), whose insert corresponds to nucleotides 2,396,530 to 2,441,543 from the PAO1 single contig sequence (www.pseudomonas.com). This *XhoI* fragment was cloned in both orientations into the *SalI* site of pUCP18 (Schweizer, 1991) to obtain pLG2 (Figure 4) and pLG3. pLG3 was then digested with *XbaI* and religated to obtain pLG4 (Figure 4). All constructs were confirmed by restriction analysis. For *hcnC* complementation assays, strain MP507 (Table 2) transformed with either pLG2, pLG4 or pUCP18 was tested in a standard worm killing assay after growth in individual chambers (see below) on BHI agar supplemented with 40 μg tetracycline per ml and 100 μg carbenicillin per ml.

The construction of IS*phoA*/hah-Tc was as follows: pCM636 (the plasmid pUT (Herrero et al., 1990) containing IS*phoA*/hah-Cm inserted internally into the *bla* gene of pUT; constructed by Jeannie Bailey) was digested with BstEII and Bsu36I, and treated with DNA polymerase Klenow fragment (New England Biolabs) to create blunt ends. This digestion excised the chloramphenicol-resistance marker from the IS*phoA*/hah-Cm portion of the plasmid. The 7,700-bp fragment from this digestion was gel purified. In a second digestion, the tetracycline-resistance cassette was excised from pUT-mTn5-Tc (de Lorenzo et al., 1990) by EcoR1 digestion. The 2,170-bp cassette was then treated with

Klenow fragment and gel purified. These two blunt-ended fragments were ligated together. Figure 13 schematically represents this construction. Note that two possible orientations for the insertion of the $Tc^R$ cassette into the former $Cm^R$-site of IS*phoA*/hah-Cm were possible. Plasmids of both orientations (F & R) were isolated (Figure 13) (the F orientation was the one used for further work in our laboratory). Subsequently, Tran Nguyen and Jeannie Bailey moved the position of the IS*phoA*/hah-Tc transposable element in the pUT vector so as to restore the full-length *bla* gene (other advantages which are beyond the scope of this description also existed for moving the position of the transposable element in the vector).

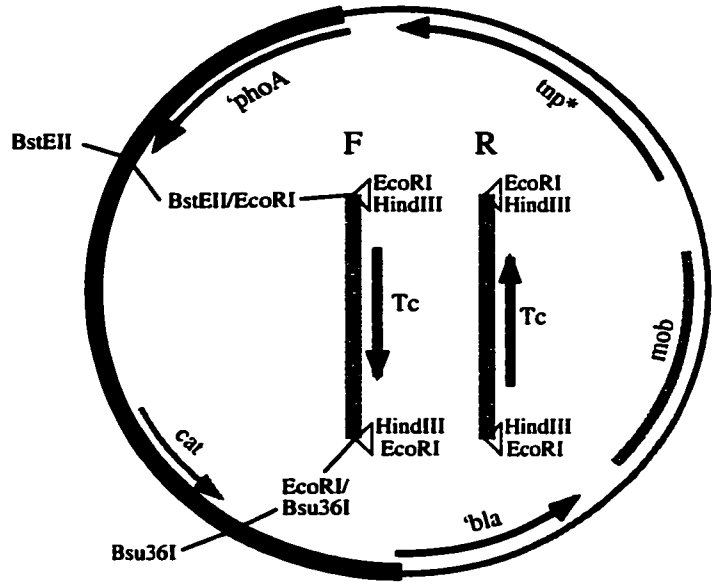


**Figure 13. Construction of IS*phoA*/hah-Tc.** The EcoRI fragment from mTn5-Tc was cloned into the gap left by digestion of pCM636 (a plasmid carrying IS*phoA*/hah-Cm with BstEII and Bsu36I (Materials and Methods), to create IS*phoA*/hah-Tc in forward (F) or reverse (R) orientation. IS*phoA*/hah-Cm is indicated by the thick part of the vector backbone. Other elements of the plasmid are labeled: tnp*, transposase; mob, mobilization functions; 'bla, truncated β-lactamase; 'phoA, partial alkaline phosphatase; cat, chloramphenicol resistance.

pJG1 (Chapter 4) was created by Jesse Goldmark by swapping the *hcnB–lacZ* transcriptional fusion allele from strain qsc128 (Whiteley et al., 1999) for the *hcnABC* fragment in either plasmid pLG4 or plasmid pLG2.

