PHYSIOLOGY AND MECHANISMS OF PYOCYANIN REDUCTION IN *PSEUDOMONAS AERUGINOSA*

Thesis by

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Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* excretes phenazines, redox-active toxins that historically have been categorized as secondary metabolites. This thesis addresses the possibility that pyocyanin, the most notorious phenazine produced by *P. aeruginosa*, acts as an electron acceptor for energy metabolism and exerts beneficial effects on *P. aeruginosa* physiology. The effects of phenazine production and exposure on *P. aeruginosa* strain PA14 were examined through a comparison of the physiological status of wild-type cells to those of a mutant defective in phenazine production, using two different techniques. Quantification of the intracellular NADH and NAD^+ pools revealed that a phenazine-null mutant maintained a more reduced intracellular redox state than the wild type; this is consistent with the capacity of *P. aeruginosa* to reduce pyocyanin. High-performance liquid chromatography of metabolites from cultures grown in defined media showed that the wild type excreted pyruvate in late stationary phase, indicating that pyocyanin alters flux through central metabolic pathways.

While mechanisms for pyocyanin redox cycling had been explored in other organisms, the mechanisms whereby *P. aeruginosa* catalyzes these reactions were largely unknown. A genetic screen was conducted to identify loci that contribute to the ability of *P. aeruginosa* PA14 to reduce ferric citrate, an activity that is phenazine dependent. This approach led to the identification of two loci with roles in pyocyanin reduction: (1) *gpsA*, encoding the soluble glycerol-3-phosphate dehydrogenase (GpsA), and (2) *fbcFBC*, encoding the respiratory cytochrome bc_1 complex. Mutants lacking functional GpsA or the cytochrome *c*-containing subunit (FbcC) of cytochrome bc_1 displayed growth defects

that correlate with the timing of pyocyanin production in batch cultures. The Δ*gpsA* mutant appeared to be unable to regulate its intracellular redox state and may be defective in pyocyanin reduction due to a lack of sufficient NADH. In contrast, the Δ*fbcC* mutant produced ample reducing power for pyocyanin reduction, raising the possibility that the cytochrome bc_1 complex directly catalyzes pyocyanin reduction.

Pyocyanin has previously been shown to affect the development of *P. aeruginosa* colonies on agar surfaces: phenazine-null mutants form highly structured, rugose colonies, while the wild type forms smooth colonies. Through experiments with this colony biofilm assay, we showed that the Δ*gpsA* mutant forms rugose colonies, consistent with a role for pyocyanin reduction in maintenance of redox homeostasis in densely packed communities. Modulation of electron acceptor availability through nitrate addition to the medium promoted smooth colony formation in rugose mutants. These results suggest roles for reduced pyocyanin and nitrate in triggering smooth colony formation in *P. aeruginosa*, and imply that colony wrinkling is an adaptation to electron acceptor limitation.

The work in this thesis has provided insight into the physiological relevance of pyocyanin reduction in *P. aeruginosa* and elaborated our understanding of the mechanisms underlying maintenance of redox homeostasis in bacteria. In addition, it has uncovered new mechanisms that may be contributing to the persistence of *P. aeruginosa* during chronic infection.

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Chapter 1

Introduction

1.1. Motivation

Bacteria surviving in the natural environment experience electron acceptor limitation, which can arise as a consequence of substrate insolubility or slow diffusion through a densely packed population. One solution that allows bacteria to circumvent this problem is the reduction of a diffusible, redox-active compound that shuttles electrons from the bacterium to its substrate, and returns to the bacterium in the oxidized form. Many bacteria have been shown to utilize colorful natural products and/or xenobiotics to catalyze this type of extracellular electron transfer. For example, the reduction of insoluble iron hydroxides by the bacterium *Shewanella oneidensis* MR-1 is facilitated by anthraquinone-2,6-disulfonate, an analog for naturally occurring humic substances that turns orange upon reduction (Newman and Kolter 2000). Planktonic and biofilm cultures of *S. oneidensis* species excrete the bright yellow cofactor riboflavin, which can facilitate electron transfer to iron hydroxides and poised-potential electrodes in fuel cells (Marsili et al. 2008; Von Canstein et al. 2008). *Escherichia coli* can also reduce these extracellular substrates, using the structurally related dye neutral red (McKinlay and Zeikus 2004).

Decades ago, microbiologists recognized the potential for phenazines, a class of redox-active antibiotics excreted by pseudomonads and other bacteria, to act as electron shuttles for their producers (Friedheim 1931). While studies were carried out to

demonstrate the reduction of these compounds by *Pseudomonas, Staphylococcus*, and other microorganisms, as well as cells and mitochondria isolated from mammalian tissue (Stewart-Tull and Armstrong 1972), the focus of research on these natural products shifted to their roles in virulence and agricultural significance (Lau et al. 2004; Mavrodi et al. 2006). Recently, researchers interested in mechanisms of reductive iron dissolution and current generation in mediator-facilitated microbial fuel cells have elucidated roles for phenazines in these processes and applications (Hernandez et al. 2004; Rabaey et al. 2005). However, the mechanisms underlying phenazine reduction by pseudomonads, and the physiological relevance of this activity, remained unstudied.

To gain a better understanding of physiology of phenazine reduction, I worked with the opportunistic pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* is the most extensively studied phenazine-producing bacterium. In particular, strain PA14 is considered a model for studies of the pathogenicity of this bacterium due, in part, to its ample production of phenazines (Wiehlmann et al. 2007; Winstanley and Fothergill 2009). The goals of my research have been (1) to determine whether *P. aeruginosa* PA14 derives a physiological benefit from the reduction of the blue pigment pyocyanin, its major phenazine product; and (2) to identify specific metabolic products and enzymes that contribute to pyocyanin reduction activity.

1.2. Overview

Chapter 2 reviews the extent of phenazine production across prokaryotic phylogeny and the roles of phenazine toxicity and redox activity in the soil environment

and during infection. It also covers a known physiological role for phenazines, i.e., in intercellular signaling, and calls into question the descriptor "secondary metabolite" for compounds such as phenazines and other quorum-sensing and iron-chelating compounds with clear and profound effects on the physiology of their producers. Chapter 3 introduces two previously unknown effects of pyocyanin reduction in *P. aeruginosa*: the oxidation of the cytoplasm, approximated using measurements of the intracellular $NADH/NAD^+$ ratio, and altered flux through central metabolism, as evidenced by the excretion of pyruvate in wild-type cultures but not in cultures unable to produce pyocyanin.

Chapter 4 covers the results of a screen for pyocyanin reduction in *P. aeruginosa* and introduces roles for two enzymes, the soluble glycerol-3-phosphate dehydrogenase and the cytochrome bc_1 complex of the respiratory chain, in stimulating this activity. I present a detailed characterization of mutants defective in production of these enzymes, including evidence that mechanisms underlying their contributions to pyocyanin reductive activity differ. Chapter 5 discusses the effects of electron acceptor availability and pyocyanin reduction on the morphological development of *P. aeruginosa* colony biofilms. Chapter 6 contains an overview of the results and conclusions covered in the thesis, and poses questions for future research into applications of these findings, particularly in clinical settings.

Three appendices are included. Appendix A demonstrates the concentration dependence of pyocyanin reduction in *P. aeruginosa* and the putative role of the periplasmic nitrate reductase enzyme in catalyzing nitrate-dependent pyocyanin

oxidation. Appendix B discusses the effect of redox state on separation of pyocyanin from bacterial cells by centrifugation and the relevance of this partitioning for NADH extraction techniques and our understanding of phenazine transport across bacterial membranes. Finally, Appendix C presents new evidence corroborating a direct mechanism for activation of the transcriptional regulator SoxR, which initiates transcription in response to pyocyanin exposure in *P. aeruginosa* PA14.

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Chapter 2

Background

This chapter is adapted from:

Price-Whelan, A., Dietrich, L.E.P., and Newman, D.K. (2006) Rethinking "secondary" metabolism: Physiological roles for phenazine antibiotics. *Nat. Chem. Biol.* **2**: 71–8.

2.1. Abstract

Microorganisms exist in the environment as multicellular communities that face the challenge of surviving under nutrient-limited conditions. Chemical communication is an essential part of the way in which these populations coordinate their behavior, and there has been an explosion of understanding in recent years regarding how this is accomplished. Much less, however, is understood about the way these communities sustain their metabolism. Bacteria of the genus *Pseudomonas* are ubiquitous, and are distinguished by their production of colorful secondary metabolites called phenazines. In this article, we suggest that phenazines, which are produced under conditions of high cell density and nutrient limitation, may be important for the persistence of pseudomonads in the environment.

2.2. Introduction

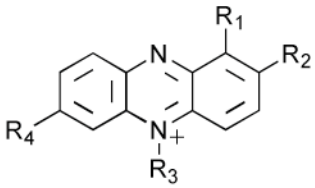
Historically, microbiologists and chemists alike have categorized as "secondary metabolites" a broad class of molecules produced at late stages of microbial growth in laboratory cultures. This nomenclature is, admittedly, pejorative, implying that these molecules are somehow less important than others to the cell that produces them. In particular, the traditional view is that secondary metabolites (i) do not contribute to the growth or survival of the producer (ii) are highly sensitive to the conditions stimulating their production (for example, medium composition) (iii) often have complex structures and (iv) have production rates that are decoupled from the doubling time of the cell (Madigan et al. 2000). Together, these leitmotifs present a conundrum: why would an organism limited for nutrients begin excreting large amounts of complex organic molecules? One reasonable and popular answer is that they function as antibiotics and are produced in copious quantities at this stage of growth to protect the producer from competitors (Firn and Jones 2003). In recent years, however, the idea that "secondary" metabolites might have other functions, ranging from controlling gene expression (Goh et al. 2002) to supporting growth or iron acquisition in microbial communities (Banin et al. 2005; Hernandez et al. 2004), has become increasingly compelling. This is due, in large part, to the recognition that microbes typically exist in nature in biofilm communities (Costerton et al. 1995) and/or in a metabolically quiescent state (Kolter et al. 1993); because the "rules of the game" for metabolism under these conditions are virtually unknown, a reexamination of the function of secondary metabolites is warranted.

To illustrate the idea that secondary metabolites have the potential to perform primary metabolic functions, we will focus this review on a class of compounds known

as “phenazines,” which have been of great interest to pharmaceutical and clinical research groups for the last fifty years (Laursen and Nielsen 2004). Phenazines are heterocyclic compounds that are produced naturally and substituted at different points around their rings by different bacterial species (table 2.1). Small modifications of the core phenazine structure give rise to a full spectrum of colors, ranging from the deep red of 5-methyl-7-amino-1-carboxyphenazinium betaine (aeruginosin A, 1) to the lemon yellow of phenazine-1-carboxylic acid (PCA, 2), to the bright blue of 1-hydroxy-5-methylphenazine (pyocyanin, 3) (figure 2.1). The combination and variety of functional groups added also determine the redox potential and solubility of these compounds, thus affecting their biological activity (Chin-A-Woeng et al. 1998; Kerr 2000; Laursen and Nielsen 2004).

Table 2.1. Structures, solubilities, and redox potentials of some of the phenazines excreted by pseudomonads

Solubilities were calculated using the KOWWIN demo program available at http://www.syrres.com/esc/est_kowdemo.htm. NA, not available. (Clark 1960; Mann 1969; Meylan and Howard 1995).



#	name	R_1	R_2	R_3	R_4	K_{ow} ox	K_{ow} red	E°' (mV)
1	aeruginosin A	COOH		CH_3	NH_2	-0.71	0.46	NA
2	phenazine-1-carboxylic acid (PCA)	COOH				2.17	3.72	-177
3	pyocyanin	OH		CH_3		1.60	2.89	-34
4	2-hydroxyphenazine-1-carboxylic acid (2-OHPCA)	COOH	OH			2.54	3.32	NA
5	1-hydroxyphenazine (1-OHPHZ)	$CONH_2$				1.04	2.19	-115
6	phenazine-1-carboxamide (PCN)	OH				1.81	2.35	-172

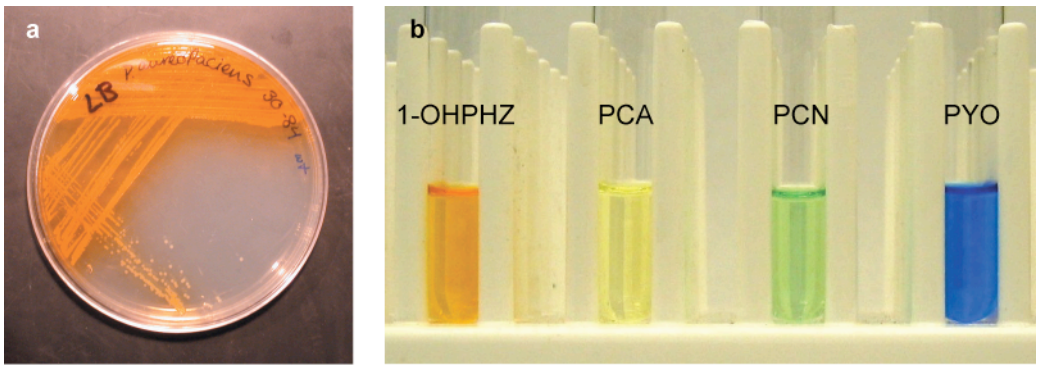


Figure 2.1. Phenazines are colorful, diffusible bacterial metabolites.

(a) Streak plate of the biocontrol strain *P. aureofaciens* 30-84. The phenazine 2-OHPCA turns the agar bright orange. (b) Aqueous solutions of some of the phenazines produced by various *Pseudomonas* strains.

The antagonistic effects of almost all of these derivatives are usually attributed to one general characteristic: redox activity. The 2-hydroxyphenazine-1-carboxylic acid (2-OHPCA, 4) produced by *Pseudomonas aureofaciens* is thought to kill off competing fungi via the production of reactive oxygen species (Chin-A-Woeng et al. 2003). Many of the effects of pyocyanin and PCA on a diversity of eukaryotic hosts as well as bacteria are thought to result from oxidative activity or the inactivation of proteins important in the oxidative stress response (Lau et al. 2004a; O'Malley et al. 2003). Regardless of whether they are acting as antibiotics in the soil or virulence factors during infection, the redox transformations of phenazines strongly influence their physiological effects in other organisms. A more detailed understanding of phenazine metabolism in competing or host cells is emerging as very recently, researchers have begun to recognize that small variations in the reactivity of phenazines can give rise to differences in their elicited response (Look et al. 2005).