β-galactosidase assays were performed as described (Miller, 1992). For the β-galactosidase assays in Chapter 4, 24-h lawns grown as for nematode killing were resuspended at an $OD_{600}$ of ~0.2 and tested for β-galactosidase activity.

The *C. elegans* strains used were wild-type Bristol strain N2 and JT330, an *egl-9* mutant (Darby et al., 1999). Nematodes were grown at 22°C and handled using standard techniques (Brenner, 1974; Wood, 1988).

Standard molecular biology protocols were used throughout (Sambrook et al., 1989).

**Nematode paralytic killing assay.**

Unless indicated otherwise all paralytic killing assays were carried out by spreading 150 µl of a two to seven day-old *P. aeruginosa* colony suspended in BHI broth at an optical density at 600 nm ($OD_{600}$) of ~0.1 onto a 3.5-cm-diameter BHI agar plate containing 4 ml BHI agar. After the plate was incubated for 24 hours at 37° C, N2 nematodes from stock plates were collected in M9 buffer and a 50-µl aliquot (containing 20–200 adult animals) was spotted onto the *P. aeruginosa* lawn. The plate was then

incubated for four hours at room temperature with the lid on, and paralytic nematode killing was scored visually using a dissecting microscope. As described previously (Darby et al., 1999), worms were scored as dead if they did not move spontaneously and did not respond detectably to tapping of the assay plate against the microscope stage. For experiments in which individual chambers were used, the 3.5-cm-diameter plate was enclosed in a 10-cm-diameter petri plate which was then either sealed with parafilm (sealed chamber) or left unsealed (unsealed chamber).

**Transposon mutagenesis of PAO1.**

Transposon insertion mutants were generated using transposons mTn*5*-Tc (de Lorenzo et al., 1990) and IS*phoA*/hah-Tc, a transposon Tn*5* derivative described in Chapter 4 and above; MP501, MP551 and the mutants generated for Table 3 were generated using IS*phoA*/hah-Tc; MP508 was generated using Tn*5* (Darby, 1998). For transposon mutagenesis, a 37° overnight aerated culture of *E. coli* SM10 λpir/pUT-mTn*5*-Tc (de Lorenzo et al., 1990) or SM10 λpir/pUT-IS*phoA*/hah-Tc grown in LB supplemented with 100 μg ampicillin per ml was diluted 1:10 into fresh L broth containing ampicillin and grown with aeration for 45 minutes at 37°. A 0.5-ml aliquot of this culture was mixed with 0.5 ml of a 42° C nonaerated overnight L broth culture of PAO1. The mixture was filtered using a Nalgene analytical test filter (pore size, 0.45

μm) and washed with 1 ml of 10 mM $Mg_2SO_4$. The filter was then removed from the apparatus, transferred to an L agar plate (cell side up), incubated at 37° for 1 hour to allow conjugation and transposition to occur, then transferred to a test tube containing 1 ml of L broth, and the cells were washed from the filter by vortexing. Cells were plated onto L agar containing 10 μg of chloramphenicol per ml to counterselect against *E. coli*, 60 μg tetracycline per ml to select for growth of *P. aeruginosa* cells carrying transposon insertions, and (when in-frame insertions were sought) 40 μg of XP (5-bromo-4-chloro-3-indolyl phosphate) per ml to indicate alkaline phosphatase activity. Individual colonies appeared after one to two days of incubation at 37°C.

For *cre*-recombinational excision of IS*phoA*/hah-Tc insertions (see text), *cre*-recombinase carried on pUT was conjugated into the recipient strain using the same method described above for transposon delivery, except that mating was performed on an L-agar plate rather than on a filter, and after mating, the mixture was streaked onto media that selected against *E. coli* and contained XP to screen for excisants (white), which appeared at a high frequency (>10%).

**Mutant screening.**

To screen for nematode-nonkilling mutants, individual transposon insertion mutants were suspended in BHI at a density sufficient to make the broth visibly turbid.

150 μl of each suspension was plated onto a 3.5-cm-diameter BHI agar plate and after 24 h of incubation at 37°C worm killing was assayed. Strains which showed at least 10% reduction in killing compared to the wild type were saved and retested. Strains arising from 37 independent mutageneses were screened. To screen for pyocyanin mutants, individual mutants were either, (i), picked into LPSM (0.2 ml) in 96-well-format and examined for growth and color differences after 2 days of growth at 37°C, or (ii), plated on 3.5-cm-diameter BHI-agar plates as for worm killing, and examined for pigmentation defects after 24 h growth at 37°C.