Concomitant with the development of ideas about phenazine activity during competition and infection, *Pseudomonas aeruginosa* and other phenazine-excreting bacteria have become popular model organisms for the study of quorum sensing and biofilm formation, two of the most active areas of research in the field of microbiology (Hall-Stoodley 2004; Juhas et al. 2005; Lazdunski et al. 2004). While pharmaceutical and clinical groups have been focused on the physiological effects of these compounds in nonproducing organisms, microbial physiologists and geneticists have typically viewed phenazines as metabolites that perform only secondary functions. As a result, despite research interest in both the biological activity of the compounds themselves, as well as the physiology of their producers, the primary functions of phenazines for producing

organisms such as the pseudomonads are still unknown. This is surprising, especially given that phenazine production and reduction is evident in many of the *Pseudomonas* cultures that microbiologists prepare for their work (figure 2.2), and that the mechanisms thought to be responsible for phenazine metabolism in nonproducers (for example, reduction by NADH or glutathione, or interaction with the respiratory chain) are present in most organisms (Armstrong and Stewart-Tull 1971; Ritz and Beckwith 2001). That phenazines and other excreted compounds (i) react with common primary metabolites, (ii) are potentially transformed by enzymes active in central metabolic pathways, and (iii) induce gene expression calls into question their categorization as secondary metabolites. We will discuss here the recent discoveries that lead to new hypotheses about the relevance of phenazine metabolism in the context of the lifestyles of their producers.

2.3. Occurrence of Phenazine Production

Phenazines are first mentioned in the literature as early as the 1860s, when French researchers and clinicians noticed a blue coloration in the pus and sputum, or respiratory secretion, of infected patients. Carle Gessard and others examined the pus microscopically and identified a rod-shaped bacterium residing in these wounds, and upon isolating the organism discovered that it was responsible for the bluish tint. For this trait, they named the species *Bacillus pyocyaneus*, and it has since been renamed *Pseudomonas aeruginosa*, for the Latin *aerugo*, which refers to the blue-green rust of copper (Villavicencio 1998). *P. aeruginosa* is widespread in terrestrial habitats, can grow in both marine and freshwater environments, and is known for its ability to infect a

diversity of hosts, ranging from plants to humans (Pirnay 2005; Turner and Messenger 1986; Villavicencio 1998). The *P. aeruginosa* laboratory strains PAO1 and PA14 are capable of producing at least 4 different phenazine derivatives (Mavrodi et al. 2001).

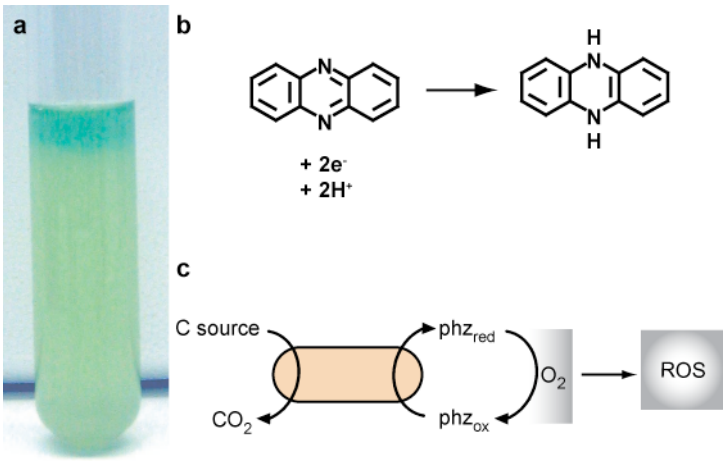


Figure 2.2. Pseudomonads stimulate phenazine reduction
(**a**) Characteristic gradient formed by standing cultures of *P. aeruginosa*. Bacterial respiration renders most of the culture anoxic. Phenazines are reduced and, in the case of pyocyanin, become colorless. The darker blue at the top represents oxidized pyocyanin. (**b**) Half reaction representing generic two-electron phenazine reduction. (**c**) Schematic of phenazine reduction and autooxidation responsible for gradient formation in standing cultures. Reduced phenazines are oxidized abiotically by oxygen, generating reactive oxygen species (ROS).

Several other *Pseudomonas* species are also phenazine producers and are known for their potential in biocontrol applications, in which an organism that inhibits the growth of crop pathogens is enriched in the soil to enhance crop yields. Representatives include the strains *P. chlororaphis (aureofaciens)* 30-84, *P. fluorescens* 2-79, and *P. chlororaphis* PCL1391. Along with *P. aeruginosa*, these isolates all produce one or more phenazines and differ in their biosynthetic capabilities with respect to phenazine derivatization. *P. chlororaphis (aureofaciens)* 30-84, for example, possesses the monooxygenase PhzO, which converts the common pseudomonad phenazine precursor

PCA into 2-OHPCA, a bright orange pigment (Delaney et al. 2001). *P. chlororaphis* PCL1391, on the other hand, expresses PhzH, a transamidase that converts this precursor into PCN, a green, sparingly soluble pigment that precipitates out of culture media (Chin-A-Woeng et al. 2001b).

Phenazine biosynthesis also has been observed in different bacterial genera, including other proteobacteria, such as *Brevibacterium*, *Burkholderia*, and *Xanthomonas*, as well as the Gram-positive genus *Streptomyces* and even the archaeal genus *Methanosarcina* (Beifuss and Tietze 2005; Rao and Sureshkumar 2000; Turner and Messenger 1986). This review will focus on the pseudomonad phenazines, because they are the best studied with respect to biosynthesis, but will also include a discussion of the methanophenazine produced by *Methanosarcina mazei* Gö1, because it is the only phenazine thus far that has been unequivocally shown to play an important role in catabolism (Beifuss and Tietze 2005).

2.4. Roles in Eukaryotic Physiology and Pseudomonad Persistence

2.4.1. Phenazines in Infection

Some of the most thorough studies that have been conducted to investigate the physiological consequences of phenazine exposure are those of Britigan and colleagues, who have reported the many effects of phenazines produced by *P. aeruginosa* during infection of the human lung (Lau et al. 2005). Phenazine production has been shown to play an important role in both acute and chronic *P. aeruginosa* lung infections, which are frequent causes of mortality in patients who have cystic fibrosis or otherwise impaired

lung function (Villavicencio 1998). Pyocyanin has been detected in the sputum of patients with chronic *P. aeruginosa* infections at concentrations as high as 27 μg/mL (Wilson et al. 1988), and the administration of purified pyocyanin at comparable concentrations in laboratory mice has been shown to induce neutrophil influx in lung tissue (Lau et al. 2004b). Phenazine production is a common trait in strains of *P. aeruginosa* isolated from patients with cystic fibrosis (Finnan 2004), and mutant versions of the *P. aeruginosa* strains PAO1 and PA14 that are unable to synthesize pyocyanin are attenuated in both acute and chronic mouse lung infection models (Lau et al. 2004a).

The oxidative activity of phenazines in particular has been shown to be important in pathogenesis during *P. aeruginosa* lung infection (Lau et al. 2004a). Both pyocyanin and PCA can increase oxidant formation in human airway epithelial cells through a number of mechanisms including the oxidation of glutathione and NADH and inhibition of antioxidant enzymes (Look et al. 2005; O'Malley et al. 2003; O'Malley et al. 2004). Once it is reduced, pyocyanin can then react with oxygen, forming superoxide radical and hydrogen peroxide (Hassan and Fridovich 1980). Pyocyanin radical is also formed as an intermediate during its redox cycling, and can further contribute to the formation of reactive oxygen species (Britigan et al. 1992; Britigan et al. 1999; Hassan and Fridovich 1979). These insults to the host cell's internal redox balance may lead to increased secretion and thereby contribute to the generation of sputum, the physical and nutritional substrate for *P. aeruginosa* in the lungs of individuals with cystic fibrosis (Ohman 1982; Palmer 2005). The generation of radical species (of phenazines and oxygen) is potentially harmful to other microbes competing for resources in the lung, such as *Staphylococcus aureus*, and may help *P. aeruginosa* to persist in this environment (Baron and Rowe

1981). However, recent work has demonstrated that phenazine production is beneficial to the growth or survival of *P. aeruginosa* in mouse infection models even in the absence of competing organisms, implying that these compounds may additionally provide a physiological benefit to their producers during infection (Lau et al. 2004b).

2.4.2. Phenazines in Soil Ecosystems

The other well-studied niche for phenazine-producing pseudomonads is the rhizosphere, the zone surrounding the roots of plants. As is the case for *P. aeruginosa* in an infected lung, species such as *P. fluorescens* and *P. chlororaphis* compete in this ecosystem with other organisms for resources. More importantly, they compete for colonization sites on the roots of agriculturally important crops, where they thrive as microcolonies (biofilms) and protect the plants from pathogenic fungi. Phenazines are thought to be important in this competition, and consistent with this, phenazine-producing strains of *P. chlororaphis (aureofaciens)* and *P. fluorescens* are better able to colonize the roots of wheat plants and persist in the rhizosphere than are phenazine-lacking mutants (Mazzola et al. 1992).

The toxicity of phenazines for bacteria and fungi typically present in the rhizosphere has been demonstrated, and again is thought to be mostly due to the generation of reactive oxygen species (Chin-A-Woeng et al. 2003). If biocontrol strains did use phenazine toxicity as a weapon to compete with indigenous soil populations for resources, one would expect the composition of rhizosphere communities to change dramatically after exposure to phenazines; however this does not occur. The overall

number of organisms competing with *P. fluorescens* for resources does not decline after this strain has colonized the root. This implies that it is not just the antibiotic activity of phenazines that is important for the ability of their producers to compete in the soil (Bankhead et al. 2004). As is the case for phenazines produced by *P. aeruginosa*, there is evidence indicating that phenazines have roles in the physiology and thus the ecological competence of the biocontrol pseudomonads.

2.5. Biosynthesis of Phenazine Derivatives

As mentioned above, the early observation that phenazines are produced in stationary phase in typical lab cultures led to the general view that they were unimportant in metabolism. However, we now know that phenazines are produced in biofilms and as a result are present in detectable quantities in the rhizosphere and in the lungs of cystic fibrosis patients. This has fueled interest in the phenazine biosynthetic pathway and the environmental factors that influence expression of the biosynthetic genes. The complexity of the regulation of phenazine biosynthesis is only just beginning to be appreciated and is consistent with the high degree of biological activity exhibited by these compounds.

In *Pseudomonas* spp., the phenazine biosynthetic pathway branches off from the shikimic acid pathway, which is also the source for metabolites such as the aromatic amino acids, siderophores, and quinones (figure 2.3). Genes encoding the phenazine biosynthetic enzymes are arranged in one core operon, *phzABCDEFG*, in most phenazine-producing pseudomonads (Delaney et al. 2001; Mavrodi et al. 1998). Such an

operon exists in the genome of *P. aeruginosa* in duplicate, and the expression of the two copies of the operon is differentially regulated (Mavrodi et al. 2001). In many strains, additional genes involved in phenazine decoration, such as *phzM*, *phzS*, *phzO*, and *phzH*, are present in single copy and can be located proximally to the core operon or elsewhere in the genome (Chin-A-Woeng et al. 2001b); currently, little is known about how these genes are regulated.

Expression of phenazine biosynthetic genes is regulated by multiple mechanisms, which are strongly influenced by environmental conditions. One of the primary factors governing phenazine production is population density, and in *P. aeruginosa* this dependency is effected by at least three quorum-sensing systems (Deziel et al. 2004; Whiteley et al. 1999). Bacteria participating in quorum sensing release intercellular signals such as N-acyl-L-homoserine lactones (AHLs, 7 and 8) and 2-heptyl-3-hydroxy-R-quinolone (the *Pseudomonas* quinolone signal, PQS, 9) into the environment, where they can be taken up by neighboring cells of the same or different species (figure 2.4). Inside the cell, these compounds induce the expression of genes for their own biosynthesis—as well as many other genes important in virulence, competition, and behavior—when they accumulate to a threshold concentration (Lazdunski et al. 2004). The dependence of phenazine biosynthesis on cell density has also been demonstrated for biocontrol pseudomonads, and in these species is mediated by a seemingly less complex quorum-sensing network (Chin-A-Woeng et al. 2001a; Khan et al. 2005; Pierson et al. 1994).

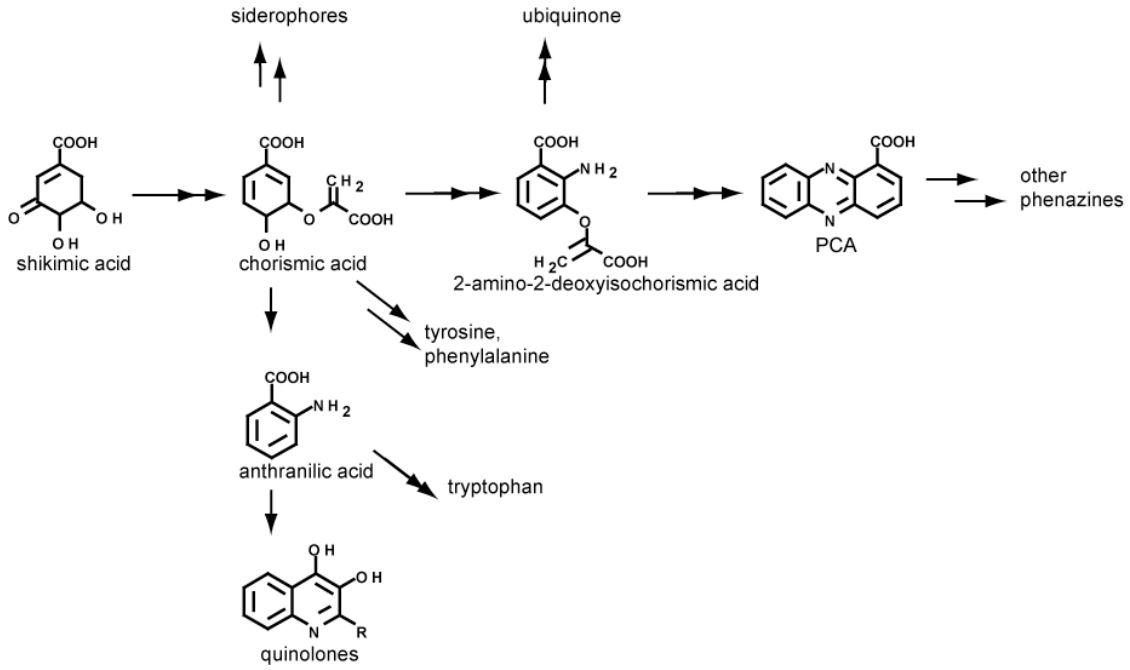


Figure 2.3. Phenazine biosynthesis and its relation to the shikimic acid pathway in pseudomonads.
(Byng et al. 1979; Dewick 1984; Mavrodi et al. 2001; Vandenende et al. 2004).

In addition to cell density, numerous environmental factors have been identified that affect the regulation of phenazine biosynthesis, including oxygen, iron, and phosphate concentration as well as the nature of the carbon sources available (Van Rij et al. 2004). For many of these effects, it is not entirely clear whether they are above quorum sensing in the regulatory cascade (for example, iron limitation induces quorum signal production, which in turn induces phenazine biosynthesis) or are the result of regulators acting independently of quorum sensing (Kim et al. 2005). The GacA/S two-component system, which effects global changes in transcription, has been implicated in control of phenazine biosynthesis and is thought to act by regulating quorum sensing, but there is also evidence that it affects phenazine gene expression through other mechanisms (Chancey 1999; Chancey et al. 2002; Whistler and Pierson 2003). Repressors of

phenazine biosynthesis have been identified in the plant symbionts *P. chlororaphis (aureofaciens)* 30-84 and *P. chlororaphis* PCL1391. Mutations in these repressors seem to override the quorum-sensing regulation of phenazine biosynthesis in these organisms, resulting in constitutive phenazine production (Chin-A-Woeng et al. 2005; Whistler and Pierson 2003).

2.6. Intercellular Signaling: A Regulatory Role for Phenazines

Recent work from our laboratory has contributed to our understanding of the complexity of the *P. aeruginosa* quorum-sensing system and the place of phenazines in this cell density-dependent cascade. We have found that, in addition to being regulated by cell-cell communication, phenazines themselves can act as intercellular signals. Our work indicates that pyocyanin is the physiological inducer of a set of genes previously identified as members of the quorum-sensing regulon. Pyocyanin acts downstream of PQS, which previously had been deemed the terminal signal in the *P. aeruginosa* quorum-sensing cascade. Pyocyanin's function as a quorum signal explains what was thought to be a delayed response in the expression of a specific set of genes in response to PQS (Whiteley et al. 1999). We now understand that these genes are expressed later than those induced directly by PQS because PQS first has to upregulate the biosynthesis of phenazines so that pyocyanin can subsequently induce its stimulon (Cases 2005; Dietrich et al. 2006) (figure 2.4).

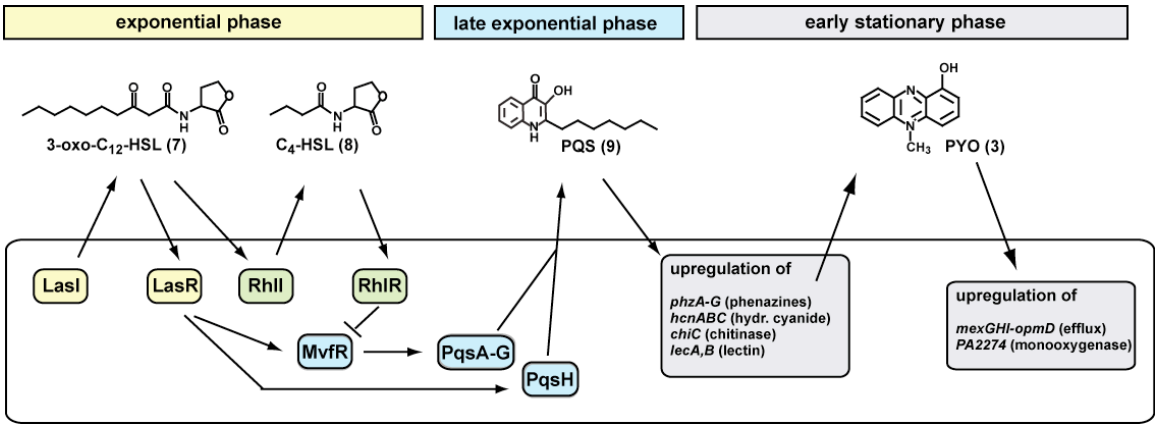


Figure 2.4. Model of the quorum-sensing network in *P. aeruginosa*.
The quorum-sensing network in *Pseudomonas aeruginosa* comprises a cascade of three types of signaling molecules that function in a growth-stage-dependent manner. The AHLs 3-oxo-C_{12}-HSL and C_4-HSL are released in exponential phase and control the production of the quinolone PQS. PQS accumulates in late exponential phase and is required for the synthesis of phenazines. Recent findings from our laboratory show that the phenazine pyocyanin upregulates genes that have previously been demonstrated to be QS controlled, establishing pyocyanin as a signaling molecule (Deziel et al. 2004; Wade et al. 2005).

The signaling function of pyocyanin makes it the newest addition to the growing list of small molecules excreted by *P. aeruginosa* that have been shown to perform multiple functions (Hooi 2004). PQS, like pyocyanin, was also long recognized for its antibiotic and virulence properties before its role in signaling was elucidated (Pesci et al. 1999). Recent studies have demonstrated that certain AHL and quinolone derivatives chelate iron (Kaufmann 2005; Oliphant 2002), raising the possibility that these metabolites facilitate iron uptake *in vivo*. The accumulating knowledge about the chemistry and biological activity of small molecules excreted by *Pseudomonas* spp. calls for a reevaluation of our categorizations of these compounds. Rather than clearly performing one dedicated purpose, they seem to be capable of multiple roles. To determine the most important physiological effects of phenazines and other small metabolites in the environment and during infection, we will need to understand the

physiological conditions allowing, requiring, and regulating their activities. Elucidating their mechanisms of action at the molecular level may also provide indications of the conditions relevant for activity.

2.7. Other Physiological Roles

In addition to inducing gene expression, phenazines also act as substrates in intracellular redox transformations. This further metabolism of phenazines, subsequent to their biosynthesis, can be observed as a color change in pseudomonad cultures that have become limited for terminal electron acceptors. This is because pseudomonads reduce their own phenazines, and changing the oxidation state of a phenazine changes its absorbance spectrum. In cultures of the bacterium *P. aeruginosa*, this is observed as a loss of blue coloration, because the main phenazine produced by this organism, pyocyanin, changes from blue to colorless upon reduction at neutral pH (figure 2.2) (Friedheim and Michaelis 1931). This activity has also been demonstrated in oxygen-limited cultures of the bacterium *P. chlororaphis*, which can use its phenazine product, phenazine-1-carboxamide (PCN, 5) to reduce extracellular iron oxides (Hernandez et al. 2004). Although a good deal of research effort has gone toward understanding phenazine reduction by mammalian cells, less work has been done to elucidate the mechanisms of the phenazine reduction that are readily observed in pseudomonad cultures. Here, we will discuss what is known about phenazine reduction and its physiological functions in phenazine producing and nonproducing prokaryotes.

2.7.1. Phenazines as "Respiratory Pigments"

Based on the redox potentials, metabolism, and solubilities of phenazines, it has been proposed that phenazines act as electron acceptors in cellular energy generation or the maintenance of the intracellular redox balance (Hernandez and Newman 2001). Studies conducted by Ernst Friedheim in the 1930s, in which he observed that pyocyanin increased the oxygen consumption of cell suspensions of *P. aeruginosa* (Friedheim 1931), support this idea. Several reports on the interactions of pyocyanin and 1-hydroxyphenazine (1-OHPHZ, 6) with the mammalian respiratory chain were published in the decades that followed. 1-OHPHZ, but not pyocyanin, was shown to inhibit respiration at the level of ubiquinone in the electron transport chain of mammalian cells. The authors concluded from their measurements of oxygen depletion (which did not decrease in the presence of pyocyanin) that pyocyanin did not inhibit mammalian cell respiration (Armstrong and Stewart-Tull 1971; Stewart-Tull and Armstrong 1972). We question this interpretation, however, given that pyocyanin can accept electrons from NADH and transfer them to oxygen; accordingly, what was thought to be normal respiration may actually have been short-circuiting of the electron transport chain by pyocyanin. In contrast, it makes sense that oxygen depletion was not observed in the presence of 1-OHPHZ given that reduced 1-OHPHZ does not react with oxygen at appreciable rates (Muller 1995).

Notably, the interactions of phenazines with the pseudomonad respiratory chain are largely unknown. Numerous groups have observed that both synthetic and natural phenazines are reduced by prokaryotes, but in most cases the physiological effect of this reduction has not been evaluated (Learoyd et al. 1992; McKinlay and Zeikus 2004). One

exception is the role of phenazine reduction in the respiratory chain of *Methanosarcina mazei* Gö1 (Deppenmeier 2004). This archaeon produces methanophenazine, a phenazine derivative with a pentaisoprenoid side chain, and can utilize phenazines in lieu of quinones in electron transport. *In vitro*, methanophenazine has been shown to accept electrons from hydrogen or a reduced coenzyme via the activity of either of two membrane-bound dehydrogenases, one of which is homologous to the NADH dehydrogenase found in bacteria and mitochondria. Reduced methanophenazine can then donate electrons to another cofactor in a reaction catalyzed by a membrane-bound heterodisulfide reductase. *In vivo*, these respiratory enzymes couple electron transport to the translocation of protons, generating a proton gradient that can be used to make ATP. In *M. mazei*, therefore, phenazine reduction is not only crucial to energy metabolism in that it reoxidizes the NADH analogue found in methanogens, but it is also required for ATP synthesis (Abken et al. 1998; Deppenmeier 2004).

2.7.2. Phenazines in Redox Homeostasis and Iron Acquisition

Advances in our understanding of bacterial communities have provided an environmental context for the hypothesis that pseudomonads benefit by reducing phenazines. It has been proposed that the reduction of diffusible small molecules is advantageous during growth in a biofilm, a surface-attached population of bacteria suspended in an excreted matrix (Hernandez and Newman 2001). The diffusion rate of oxygen through a biofilm is thought to be slow, and cells at the base of an aerobic biofilm become limited for oxidants (Fu et al. 1994; Stewart 2003). Under this condition,

phenazines could allow bacteria to generate energy for growth or help maintain redox homeostasis by acting as electron acceptors for the reoxidation of accumulating NADH. Indeed, maintaining a proper redox balance in the pyridine nucleotide pool is essential for metabolism (De Graef et al. 1999), and recent work from our lab indicates that *P. aeruginosa* phenazine-negative mutants have higher intracellular NADH/NAD^+ ratios in stationary phase than does the parent strain in planktonic cultures (Price-Whelan et al. 2007). This suggests that an important role for phenazines could be to serve as intracellular redox "buffers."

Various research groups have recently become more interested in phenazine reduction by biofilm-forming bacteria because phenazines make excellent electron transfer mediators to electrodes in biological fuel cells (Fultz and Durst 1982). In biological fuel cells deployed in the environment as well as those set up in laboratories, bacteria often grow as biofilms attached to the electrode surface (Bond et al. 2002; Kim et al. 2004). That phenazines facilitate electron transfer to electrodes has been demonstrated by Zeikus and colleagues, who investigated the ability of *Escherichia coli* to reduce the synthetic phenazine neutral red. They showed that *E. coli* is able to use this synthetic phenazine as an electron transfer mediator in the reduction of iron oxide, and presented evidence indicating that hydrogenase is at least partially responsible for this capability (McKinlay and Zeikus 2004). Our group as well as others have shown that other synthetic dyes, with structures resembling those of phenazines, are reduced by *Bacillus*, *Lactococcus*, and *Shewanella* species (Hernandez et al. 2004; Learoyd et al. 1992; Lies et al. 2005). The Verstraete group has shown that phenazine production enhances power output from microbial fuel cells, and that biofuel cells enrich for

phenazine-producing organisms; whether phenazine production influences the growth or survival of these bacteria in this context remains unclear (Rabaey et al. 2004; Rabaey et al. 2005).

Aside from these proposed roles in energy generation, it has been suggested that phenazine reduction could act to make iron more available to the producing organism. Pyocyanin may assist infectious *P. aeruginosa* in the acquisition of iron by reducing it and freeing it from transferrin, a protein that normally sequesters iron such that it is available only to the human host (Cox 1986). As mentioned above, *P. chlororaphis* has been shown to reduce iron oxides via electron transfer to PCN, and it is thought that this ability may be important in the rhizosphere, where iron is predominantly present in an insoluble form (Hernandez et al. 2004). An examination of the relationship between iron availability and the regulation of phenazine biosynthesis, however, presents a complicated picture that neither refutes nor supports a role for these compounds in iron acquisition. Although in many cases it has been reported that phenazine production is enhanced in iron-deprived cultures, other studies have demonstrated a requirement for iron in media optimized for phenazine biosynthesis (Cox 1986; King 1954; Van Rij et al. 2004). These differences probably arise from the high degree of variability with respect to other parameters, such as carbon source and the concentrations of oxygen and various salts. Perhaps the best way to ascertain whether or not iron availability bears relevance to phenazine production will be to observe its effects under conditions that imitate the most common habitats for phenazine-producing pseudomonads (Palmer 2005).

2.7.3. How Are Phenazines Reduced?

Although we are beginning to recognize the potential physiological importance of phenazine redox cycling, we have yet to identify the specific mechanisms by which pseudomonads catalyze the reduction of these compounds. On the basis of their low redox potentials and the mechanisms for phenazine reduction identified in eukaryotic cells, we might predict that NADH or glutathione would act as electron donors in these reactions. Using ferric citrate reduction as a proxy for pyocyanin reduction, we have identified genetic loci required for full pyocyanin reduction activity in *P. aeruginosa*, including *gpsA*, encoding a glycerol-3-phosphate dehydrogenase, and the *fbcFBC* operon, encoding the cytochrome bc_1 complex of the respiratory chain (chapter 4). The *gpsA* gene is required for maintenance of redox homeostasis; without functional GpsA, the intracellular $NADH/NAD^+$ pool becomes too oxidized for pyocyanin reduction to proceed. The cytochrome bc_1 complex is a coupling site in the aerobic respiratory chain. Identification of this locus in a pyocyanin reduction screen raises the intriguing possibility that pseudomonads could be able to couple the reduction of their own excreted metabolites to the generation of a proton-motive force.

2.8. Conclusions

As we learn more about the chemistry and biological activity of phenazines, we begin to question their categorization as "secondary" metabolites. This compels us to rethink secondary metabolism as a phenomenon more generally. It is striking that the conditions under which secondary metabolites are produced in laboratory cultures (that

is, stationary phase) are effectively the same as those that define many microbial habitats in nature (Kolter et al. 1993). Consistent with this, gene expression and physiological attributes appear to be very similar in stationary-phase planktonic cultures and biofilms (Fux 2005; Waite 2005). As we have discussed for phenazines, stationary-phase metabolites can allow bacteria to sense the conditions of their surroundings and induce appropriate changes in gene expression; moreover, they can facilitate extracellular electron transfer to oxidants such as insoluble iron (Hernandez et al. 2004; Mavrodi et al. 2001) and play a role in the maintenance of redox homeostasis. Notably, phenazines are only one class of myriad natural products made by microorganisms (Handelsman and Wackett 2002), many of which bear intriguing structural resemblances to cofactors that play important roles in primary metabolism (figure 2.5). Now that we are beginning to understand stationary phase physiology and its ecological relevance, it is time to revisit the roles of these compounds in gene expression and survival. We suspect that such studies will further blur the line between primary and secondary metabolism, and lead to a more complete picture of the mechanisms allowing organisms to persist in dynamic environments.