**DNA sequencing.**

The chromosomal DNA flanking the transposon insertions was sequenced after semirandom PCR amplification or cloning. For semirandom PCR, a variation on a protocol described by Chun et al. (Chun et al., 1997) was used. For mTn5-Tc insertions, one μl of a 50-μl boiled single colony suspension in distilled $H_2O$ was used as the template DNA in a 20-μl PCR using primers MTN5I.1 (5'-CGAGGGCTTTACTAAGCTG-3') and either CEKG 2A (5'-GGCCACGCGTCGACTAGTACN$_{10}$AGAG-3'), CEKG 2B (5'-GGCCACGCGTCGACTAGTACN$_{10}$ACGCC-3') or CEKG 2C (5'-GGCCACGCGTCGACTAGTACN$_{10}$GATAT-3'); 1 μl of a 1:5 dilution of this reaction was used as the template DNA for a second PCR using primers MTN5O.1 (5'-

ATTCGTCGACAAGCTTCGG-3') and CEKG 4 (5'-GGCCACGCGTCGACTAGTAC-3'). For the first reaction, the thermocycler conditions were 94°C for 2 min, followed by 6 cycles of 94°C for 30 s, 42°C for 30 s (with the temperature reduced 1°C per cycle), and 72°C for 3 min and then 25 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 3 min; for the second reaction, the thermocycler conditions were 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 3 min. Samples that produced distinct bands on an agarose gel after the second reaction were treated with a PCR purification kit (Qiagen) to remove oligonucleotide primers, and were sequenced by using primer MNT5S.1 (5'-GACAAGCTTCGGCCGCCT-3'). For IS*phoA*/hah-Tc insertions, the identical protocol was used, only primer Tn*PhoA*-II (5'-GTGCAGTAATATCGCCCTGAGCA-3') replaced MTN5I.1, primer HAH-1 (5'-ATCCCCCTGGATGGAAAACGG-3') replaced MTN5O.1 and primer HAH-2 (5'-AAACGGGAAAGGTTCCGTCCA-3') replaced MNT5S.1. For cloning, chromosomal DNA digested with *PstI* was ligated into *PstI*-digested pUC18 (Yanisch-Perron et al., 1985). The ligation mixture was electroporated into DH5α, and transformants were selected with tetracycline. The chromosomal locations of the insertions were determined by BLAST analysis of the transposon-adjacent chromosomal DNA sequences compared with the complete PAO1 genome (www.pseudomonas.com).

**Chromosomal allelic replacement.**

For replacing chromosomal loci with homologous selectable alleles, the plasmid pNB1 (a shuttle vector carrying the λ-phage Red recombinase functions, based on pKD46 (Datsenko and Wanner, 2000)) was transformed into the recipient strain of interest and maintained with carbenicillin. The recipient strain carrying pλred was grown overnight without aeration at 42°C in LB supplemented with 25 mM $MgCl_2$ and 200 μg carbenicillin per ml, then subcultered (1:50) into the same media with 0.2% arabinose added to induce expression of the λred functions. 5 ml cultures were incubated at 37°C with aeration until the culture reached an $OD_{600}$ of 0.8 to 1.2, pelleted twice, resuspended in 1 ml of a 2% sucrose, 25 mM $MgCl_2$ solution, then pelleted and resuspended in 0.2 ml of a 2% sucrose solution (no Mg). Cells were kept cold throughout. ~1 μg of genomic DNA carrying the selectable donor allele (usually $Tc^R$ transposon insertion alleles) was electroporated into these cells (1.1 KV, 400 Ω, 24 μF). The cells were incubated 1 h at 37°C with aeration in LB supplemented with 25 mM $MgCl_2$, then plated on selective media. Most transformations yielded one to fifty transformants. For all assays using these transformants, the pλred plasmid was first cured from the strain by growing overnight in LB lacking carbenicillin but supplemented with tetracycline (for $Tc^R$ mutants) and containing 0.2% arabinose. Cells were screened for loss of the plasmid by replica plating.