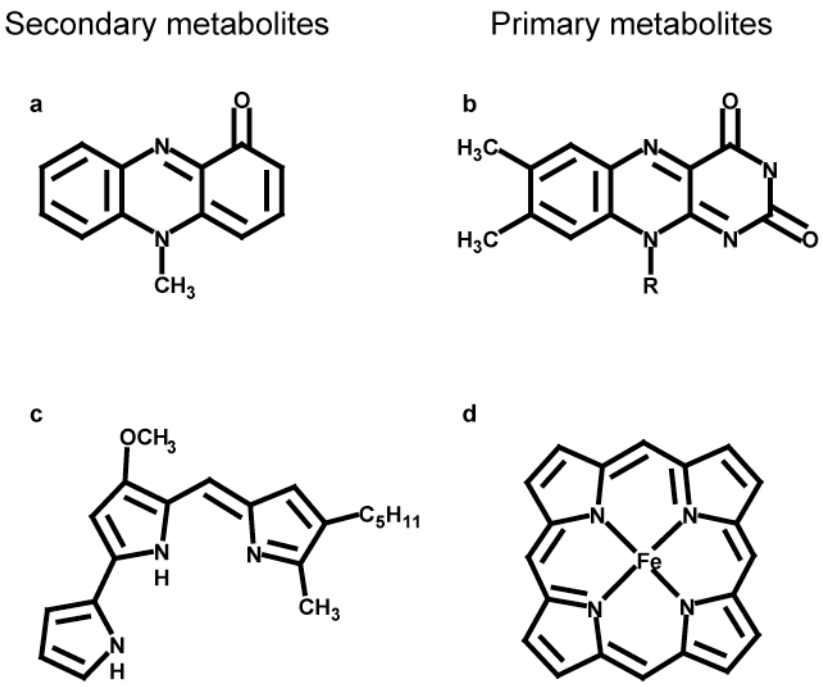


Figure 2.5. Key structural elements of secondary metabolites resemble those of cofactors that play critical roles in energy metabolism.
(a) Pyocyanin. (b) Generic flavin. (c) Prodigiosin, an antibiotic produced by *Serratia marcescens*. (d) Generic heme (White 2000; Williamson 2005).

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Chapter 3

Physiological Effects of Pyocyanin in *Pseudomonas aeruginosa*

This chapter is adapted from:

Price-Whelan, A., Dietrich, L.E.P., and Newman, D.K. (2007) Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14. *J. Bacteriol.* **189**: 6372–81.

3.1. Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* produces colorful, redox-active antibiotics called phenazines. Excretion of pyocyanin, the best-studied natural phenazine, is responsible for the bluish tint of sputum and pus associated with *P. aeruginosa* infections in humans. Although the toxicity of pyocyanin for other bacteria, as well as its role in eukaryotic infection, has been studied extensively, the physiological relevance of pyocyanin metabolism for the producing organism is not well understood. Pyocyanin reduction by *P. aeruginosa* PA14 is readily observed in standing liquid cultures that have consumed all of the oxygen in the medium. We investigated the physiological consequences of pyocyanin reduction by assaying intracellular concentrations of NADH and NAD^+ in the wild-type strain and a mutant defective in phenazine production. We found that the mutant accumulated more NADH in stationary phase relative to the wild type. This increased accumulation correlated with a decrease in oxygen availability and was relieved by the addition of nitrate. Pyocyanin addition to a

phenazine-null mutant also decreased intracellular NADH levels, suggesting that pyocyanin reduction facilitates redox balancing in the absence of other electron acceptors. Analysis of extracellular organic acids revealed that pyocyanin stimulated stationary-phase pyruvate excretion in *P. aeruginosa* PA14, indicating that pyocyanin may also influence the intracellular redox state by decreasing carbon flux through central metabolic pathways.

3.2. Introduction

Redox transformations are a defining feature of the creation of biomass. To form precursors for incorporation into cellular material, heterotrophic organisms catalyze the oxidation of organic carbon sources, generating reducing power. This reducing power can be released in fermentation products or transferred to an externally supplied oxidant via the respiratory chain. The fluid exchange of electrons between intra- and extracellular environments permits organisms to maintain a buffered intracellular redox state, a condition required for the stability and function of biological macromolecules (Bessette et al. 1999; Mossner et al. 1999). Under traditional laboratory culture conditions, electron donors and acceptors are often provided in excess, allowing microorganisms to control intracellular redox conditions. However, it is becoming clear that energy starvation, the limitation of substrates for oxidative or substrate-level phosphorylation, more closely mirrors conditions encountered by many bacteria in their natural habitats (Kolter et al. 1993). How do bacteria maintain redox homeostasis under these conditions?

Bacteria of the genus *Pseudomonas*, like most heterotrophic bacteria (Conway 1992; Fuhrer et al. 2005), oxidize organic carbon sources via the activity of the Entner-Doudoroff pathway and the citric acid cycle. Several of the oxidative steps in these pathways are coupled to the reduction of NAD^+ to NADH, and NADH must be reoxidized so that these pathways can proceed and generate anabolic precursors. In pseudomonads, the primary mechanism whereby this is accomplished is through reduction of one of the NADH dehydrogenases at the start of the respiratory chain, which ultimately transfers the electrons to oxygen or nitrate (Williams et al. 2007). It has therefore been assumed that pseudomonads, organisms that rely on respiration for growth under most conditions, accumulate NADH when terminal electron acceptors become limiting.

Given that the $NADH/NAD^+$ redox couple plays a major role in central metabolism, the ratio of the reduced to oxidized forms is thought to be representative of the intracellular redox state (De Graef et al. 1999). In previous studies, measurements of $NADH/NAD^+$ in a variety of bacterial species have distinguished the opportunistic pathogen *Pseudomonas aeruginosa* from organisms such as *Clostridium welchii*, *Klebsiella aerogenes*, *Escherichia coli*, and *Staphylococcus albus* as the only species with a steady-state $NADH/NAD^+$ ratio greater than one (Wimpenny and Firth 1972). However, another characteristic feature of some pseudomonad strains, which distinguishes them from all of the other genera mentioned above, is the ability to produce redox-active antibiotics known as phenazines (Mavrodi et al. 2006). Some of these compounds, including pyocyanin, the best-studied phenazine due to its role in the pathology of *P. aeruginosa* infections (Lau et al. 2004), have been shown to react with

NADH *in vitro* (Davis and Thornalley 1983; Kito et al. 1974). This has led to the hypothesis that electron transfer to phenazines may represent an adaptation that allows bacteria to modulate their intracellular redox state (Friedheim 1931; Price-Whelan et al. 2006; Trutko 1989; Trutko et al. 1989). This physiological role would be consistent with the fact that phenazine biosynthesis is regulated such that phenazines are produced at high cell densities (Byng et al. 1979; Ingledew and Campbell 1969; Pierson et al. 1994; Whiteley et al. 1999), a condition that typically correlates with electron acceptor limitation (Sweet and Peterson 1978).

Much research has focused on the toxic effects of pyocyanin as a virulence factor in the eukaryotic host (Lau et al. 2004; Look et al. 2005; O'Malley et al. 2003; Reszka et al. 2004; Stewart-Tull and Armstrong 1971) as well as in microorganisms (Baron and Rowe 1981; Baron et al. 1989; Hassan and Fridovich 1980; Kerr et al. 1999; Ran et al. 2003). These effects have been attributed to the production of reactive oxygen species such as superoxide in the presence of pyocyanin (Gardner 1996; Hassan and Fridovich 1979), and physiological studies have shown that *P. aeruginosa* resists the toxicity of this compound with increased superoxide dismutase and catalase activities under pyocyanin-producing conditions (Hassett et al. 1992; Hassett et al. 1995). Additionally, recent gene expression studies have uncovered a role for pyocyanin in intercellular signaling (Dietrich et al. 2006). However, little is known about the role of this compound in pseudomonad metabolism, or whether *P. aeruginosa* derives a benefit from the utilization of pyocyanin as an electron acceptor.

The facile reversibility of phenazine redox reactions allows these compounds to oxidize major intracellular reductants and subsequently reduce extracellular oxidants, thereby acting as redox mediators (Fultz and Durst 1982; Hernandez et al. 2004; Learoyd et al. 1992; McKinlay and Zeikus 2004; Rabaey et al. 2005; Stams et al. 2006). The redox potentials of pyocyanin and phenazine-1-carboxylic acid, the two major phenazines produced by *P. aeruginosa* PA14, are −34 mV (Friedheim and Michaelis 1931) and −116 mV (Wang and Newman 2008), respectively, versus the standard hydrogen electrode at pH 7. These potentials are high enough to allow reduction by NADH ($E^{\circ\prime}$ = −320 mV) and glutathione ($E^{\circ\prime}$ = −240 mV) (Aslund et al. 1997; Thauer et al. 1977), but low enough to allow electron transfer to environmentally relevant oxidants, including oxygen, nitrate, and ferric iron. Therefore, electron shuttling via phenazines may be a mechanism whereby pseudomonads can utilize electron acceptors that, due to low concentrations or solubility, might otherwise be inaccessible via conventional biochemical and enzymatic routes (Cox 1986; Hernandez and Newman 2001; Price-Whelan et al. 2006). The role of this electron transfer in *P. aeruginosa* energy metabolism has yet to be elucidated, but work carried out in another γ-Proteobacterium, *Shewanella oneidensis* MR-1, indicated that pyocyanin, as well as similarly structured small molecules, facilitates reduction of insoluble ferric oxyhydroxides and stimulates growth (Hernandez et al. 2004; Lies et al. 2005). Furthermore, a critical electron carrier function has been demonstrated for a membrane-bound phenazine derivative present in the electron transport chains of methanogenic archaea (Beifuss and Tietze 2005). In an effort to better understand the physiological significance of phenazine reduction in pseudomonads, we characterized the

effects of pyocyanin on redox homeostasis and central metabolism in *P. aeruginosa* PA14.

3.3. Materials and Methods

3.3.1. Bacterial Strains and Culture Conditions

For this study, we used *P. aeruginosa* strain UCBPP-PA14 (Rahme et al. 1995), which produces approximately ten times more pyocyanin in LB batch cultures than strain PAO1 (Dietrich et al. 2006). The *P. aeruginosa* PA14 mutant containing the *MAR2xT7* transposon inserted in the *ldhA* gene in a Δ*exoU* background was obtained from a publicly available mutant library (Liberati et al. 2006) and is mutant ID# 5174. Generation of the *P. aeruginosa* PA14 Δ*phzA1-1G1 ΔphzA2-2G2* deletion mutant (hereafter referred to as the Δ*phz* mutant) was described previously (Dietrich et al. 2006). *P. aeruginosa* PA14 wild type and mutants were grown aerobically at 37 °C in Luria-Bertani Broth, Miller (Fisher Scientific) or modified MOPS synthetic medium (Palmer et al. 2005). Our modified MOPS synthetic medium contained 50 mM morpholinepropanesulfonic acid (MOPS, Sigma) at pH 7.2, 93 mM NH_4Cl, 43 mM NaCl, 2.2 mM KH_2PO_4, 1mM $MgSO_4 \bullet 7H_2O$, and 3.6 μM $FeSO_4 \bullet 7H_2O$. Unless otherwise noted, 50 mM D-glucose was added to this medium as the sole carbon and energy source. Aerobic conditions were generated either through incubation with vigorous shaking at 250 rpm, or in a BioFlo 110 fermentor (New Brunswick Scientific) set to agitate at 250 rpm and bubble with 100% air at a rate of 2 L/minute. Aerobic culture volumes relative to vessel size are described below for specific experiments.

Culture densities were followed at 500 or 600 nm in a Thermo Spectronic 20D+ or Beckman Coulter DU 800 spectrophotometer. Cultures with optical densities greater than 0.8 were diluted 1:10 in fresh medium to allow accurate measurements.

3.3.2. Preparation of Pyocyanin for Reduction Assays and NADH/NAD^+ Studies

To maximize pyocyanin yields from *P. aeruginosa* cultures, we utilized a mutant, strain DKN370, which contains two copies of the gene *phzM*. PhzM converts phenazine-1-carboxylic acid to the precursor for pyocyanin, 5-methylphenazinium carboxylate (Mavrodi et al. 2001). Purification of pyocyanin by organic extractions was carried out as described previously (Dietrich et al. 2006). HPLC analysis verified the purity of pyocyanin after the extraction step, so the HPLC purification step described in Dietrich et al. (2006) was omitted. Purified pyocyanin was dissolved in MOPS buffer (MOPS synthetic medium without $FeSO_4$, $MgSO_4$, or glucose), and filtered (0.2 μm).

3.3.3. Whole Cell Suspension Assay for Pyocyanin Reduction

Cell culture samples were concentrated or diluted in filtrates of supernatants from the same culture to normalize optical density at 600 nm to 0.8. In an anaerobic chamber, the samples were transferred to cuvettes, and an anoxic solution of oxidized pyocyanin (in MOPS buffer) was added for a final pyocyanin concentration of about 0.1 mM. The cuvettes were stoppered to minimize oxygen exposure. Pyocyanin reduction was then followed as a decrease in absorbance at 690 nm over time in a DU 800 Beckman Coulter spectrophotometer. The rate of reduction could be calculated by converting the change in

absorbance to micromoles pyocyanin reduced using the extinction coefficient for pyocyanin at this wavelength (ε =4310 M^{-1} cm^{-1} at pH 7 (O'Malley et al. 2004)) and the volume of sample in the cuvette (1 mL).

3.3.4. Quantification of Pyocyanin

Pyocyanin concentrations in filtrates (0.2 μm pore) from LB and MOPS synthetic medium cultures were quantified as described previously (Dietrich et al. 2006). Briefly, absorbance in LB culture filtrates was measured spectrophotometrically at 690 nm and pyocyanin concentrations were calculated using the extinction coefficient for pyocyanin (above). Pyocyanin concentrations in 200-μl sample filtrates from MOPS synthetic medium cultures were determined by HPLC analysis on a Waters Symmetry C18 reverse-phase column with a gradient method (water versus acetonitrile containing 0.1% trifluoroacetic acid) and calculated based on absorbance values for purified standards diluted into MOPS buffer.

3.3.5. Extraction and Quantification of Intracellular NADH and NAD^+

Extraction of NADH and NAD^+ was carried out according to the method described in San et al. (2002). Two × 1 mL of culture were sampled into two separate microcentrifuge tubes and centrifuged at 16,000 × g for 1 minute. Supernatant was removed and pellets were resuspended in 300 μl of 0.2 M NaOH (for NADH extraction) or 0.2 M HCl (for NAD^+ extraction). These extracts were incubated for 10 minute at

50 °C, then for 10 minute on ice. While vortexing, 300 μl of 0.1 M HCl (for NADH) or 0.1 M NaOH (for NAD^+) were added drop wise to neutralize the solutions. They were then centrifuged for 5 minute at 16,000 × g. Supernatants were transferred to fresh tubes and stored at −80 °C until quantification.

Relative or absolute NADH and NAD^+ were quantified using a modification (San et al. 2002) of the enzyme cycling assay developed by Bernofsky and Swan (1973), adapted for measurement in a microtiter plate. A master reagent mix was prepared with 1× Bicine buffer (2.0 M, pH 8.0), 8× water, 1× 80 mM EDTA, 2× 100% EtOH, 2× 4.2 mM thiazolyl blue (MTT), and 4× 16.6 mM phenazine ethosulfate. The reagent mix was warmed to 30 °C, then 90-μl aliquots were dispensed into individual wells of a 96-well microtiter plate. Five microliters of standard or sample were added to each well, then the cycling reaction was started by the addition of 5 μl of alcohol dehydrogenase (Sigma #A-3263) prepared at 347 units/mL in 0.1 M Bicine (pH 8.0). The microtiter plate was incubated at 30 °C, mixed by brief shaking, and read every 30-60 seconds for absorbance at 570 nm, which is the spectral peak of MTT that increases upon reduction. Slopes arising from plots of absorbance at 570 nm over time were generated for NADH and NAD^+ standards as well as all samples. Standard curves were used to calculate the absolute concentrations in μM, and values were normalized to optical density of the original cell culture sample.

3.3.6. Relative Quantification of Dissolved Oxygen in Batch Cultures

Oxygen was measured in batch fermentor cultures using a Clark electrode (Clark et al. 1953). The electrode was calibrated such that the reading obtained by the computer without the probe attached was equal to zero, while the initial reading for the uninoculated medium (after aeration and agitation for 12 hours) was set to 100 percent.

3.3.7. Analysis of Small Organic Acids in Culture Filtrates

Two hundred microliters were sampled from MOPS-glucose cultures (10 mL in an 18 × 150 mm test tube) at regular intervals and filtered (0.2 μm pore). In cases where repeated sampling from the same culture would alter the total culture volume by more than 10%, multiple identical cultures were inoculated from the same preculture and sampled sequentially. Twenty microliters of each filtrate were loaded onto a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) and subjected to an isocratic method in 5 mM H_2SO_4 at 35 °C, using a Waters HPLC system. Compounds were detected by UV absorbance at 210 nm. Absolute concentrations of pyruvate were calculated using a standard curve for pyruvate diluted in MOPS buffer. The identity of the pyruvate peak was verified by coelution of an internal standard.

3.3.8. Pyruvate Fermentation Experiments

Strains were tested for the ability to survive via pyruvate fermentation using a method similar to that described by Schreiber et al. (2006). Stationary-phase LB cultures of wild-type PA14 and the *ldhA::MAR2xT7* mutant were centrifuged (8000 × g, 5

minutes) and resuspended at an OD (500 nm) of 11 in fresh LB. One milliliter of this suspension was used as an inoculum for 55 mL phosphate (100 mM, pH 7.4)-buffered LB in a 60-mL stoppered serum bottle. Cultures were amended with pyruvate to a concentration of 20 mM, or with water for negative controls, and incubated at 37 °C with shaking at 250 rpm. At regular intervals, 100 μl samples were drawn from anaerobic cultures using needles and syringes flushed with N_2. These samples were diluted and plated for CFU as previously described.

3.4. Results

3.4.1. *P. aeruginosa* PA14 Catalyzes Pyocyanin Reduction

Stationary-phase LB cultures of *P. aeruginosa* PA14 turn bright blue-green due to the production of the blue pigment pyocyanin specifically during this growth phase. *P. aeruginosa* PA14 also catalyzes the reduction of pyocyanin, a process that is readily observed when a stationary-phase culture is left standing without mixing or aeration by bubbling. Pyocyanin is converted from its blue (oxidized) form to a colorless (reduced) form (Cox, 1986). At the air-liquid interface, pyocyanin remains oxidized or becomes re-oxidized by an abiotic reaction with oxygen, but respiration by the bacteria creates a steep oxygen gradient just below this interface such that pyocyanin below a few millimeters remains colorless. A demonstration of this process is depicted in Figure 3.1.A (tube 3). We centrifuged a stationary-phase culture and resuspended the cell pellet in a 100 μM solution of pyocyanin in MOPS buffer, then allowed the culture to sit without shaking for 5 minutes at room temperature. A gradient formed that resembled those observed for cultures in growth media. After vortexing, the entire suspension regained its

original blue color (figure 3.1.A, tubes 5 and 6). A filtrate from this suspension had the absorbance spectrum characteristic of pyocyanin in the oxidation state most stable under atmospheric conditions. When we moved the culture into an anaerobic chamber and used a stoppered anaerobic cuvette to measure the absorbance spectrum of anaerobic culture filtrate, the sample showed decreased absorbance, indicating that pyocyanin had been reduced (figure 3.1.B).

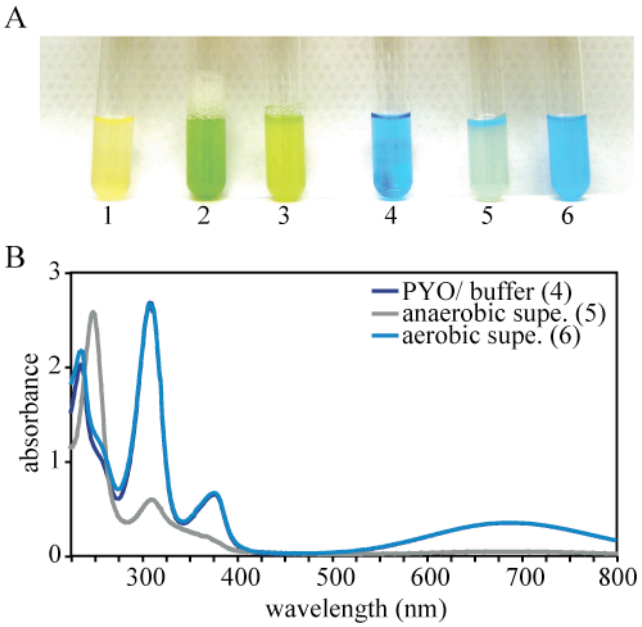

Figure 3.1. Stationary-phase *P. aeruginosa* PA14 cultures produce pyocyanin and directly catalyze its reduction.
(A) Tube 1, exponential-phase LB culture; tube 2, stationary-phase LB culture, immediately after removal from a shaking incubator; tube 3, stationary-phase LB culture, left standing at room temperature for ~5 minutes; tube 4, 100 μM pyocyanin in MOPS buffer, left standing at room temperature for ~5 minutes; tube 5 same culture as in tubes 2 and 3, resuspended in buffer shown in tube 4 and left standing at room temperature for ~5 minutes; tube 6, same suspension as in tube 5, after vortexing. (B) Absorbance spectra of buffer and supernatants from (A), tubes 4-6. The suspension from tube 5 was centrifuged and placed in a stoppered cuvette under anaerobic conditions. The pyocyanin/buffer spectrum overlaps almost completely with that of the supernatant from the aerated culture.

3.4.2. Pyocyanin Reduction Rates Increase in Stationary Phase

To quantify the rate of pyocyanin reduction by whole cells and test whether this process, like the biosynthesis of phenazines, was growth-phase dependent, we sampled an LB culture at different stages of growth. Samples were diluted into their own supernatant, amended with pyocyanin, and transferred to an anaerobic cuvette. We followed the decrease in oxidized pyocyanin absorbance over time for each sample, and observed a marked increase in the rate of pyocyanin reduction after the appearance of pyocyanin in stationary phase. This result indicates that the rate of pyocyanin reduction by whole cells is growth-phase dependent (figure 3.2).

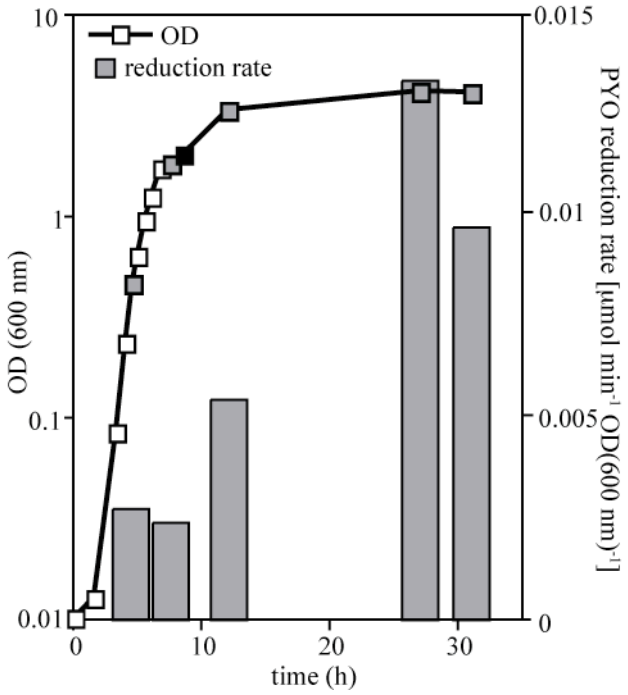

Figure 3.2. The rate of pyocyanin reduction increases in stationary phase in *P. aeruginosa* PA14.
A 100-mL *P. aeruginosa* LB culture was grown in a 500-mL Erlenmeyer flask and sampled at various points in the growth curve. Cells were concentrated or diluted in culture supernatant to normalize their OD (600 nm) to 0.8, amended with pyocyanin, then transferred to anaerobic cuvettes and stoppered. Absorbance at 690 nm was measured over time and was converted to the concentration of oxidized pyocyanin remaining in the cuvette. Gray squares indicate time points at which samples were taken for cell suspension assays. The black square indicates the first

appearance of pyocyanin in the culture. Data shown is representative of three separate experiments. OD, optical density.

3.4.3. Pyocyanin Exposure Balances the Intracellular Redox State

Strains of *P. aeruginosa* have been shown to vary in the timing and extent of phenazine production relative to the growth phase (Byng et al. 1979; Chang and Blackwood 1969; Dietrich et al. 2006). We have observed that the appearance of pyocyanin in wild-type *P. aeruginosa* PA14 LB cultures correlates with entry into stationary phase and that pyocyanin production plateaus in late stationary phase, reaching concentrations ranging from ~100 to 300 μM depending on the growth conditions (figures 3.3.A and 3.4.C).

Given that NADH reacts with pyocyanin *in vitro* (Kito et al. 1974), one potential consequence of pyocyanin production and/or exposure would be a decrease in intracellular NADH levels. We tested this by growing cultures of *P. aeruginosa* wild type and a Δ*phz* mutant (with in-frame deletions of both phenazine biosynthetic loci (Dietrich et al. 2006)) and measuring intracellular NAD(H) approximately 4 hours after the onset of stationary phase. The intracellular NADH/NAD^+ ratio in the wild type was less than half that observed for the Δ*phz* mutant. The growth curves for these cultures were virtually identical under the incubation conditions for this experiment (data not shown). Addition of 90 μM oxidized pyocyanin (the approximate concentration of pyocyanin produced by wild-type cultures under these conditions) to Δ*phz* mutant cultures reduced the NADH/NAD^+ratio to the wild-type level (figure 3.3.B). As a negative control, supernatant from the Δ*phz* mutant was treated similarly and tested for an effect on

intracellular NAD(H) concentrations; no difference was observed between cultures treated with "pyocyanin" preparations from the Δ*phz* mutant and those treated with water (data not shown). In titration experiments, an inverse relationship was found to exist between the concentration of pyocyanin added to a Δ*phz* mutant culture and the $NADH/NAD^+$ ratio (figure 3.3.C).

To test whether the effect of pyocyanin is similar to that of a physiologically relevant terminal electron acceptor, we added 30 mM nitrate (a concentration sufficient to support growth of *P. aeruginosa* via anaerobic nitrate respiration (Williams et al. 1978)) to a wild-type culture, and nitrate with or without pyocyanin to Δ*phz* mutant cultures in stationary phase (figure 3.3.B). Nitrate and pyocyanin both effected decreases in intracellular $NADH/NAD^+$ ratios, apparently by catalyzing NADH oxidation, since decreases in absolute NADH concentrations correlated with increases in absolute NAD^+ concentrations (figure 3.3.D). Whereas pyocyanin effected a decrease when added in the micromolar range, nitrate did only when added at millimolar concentrations (data not shown). Together, these results suggested that NADH can act as a source of electrons for pyocyanin reduction.