**Exoproduct assays.**

To measure cyanide production, we used a protocol modified from a protocol generously supplied by D. Haas and based on the method of Gewitz et al. (Gewitz et al., 1976). Strains were grown on 3.5-cm-diameter BHI agar plates in individual unsealed chambers for 24 h at 37°C, then enclosed without lids in individual sealed chambers which also contained a 1-ml reservoir of 4 M NaOH (in an inverted 3.5-cm-diameter plate lid). After 4 hours incubation at 30°C, the NaOH was collected and diluted to 0.09 M with $ddH_2O$. If necessary, the sample was further diluted with 0.09 M NaOH to bring the cyanide concentration to within the linear range of the detection procedure (0-10 μM). The cyanide in the sample was quantified by comparison to standards of KCN dissolved in 0.09 M NaOH: 105-μl aliquots of the samples were mixed with 350-μl aliquots of a fresh 1:1 mixture of 0.1 M *o*-dinitrobenzene (Sigma) in ethylene-glycol monomethyl ether (Sigma) and 0.2 M *p*-nitrobenzaldehyde (Sigma) in ethylene-glycol monomethyl ether. After exactly thirty minutes of incubation at ambient temperature (22°C), the $OD_{578}$ was measured.

Pyocyanin production was assayed using a modification of the method of Essar et al. (Essar et al., 1990): 24-h plate cultures were grown as described for the nematode killing assay in unsealed individual chambers. The lawn-bearing agar from each plate was diced and extracted for 3 h with 4 ml of chloroform. The chloroform was then

extracted with one-seventh volume 0.2 M HCl, and pyocyanin in the aqueous phase was quantified by measuring the $OD_{520}$.

Pyoverdine production was assayed according to published methods (Cunliffe et al., 1995; Stintzi et al., 1998) by measuring the $OD_{404}$ (relative to that of *pvd* mutant strains generously supplied by David D'Argenio) of cell-free supernatants from saturated overnight 37°C aerated cultures grown in King's B medium and adjusted for culture density (King et al., 1954). Exoprotease production was assessed by spotting 5-$\mu$l aliquots of cultures at an $OD_{660}$ of ~0.1 onto skim-milk agar plates, incubating the plates overnight at 37°C, and measuring zones of clearance from the edges of the growth spots.

**Treatment of nematodes with exogenous compounds.**

For direct exposure to exogenous cyanide, nematodes were placed on a 3.5-cm-diameter BHI-agar plate without a lid, and this plate was sealed in a 10-cm-diameter petri plate containing an inverted 3.5-cm-diameter lid. The inverted lid contained separated 0.25-ml aliquots of 0.18 M HCl and defined amounts of KCN dissolved in 0.09 M NaOH. After sealing the 10-cm plate, the aliquots were mixed by tipping the plate, thus acidifying the cyanide solution and releasing HCN gas. For experiments in which cyanide exposure in the presence of bacteria was examined (Figure 6), worms were placed on a standard 24-h pregrown lawn of bacteria rather than an empty BHI-agar plate.

For exposure to methyl viologen, adult worms were allowed to lay eggs for three hours on standard NGM agar plates (Wood, 1988) containing methyl viologen and seeded with *E. coli* OP50. The adults were then removed and the larvae were scored for developmental stage over time.

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# Vita

Larry Gallagher, son of physicists, son of the mountains, was born, grew up and went to high school in Boulder, Colorado. He skied, rock climbed, ice skated, computer programmed, studied stars, attempted poetry, bicycled, tap-danced, ran thousands of pick-up basketball games at the park, and thrashed thousands of matches of 21 in the dirt court with the big bush inside the baseline. He went to college here and there, fell in love more than once, took some time off, hit Europe, entertained kids, owned a motorcycle, and graduated from the University of Colorado in May, 1992. His major, theatre and dance. After a brief stop in Denver he moved to New York City, visited Indonesia, then enrolled in the Bahá'í Faith. He trekked back to the University of Colorado. He studied hard, climbed more, made some mistakes, made some progress, and eventually got a second bachelor's degree in 1996. His major was molecular, cellular and developmental biology. He tried and failed to get into med school, then headed for grad school and the University of Washington. He did some studying, lots of lab work and lots of writing, climbed some more, made good friends, visited Latvia, Ecuador and Paraquay, went on pilgrimage to Bahjí and Mount Carmel, and served on the Spiritual Assembly of the Bahá'ís of Seattle. The adventure continues...