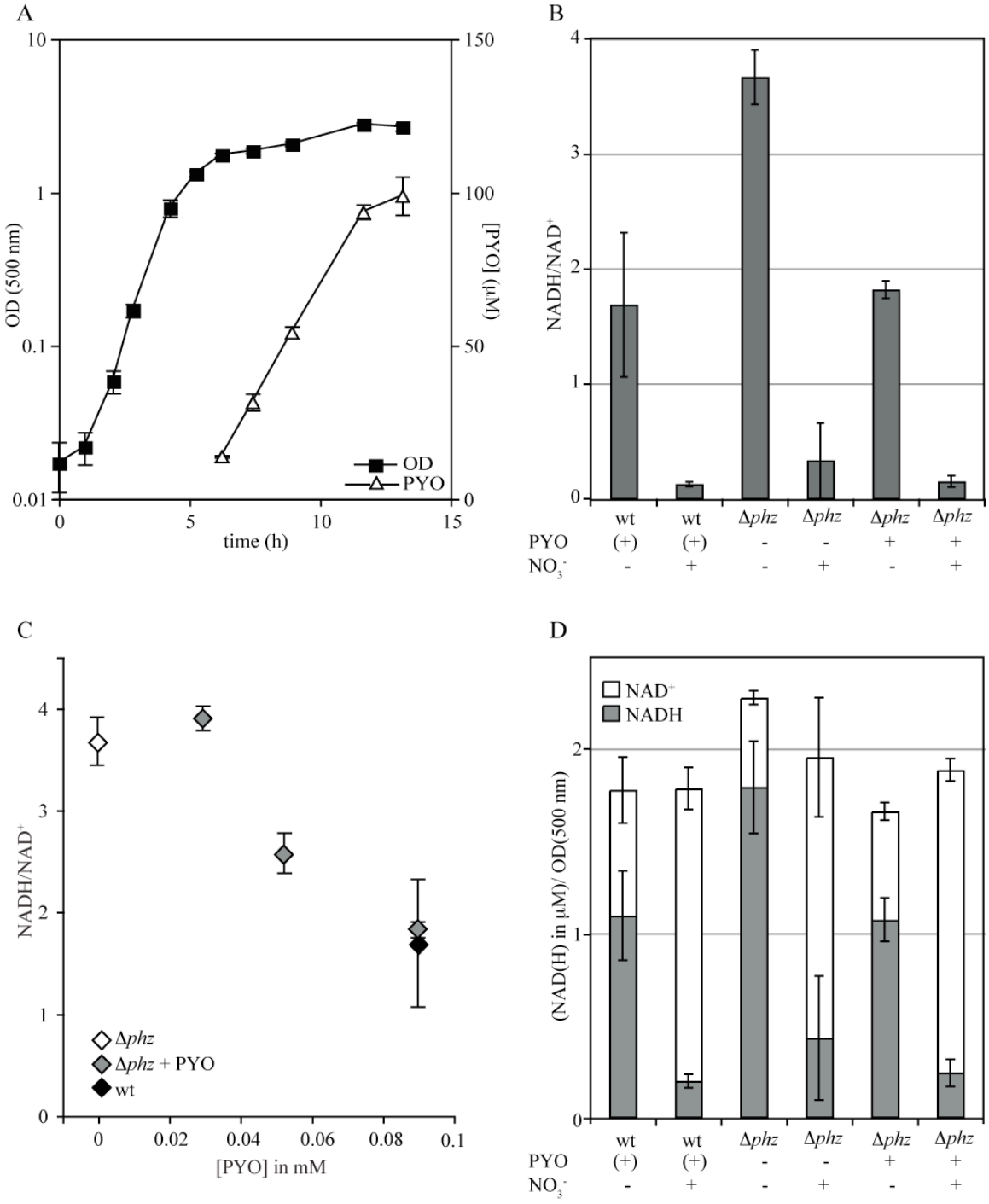

Figure 3.3. Pyocyanin exposure effects redox balancing in stationary phase in a manner analogous to that of a known physiological electron acceptor.
(A) Growth and pyocyanin production for wild-type *P. aeruginosa* PA14 grown aerobically in 10 mL LB in 18×150 mM tubes. (B) NADH/NAD^+ratios for cultures grown under the same conditions as those described in part (A). At 7 hours, pyocyanin production in the wild-type cultures was visible by eye. 45 μM (half the expected final concentration) was added to the Δ*phz* cultures to be tested for complementation, and 15 mM KNO_3 was added to cultures to be tested

for the effect of an additional electron acceptor. At 9 hours, pyocyanin in the wild-type cultures had increased to near-maximum concentrations, so a second dose of pyocyanin or KNO_3 was added to the appropriate cultures, for final concentrations of 90 μM and 30 mM, respectively. Water was added to negative controls. Eleven hours after inoculation, and 2 hours after the addition of the final dose of pyocyanin, NAD(H) was extracted and assayed for each culture. (C) $NADH/NAD^+$ ratios for cultures treated as in part (B), but with varying concentrations of pyocyanin added. (D) NADH and NAD^+ concentrations for cultures described in (B), normalized to OD (500 nm). Error bars represent the standard deviations of triplicate samples. OD, optical density. wt, wild-type.

3.4.4. The Intracellular $NADH/NAD^+$ Ratio is Influenced by the Relative Availability of Electron Donor and Acceptor

Our observation that other electron acceptors, i.e., pyocyanin and nitrate, decreased the $NADH/NAD^+$ ratio suggested that oxygen was limiting during stationary phase in our cultures. This could explain the accumulation of NADH 4 hours after the onset of stationary phase in the Δ*phz* mutant (figure 3.3.B). To confirm this, we grew a batch culture of the Δ*phz* mutant in a fermentor, which allowed us to control temperature and aeration while simultaneously measuring dissolved oxygen in the culture. We sampled at regular intervals to measure optical density and extract NAD(H). As predicted, oxygen levels decreased slowly until the culture reached mid- to late-exponential phase, at which time it plummeted to zero. This drop in oxygen correlated with an increase in the intracellular $NADH/NAD^+$ ratio (figure 3.4.A). To test whether the drop in oxygen depended on the availability of electron donors for oxygen reduction, we repeated the experiment and added 20% of the glucose concentration added to the medium in the initial experiment (10 mM versus 50 mM). When less electron donor was available, the oxygen concentration decreased in mid-exponential phase, but never reached zero and rapidly increased again upon entry into stationary phase (figure 3.4.B). This culture never reached the same growth yield achieved by the culture containing 50

mM glucose, implying that the carbon source was the limiting factor that led it to enter stationary phase. The culture experienced oxygen limitation only transiently, if at all, due to the lower ratio of electron donor to electron acceptor in the experiment depicted in Figure 3.4.B compared to Figure 3.4.A. As a result, the $NADH/NAD^+$ ratio never reached the high level observed for the culture containing excess glucose.

Finally, we tested the wild-type strain in the presence of 50 mM glucose, and sampled for pyocyanin concentrations in addition to NAD(H) and cell density. The wild-type strain also exhibited increased $NADH/NAD^+$ ratios upon entry into stationary phase, and these ratios correlated with oxygen limitation. However, unlike the Δ*phz* mutant, the wild type showed a decrease in intracellular $NADH/NAD^+$ that correlated with the appearance of pyocyanin in the culture. These results further support the hypothesis that pyocyanin can act as an alternate oxidant under conditions where the terminal electron acceptor for respiration has become limiting. This interpretation derives from the large difference in NADH levels observed between the wild-type strain and Δ*phz* after about 12 hours of incubation, and the correlation between decreasing NADH levels and increasing pyocyanin concentrations in culture filtrates observed upon entry into stationary phase (figure 3.4.C).

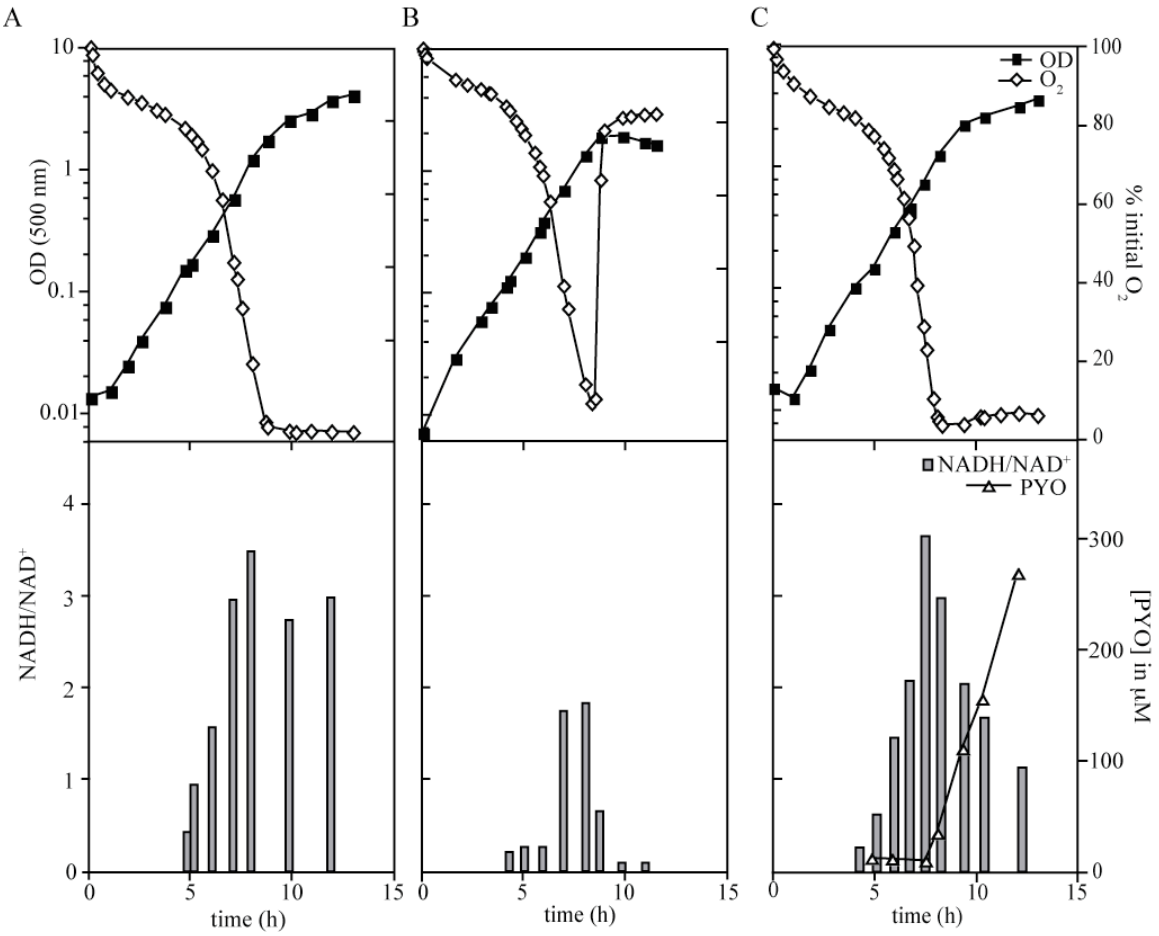

Figure 3.4. NADH accumulates in stationary phase in cultures limited for oxygen and defective in pyocyanin production.
P. aeruginosa wild type and Δ*phz* cultures were grown in 1 L MOPS synthetic medium supplemented with either 50 or 10 mM glucose in a 3-L fermentor with constant aeration and agitation. Cultures were sampled at various points in the growth curve to allow measurement of the optical density (OD) at 500 nm and extraction of NAD(H). Relative dissolved oxygen concentrations were measured throughout growth using a polarographic oxygen electrode. OD (500 nm), dO_2, and $NADH/NAD^+$ are shown for (A), the Δ*phz* mutant grown in medium containing 50 mM glucose, and (B) the Δ*phz* mutant grown in medium containing 10 mM glucose. For (C), wild-type *P. aeruginosa* PA14 grown in medium containing 50 mM glucose, these parameters plus the concentration of pyocyanin produced by the culture are shown.

3.4.5. *P. aeruginosa* PA14 Excretes, and then Consumes, Pyruvate in Late Stationary Phase

For fermentative organisms such as *E. coli* and *Propionibacterium freudenreichii*, the addition of the synthetic redox-cycling compound ferricyanide has been shown to alter carbon flux through central metabolic pathways. Particularly when the reoxidation of this compound is coupled to electron transfer to an electrode, ferricyanide shifted the fermentation balance away from ethanol and propionate, products that require NADH for their formation, toward acetate, a more oxidized product (Emde et al. 1989; Emde and Schink 1990). This implies that the ferricyanide acts as an electron shuttle from major pools of reductant inside the cell, such as NADH, to the electrode, thereby lessening the need for formation of more reduced fermentation products to dissipate cellular reductant.

To determine whether pyocyanin could play a similar role in *P. aeruginosa*, we analyzed filtered culture supernatants for small organic acids that are known fermentation products of *P. aeruginosa* metabolism. *P. aeruginosa* has been shown to ferment pyruvate under energy-starved conditions, converting it to lactate, acetate, and/or succinate. The production of lactate or succinate from pyruvate requires NADH as a substrate, while the conversion of pyruvate to acetate requires NAD^+ (Eschbach et al. 2004). Therefore, the NADH/NAD^+ ratio in the wild type would be more favorable for acetate production, whereas the NADH/NAD^+ ratio in the Δ*phz* mutant would favor production of lactate and succinate.

Surprisingly, we observed a marked difference between the wild type and the Δ*phz* mutant with respect to the production of pyruvate itself. In late stationary-phase

(about 30 hours after inoculation) after growth in a defined medium with 50 mM glucose, we observed pyruvate concentrations as high as 6 mM in wild-type culture filtrates (as indicated by a peak eluting at about 10.5 minutes), but were unable to detect any pyruvate in filtrates from Δ*phz* mutant cultures. Adding pyocyanin to the Δ*phz* mutant upon entry into stationary phase complemented the pyruvate excretion phenotype (figure 3.5), although incompletely because we added only about half the final concentration of pyocyanin produced by the wild type under these conditions (50 versus 100 μM). We also detected citrate, lactate and acetate in both wild-type and Δ*phz* mutant culture filtrates at similar concentrations, eluting at ~9.1, 14.3 and 17.0 minutes, respectively. The peak eluting at 7.1 minutes was the MOPS buffer from the medium. We were unable to identify the compounds represented by the peaks eluting at approximately 7.3 minutes (wild-type filtrate only), and 9.9 and 12.2 minutes (both wild-type and Δ*phz* mutant filtrates). Standards containing 2-oxoglutarate and malate were run with the same method, but did not co-elute with any of these peaks.

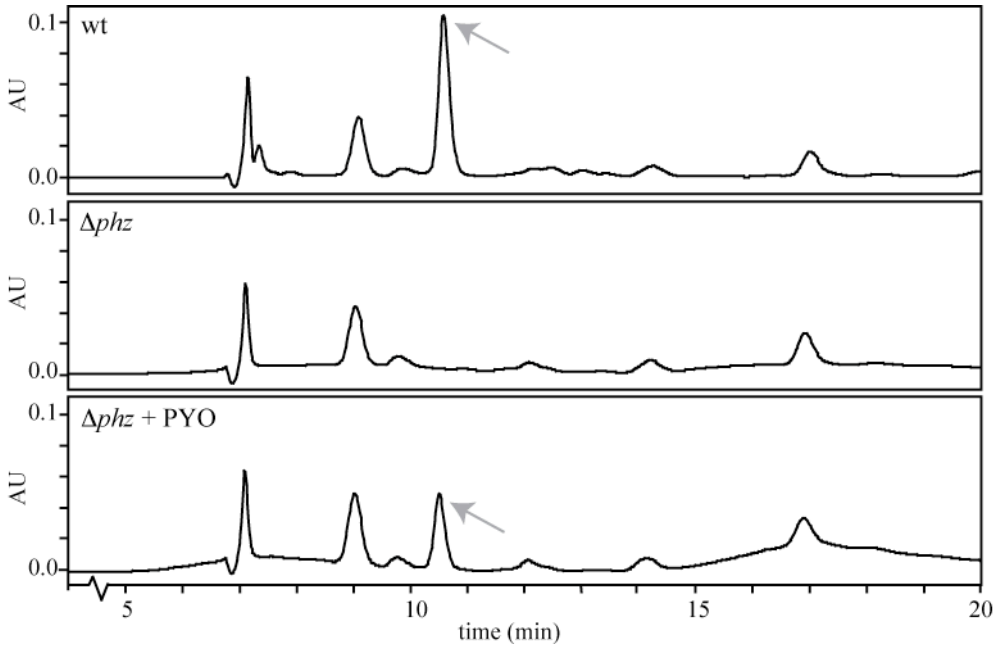

Figure 3.5. Wild-type *P. aeruginosa* PA14 excretes pyruvate in stationary phase, and addition of pyocyanin to Δ*phz* mutant cultures restores the pyruvate excretion phenotype. Cultures were inoculated into MOPS synthetic medium amended with 50 mM glucose (initial OD (500 nm) = 0.03). To complement pyruvate excretion, 50 μM pyocyanin was added to the Δ*phz* culture at the time when pyocyanin reached its maximum concentration in the wild-type cultures (approximately 12 hours after inoculation). 20 μl of culture filtrates at the 24-hour time point were loaded onto an anion exchange column and subjected to an isocratic gradient in 5 mM H_2SO_4. Pyruvate peaks are indicated by arrows. The elution time of pyruvate drifts slightly but averages around 10.5 minutes. Results shown are representative of three separate experiments. Other peak identities are described in the text.

To better constrain the timing of metabolite excretion in the wild type and the Δ*phz* mutant, we sampled every 4 hours from duplicate cultures over the course of approximately 30 hours in stationary phase (figure 3.6). Pyruvate appeared at detectable levels in wild-type cultures between 22 and 26 hours after inoculation, and had increased to ~5 mM after 38 hours. However, by the 42-hour time point, the pyruvate in both replicates had decreased to levels below the detection limit (~0.05 mM) (figure 3.6.C). Abiotic degradation of pyruvate generates a peak eluting at approximately 8 minutes, which does not co-elute with any of the peaks observed in traces from our culture filtrates

(data not shown). Therefore, the disappearance of the pyruvate peak at the 42-hour time point implied that it had been metabolized by the bacteria.

Another phenotype that became apparent under these growth conditions was the reproducible difference in cell yields between wild-type and Δ*phz* mutant cultures. The optical densities of wild-type cultures were typically lower than those of the Δ*phz* mutant cultures in stationary phase, a phenotype that becomes more apparent when the optical density is plotted on a linear scale (figures 3.6.A and 3.6.B).

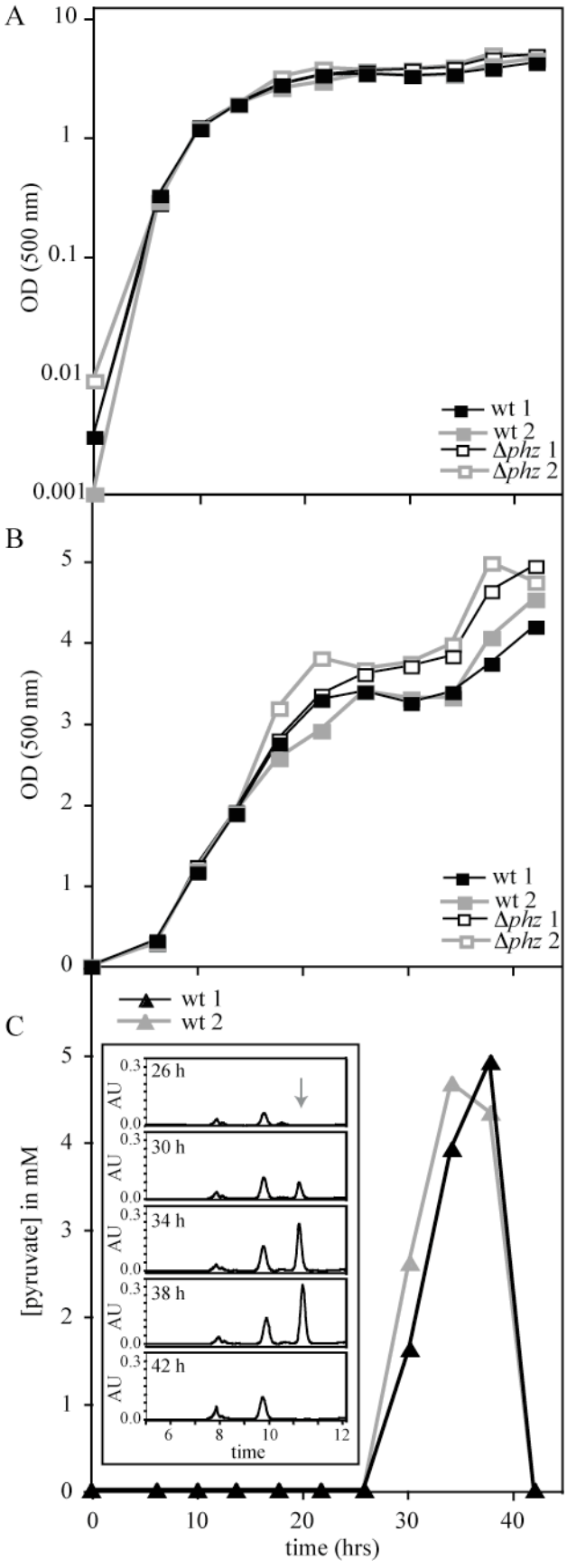
A
10
1
0.1
0.01
0.001
OD (500 nm)
wt 1
wt 2
Δphz 1
Δphz 2
B
5
4
3
2
1
0
OD (500 nm)
wt 1
wt 2
Δphz 1
Δphz 2
C
wt 1
wt 2
[pyruvate] in mM
26 h
30 h
34 h
38 h
42 h
AU
0.3
0.0
6
8
10
12
time
0
10
20
30
40
time (hrs)

Figure 3.6. *P. aeruginosa* PA14 cultures consume excreted pyruvate in very late stationary phase.
Duplicate cultures were inoculated at OD (500 nm) ~ 0.001 in MOPS synthetic medium amended with 50 mM glucose. Approximately every 4 hours, 100-200 μl culture were sampled and filtered for HPLC analysis as described for figure 3.4. (A), OD 500 for wild-type and Δ*phz* cultures plotted on a logarithmic scale. (B), same data as in (A) plotted on a linear scale to show the lower growth yields consistently observed for wild-type PA14 under this condition. (C) Quantification of pyruvate production for the "wt 1" and "wt 2" cultures, and inset, chromatograms demonstrating the disappearance of pyruvate at 42 hours for the "wt 1" culture. The arrow indicates the elution time of the pyruvate peak. wt, wild type.

3.4.6. Pyruvate Fermentation Facilitates Survival in Energy-Starved *P. aeruginosa* PA14 Cultures

Recently, Schobert and colleagues have characterized genes implicated in a pyruvate fermentation pathway in *P. aeruginosa* strain PAO1 (Eschbach et al. 2004; Schreiber et al. 2006). In this pathway, pyruvate is converted by multiple enzymes to succinate, acetate, and/or lactate. We do not suspect that these reactions were responsible for the consumption of pyruvate in late stationary phase in our cultures, because these compounds are detectable by our analytical HPLC method, and we did not see their concentrations increase as pyruvate disappeared (data not shown). We therefore hypothesize that pyruvate was completely oxidized through the utilization of the small amount of oxygen available to the cells. However, in environments with steep gradients of electron acceptor availability, such as those encountered in surface-attached or aggregated bacterial communities, excreted pyruvate may be utilized for substrate-level phosphorylation when respiratory electron acceptors become limiting. To verify that *P. aeruginosa* strain PA14 can utilize pyruvate for survival under strict anaerobic conditions, we incubated the wild type and an *ldhA* mutant, defective in the ability to reduce pyruvate to lactate, in stoppered serum bottles containing buffered LB amended

with 20 mM pyruvate. As a control, we set up a wild-type culture with no pyruvate. We followed colony-forming units in samples from these cultures over more than three weeks, and found that, as had been previously reported for *P. aeruginosa* PAO1 (Eschbach et al. 2004; Schreiber et al. 2006), a mutant with a disruption in the gene *ldhA* was defective in survival on pyruvate (figure 3.7). The decline of this mutant was similar to that of the wild-type culture containing no added pyruvate. *P. aeruginosa* PA14 is therefore also able to survive under conditions of energy starvation through utilization of a lactate dehydrogenase-dependent pathway for pyruvate fermentation.

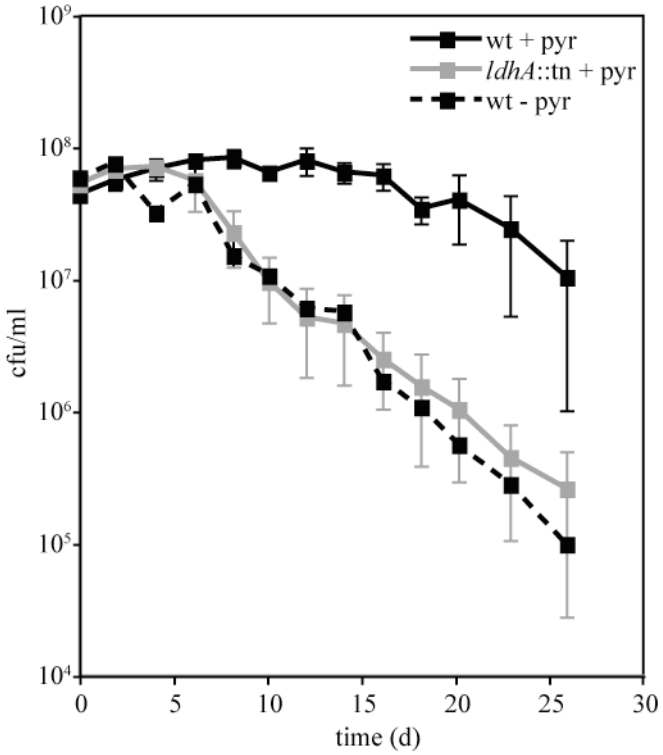

Figure 3.7. Pyruvate fermentation facilitates survival under anaerobic conditions in *P. aeruginosa* PA14.
Triplicate cultures of the wild type and *ldhA::MAR2xT7* Δ*exoU* mutant were incubated in stoppered serum bottles containing phosphate-buffered LB medium amended with 20 mM pyruvate. At regular intervals, 100 μl of each culture were sampled with N_2-flushed needles, diluted, and plated for colony-forming units. A single replicate of the wild type lacking pyruvate showed a similar survival defect as that observed for the *ldhA* mutant. Since the *ldhA* mutant utilized in this study also contains a deletion in the *exoU* gene, we have compared a transposon insertion mutant in *exoU* to the wild type and found that a defect in ExoU production does not affect the ability of *P. aeruginosa* PA14 to survive under pyruvate fermentation conditions (data not shown). tn, transposon *MAR2xT7*. pyr, pyruvate.

3.5. Discussion

In this study, we have characterized the effects of a stationary phase-specific metabolite on the carbon and energy metabolism of *P. aeruginosa* cultures. Metabolites formed in stationary phase historically have been categorized as products of "secondary" forms of metabolism that bear little relevance to energy generation. However, we have shown that the redox activity of pyocyanin, a phenazine produced after the exponential

phase of growth in batch cultures, affects the metabolic status of its producer. Bacteria such as the pseudomonads, with limited capacities for fermentation, are generally thought to depend on terminal electron acceptors, and on the function of their membrane-bound respiratory chains, for the ability to maintain a balanced intracellular redox state. The redox-balancing effect of pyocyanin may be particularly important in bacterial communities limited for oxygen, an electron acceptor whose uptake rate outpaces its diffusion rate through dense cultures of respiring bacteria (Sweet and Peterson 1978; Xu et al. 1998).

As part of our characterization of the physiological effects of pyocyanin reduction in *P. aeruginosa*, we found that pyocyanin reductive activity in whole cells increases after entry into stationary phase and the appearance of phenazines in batch cultures (figure 3.2). While we cannot rule out that this increase is due merely to an increase in the concentration of the electron donor for this reaction, we know that pyocyanin induces expression of genes encoding multidrug efflux pumps and oxidoreductases that could be involved in the redox cycling of this compound (Dietrich et al. 2006). Such gene products may contribute to the observed increase in the pyocyanin reduction rate. We also found that pyocyanin exposure in stationary phase cultures decreases the intracellular $NADH/NAD^+$ ratio (figure 3.3). The increase in the $NADH/NAD^+$ ratio that we observe for a mutant defective in phenazine production correlates with oxygen limitation (figure 3.4), and is relieved by the presence of pyocyanin (figure 3.3), suggesting that pyocyanin plays a role in redox balancing.

Given that the reduction of pyocyanin by NADH is a thermodynamically favorable reaction, one could attribute the observed inverse relationship between intracellular [NADH] and pyocyanin production (figure 3.4.C) to a model such as that shown in Figure 3.8. In this model, stationary-phase cells accumulate NADH as a consequence of oxygen limitation (figure 3.8.A), and transfer electrons to pyocyanin when it becomes available (figure 3.8.B). Pyocyanin can subsequently be reoxidized through abiotic electron transfer to oxygen. Differences in central metabolism depending on the presence or absence of synthetic and environmental electron shuttles have been reported for fermentative bacteria (Benz et al. 1998; Emde et al. 1989; Emde and Schink 1990), and apparently arise from the effects of extracellular electron shuttling on the intracellular redox state. If the mechanism of redox balancing depicted in Figure 3.8.B were operational, we might expect a phenazine-null mutant (lacking the naturally produced pseudomonad electron shuttle) to be defective in complete oxidation of its carbon source, since flux through the citric acid cycle would be inhibited by a relatively reduced NAD(H) pool. Instead, we observed that the wild-type strain appears to be affected in its ability to mineralize its carbon source, based on the excretion of pyruvate in late stationary phase (figures 3.5 and 3.6). This phenomenon thus suggests an alternative mechanism for lowering the NADH/NAD^+ ratio, in which NADH accumulation is avoided in the wild type by excretion of pyruvate before it can enter the citric acid cycle and reduce NAD^+ (figure 3.8.C). Because stationary-phase *P. aeruginosa* cells both catalyze pyocyanin reduction and excrete pyruvate, maintenance of redox homeostasis in stationary-phase *P. aeruginosa* PA14 could be due to a combination of the mechanisms shown in Figures 3.8.B and 3.8.C.

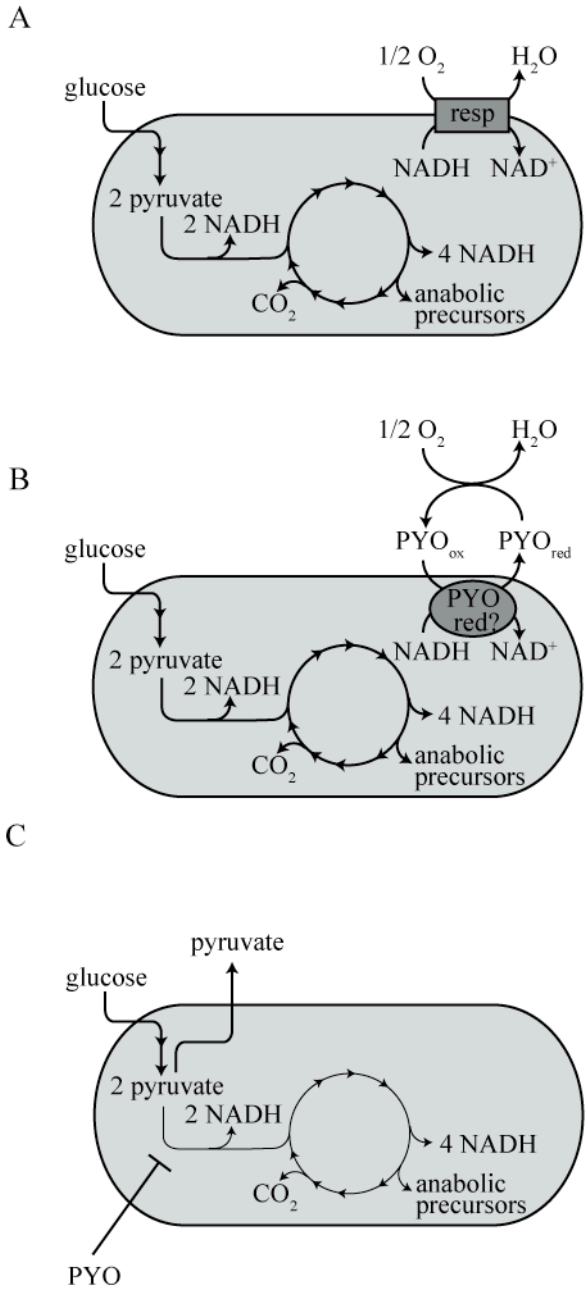

Figure 3.8. Model: Pyocyanin reduction allows *P. aeruginosa* PA14 to maintain redox homeostasis under oxygen-limited conditions.
When sufficient oxygen is available for growth (A), the aerobic respiratory chain ("resp") can catalyze the reoxidation of NADH. Under conditions in which terminal electron acceptors for respiration are limiting (B), *P. aeruginosa* can couple the reoxidation of NADH to the reduction of pyocyanin, either directly or through an enzyme-mediated reaction as represented by "pyocyanin red," a putative phenazine reductase. The electrons could be transferred from pyocyanin to oxygen through an abiotic extracellular reaction. (C) Also under conditions of oxygen limitation, the NADH/NAD^+ ratio could be balanced through inactivation of the pyruvate

dehydrogenase complex by pyocyanin. NAD^+ reduction (and therefore NADH production) would be avoided because pyruvate would be excreted without further oxidation.

Although pyruvate excretion has been observed in cultures of other bacteria, such as *Aerobacter aerogenes* and *Photobacterium fischeri* (since reclassified as *Enterobacter* and *Vibrio*, respectively), the mechanisms underlying its regulation have not been elucidated (Ruby and Nealson 1977; Webb 1968). An understanding of the pyruvate oxidation machinery in pseudomonads provides insight into the potential mechanisms whereby this reaction may be inhibited in pyocyanin-producing cells. The conversion of pyruvate to acetyl-CoA is catalyzed by pyruvate dehydrogenase, a large multienzyme complex prevalent during aerobic growth in bacteria and eukarya, though there is some evidence for its occurrence in archaea as well (Jolley et al. 2000). All pyruvate dehydrogenase multienzyme complexes require dihydrolipoamide, a cofactor which is covalently bound to the E2 subunit, to transfer an acyl group derived from pyruvate to coenzyme A (CoA) and produce acetyl-CoA. Dihydrolipoamide contains a disulfide bond that is broken and reformed during the three-step pyruvate decarboxylation and oxidation mechanism (Cronan et al. 2005; De Kok et al. 1998). The one-electron reduction of each suflhydryl group of the lipoamide cofactor on the E2 subunit, catalyzed by superoxide (Bunik and Sievers 2002; Tabatabaie et al. 1996), is thought to inactivate the enzyme. The generation of superoxide by pyocyanin, or even the formation of pyocyanin radical itself (Hassett et al. 1992), may therefore inhibit the pyruvate dehydrogenase complex, leading to accumulation of pyruvate.

Regardless of whether the excretion of pyruvate by wild-type *P. aeruginosa* results from an apparently toxic side reaction, it may be beneficial under pyocyanin-producing conditions. Excreted pyruvate that remains in the immediate environment could potentially be accessed later during a "last gasp" under conditions of extreme energy starvation. While this scenario is unlikely to occur in the soil, where other microorganisms might consume the pyruvate before it was metabolized by the producer, it may be relevant for conditions encountered by *P. aeruginosa* during chronic infection of the lung. Individuals with chronic *P. aeruginosa* infections resulting from impaired lung function harbor monocultures of this bacterium at cell densities as high as 10^7 CFU/g sputum (Lyczak et al. 2002). It is thought that bacteria in the lung cavity experience steep gradients of electron acceptor availability (Worlitzsch et al. 2002); therefore, the ability to reserve a pool of substrate or transfer it to an energy-starved neighbor may contribute to the ability of *P. aeruginosa* populations to persist throughout the lifetime of the individual host (Schreiber et al. 2006). Pseudomonad phenazines as well as other high cell-density signals have been detected in the sputum of chronically infected patients (Singh et al. 2000; Wilson et al. 1988). An interesting avenue for future research, therefore, its to determine whether the physiological effects of pyocyanin on *P. aeruginosa* contribute to its long-term survival during chronic colonization of the lung.

3.6. Acknowledgements

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Chapter 4

Identification and Characterization of *Pseudomonas aeruginosa* Mutants Defective in Pyocyanin Reduction

4.1. Abstract

Pseudomonas aeruginosa catalyzes electron transfer to its own, endogenous phenazine antibiotics. Using ferric citrate reduction as a proxy for phenazine reduction, we screened a *Pseudomonas aeruginosa* PA14 transposon insertion mutant library for defects in reduction of the blue phenazine pyocyanin. In addition to the expected hits in pyocyanin biosynthesis and regulation, this screen uncovered genes potentially involved in pyocyanin transport and reduction. Our findings suggest roles in pyocyanin redox cycling for two metabolic enzymes that have been well characterized in other organisms: the biosynthetic glycerol-3-phosphate dehydrogenase, and the cytochrome bc_1 complex of the respiratory chain. Mutants lacking these enzymes retain approximately 50% of the wild-type level of pyocyanin reduction activity, while a double mutant lacking both enzymes retained less than 40% of the wild-type activity. The biosynthetic glycerol-3-phosphate dehydrogenase of *P. aeruginosa* is involved in production of cytosolic reducing equivalents, a prerequisite for pyocyanin reduction. Ubiquinol:cytochrome *c* oxidoreductases have been implicated in phenazine redox toxicity in mammalian cells,

fungi, and nonpseudomonad bacteria; our findings extend this role to the reduction of pyocyanin in its producer.

4.2. Introduction

Biochemists and physiologists routinely exploit the biological reactivities of synthetic redox-active dyes, such as tetrazolium salts, viologens, and phenazines, in colorimetric assays for *in vivo* and *in vitro* reactions. Many bacteria excrete natural products that share the properties exhibited by these synthetic reagents. This is the case for the phenazine pigments, produced by some *Pseudomonas* strains as well as a diversity of other isolates (Mavrodi et al. 2006; Turner and Messenger 1986). Synthetic phenazines, such as phenazine methosulfate, phenazine ethosulfate, and neutral red, are used in biochemical and enzymatic assays, as well as electrochemical experiments that require a mediator to facilitate electron transfer between cells or enzymes and electrodes (Kobayashi and Tagawa 2004; Park and Zeikus 2000). The phenazines naturally produced by *Pseudomonas aeruginosa* have been shown to enhance electron transfer between bacteria and electrodes (Pham et al. 2008; Rabaey et al. 2005). Pseudomonad phenazines, like synthetic phenazines, have also been shown to catalyze the reduction of extracellular iron, increasing its bioavailability (Hernandez et al. 2004; McKinlay and Zeikus 2004; Wang and Newman 2008). This has reignited earlier speculation about the physiological relevance of these compounds in the producing organism (Friedheim 1931; Price-Whelan et al. 2006; Trutko et al. 1988).

The similarities between naturally produced and synthetic phenazines have been apparent for decades, and research attention has focused primarily on the toxicity of these compounds. However, the bacteria that produce these compounds do not appear to experience toxicity. They catalyze phenazine reduction and oxidation, but rather than suffering or merely tolerating these compounds, they appear to benefit from their presence under certain conditions (Dietrich et al. 2008; Maddula et al. 2006, 2008). Understanding the nature of redox reactions between phenazines and intracellular metabolites or enzymes of the producing organism may provide insight into the physiological roles of these compounds and the ability of these bacteria to survive in diverse environments. It may also allow us to better control persistent infections established by opportunistic pathogens such as *P. aeruginosa* (Lau et al. 2004a; Lau et al. 2004b). We conducted a genetic screen to identify loci involved in the reduction of pyocyanin in *P. aeruginosa* PA14 and further characterized the roles of two enzymes—the biosynthetic glycerol-3-phosphate dehydrogenase and the cytochrome bc_1 complex of the respiratory chain—in contributing to redox homeostasis and pyocyanin reduction in this bacterium.

4.3. Materials and Methods

4.3.1. Bacterial Strains and Culture Conditions

Strains and mutants that were used in this study are listed in Table 4.1. Plasmids and primers that were used are listed in Table 4.2. Bacteria were grown either in Lysogeny Broth (LB) or MOPS defined medium [50 mM morpholinepropanesulfonic

acid (MOPS, Sigma) at pH 7.2, 93 mM NH_4Cl, 43 mM NaCl, 2.2 mM KH_2PO_4, 1mM $MgSO_4 \cdot 7H_2O$, and 3.6 mM $FeSO_4 \cdot 7H_2O$]. Twenty millimolar D-glucose was added to the MOPS-buffered medium as the sole carbon and energy source. Unless otherwise noted, cultures were grown in 10-mL volumes in 18 × 100 mm tubes and shaken vigorously at 250 rpm. Culture densities were followed at 500 nm in a Thermo Spectronic 20D+ or Beckman Coulter DU 800 spectrophotometer. Cultures with optical densities greater than 0.8 were diluted 1:10 in fresh medium to allow accurate measurements. For the purposes of this study, "early stationary phase" refers to cultures grown from an optical density of 0.01 for about 12-14 hours; this corresponds to an optical density of approximately 2.5 for the wild type. "Late stationary phase" refers to cultures grown for about 18-20 hours, corresponding to an optical density of approximately 3.5 for the wild type.

E. coli WM3064 was grown on LB amended with 0.3 mM diaminopimelic acid. For selection and maintenance of plasmid pUCP18 and derivatives in *E. coli* and *P. aeruginosa*, 100 and 300 μg/mL carbenicillin, respectively, was added to LB medium. Selection and maintenance of *E. coli* containing pSMV10 was carried out on 15 μg/mL gentamicin sulfate. *P. aeruginosa* exconjugants containing pSMV10 were selected on 100 μg/mL gentamicin sulfate. Counterselection against pSMV10 was carried out on 1% tryptone, 0.5% yeast extract, 10% sucrose plates.

Culture methods specific to the screen protocols are described in sections 4.3.2 and 4.3.3.

Table 4.1. Strains and mutants used in this study

Strain or mutant	Genotype and/or description	Source
P. aeruginosa		
PA14	Wild type	Rahme et al. 1995
PA14 Δ*phz*	Δ*phzA1-G1* Δ*phzA2-G2*	Dietrich et al. 2006
PA14 *gpsA*::tn	*gpsA*::MAR2XT7; $Gent^R$	Liberati et al. 2006
PA14 *fbcC*::tn	*fbcC*::MAR2ST7; $Gent^R$	Liberati et al. 2006
PA14 Δ*gpsA*	Δ*gpsA*	This study
PA14 Δ*fbcC*	Δ*fbcC*	This study
PA14 Δ*phz* Δ*gpsA*	Δ*phzA1-G1* Δ*phzA2-G2* Δ*gpsA*	This study
PA14 Δ*phz* Δ*fbcC*	Δ*phzA1-G1* Δ*phzA2-G2* Δ*fbcC*	This study
PA14 Δ*gpsA* Δ*fbcC*	Δ*gpsA* Δ*fbcC*	This study
PA14 Δ*phz* Δ*gpsA* Δ*fbcC*	Δ*phzA1-G1* Δ*phzA2-G2* Δ*gpsA* Δ*fbcC*	This study
E. coli		
UQ950	DH5a λ(*pir*); host for cloning	Douglas Lies, Caltech
WM3064	Δ*dapA*1341::[*erm pir*(wt)]; donor strain for conjugation	William Metcalf, U. of Illinois

Table 4.2. Plasmids and primers used in this study.

Plasmid or primer	Genotype or sequence and description	source
Plasmids		
pUCP18	broad host range vector for complementation; Cb^R	Schweizer 1991; West et al. 1994
pAPW3	pUCP18 containing *gpsA* gene cloned into EcoRI/Acc65I site	This study
pAPW5	pUCP18 containing *fbcC* gene cloned into SmaI/XbaI site	This study
pSMV10	*oriR6K mobRP4, sacB;* $Gent^R$; mobilizable suicide vector	Douglas Lies, Caltech
pAPW6	pSMV10 containing 2-kb fusion PCR fragment for *gpsA* deletion, cloned into SpeI site	This study
pAPW7	pSMV10 containing 2-kb fusion PCR fragment for *fbcC* deletion, cloned into SpeI site	This study
Primers		
gpsA 1	cggcGAATTCcgggtgatgatgttgatca	
gpsA 2	cggcGGATCCtcggtttgtagtcattgcg	
PA4429 1	cggcCCCGGGtttgcaccgtcctgtattt	
PA4429 2	cggcTCTAGAgttgattgaagccatgga	
gpsA 1 5' b	GCGactagtCGGCATAGTCGTGGGC	
gpsA 1 3'	cccatccactaaatttaaataTCCACTGGCGGACCGC	
gpsA 2 5'	tatttaaatttagtggatgggCGATCCTCTTCGAAGGC	
gpsA 2 3'	GCGactagtGATGACGCTTTTCGCGC	
PA4429 1a	cagcttgttgattgaagccaAGTGCAGCGAATTGCTTTTT	
PA4429 1b	GCGactagtGGCTGGATCATTCGCTACAT	
PA4429 2a	GCGactagtCGCTGGAATGTACAGGCTCT	
PA4429 2b	aaaaagcaattcgctgcactTGGCTTCAATCAACAAGCTG	

4.3.2. Primary Screen for Ferric Citrate Reduction in *P. aeruginosa* PA14

Cultures were inoculated from frozen glycerol stocks of the nonredundant *P. aeruginosa* PA14 transposon insertion mutant library (the PA14 NR Set; prepared as described in Liberati et al. (2006) into deep-well microtiter plates containing 500 μl LB per well. The deep-well plate was covered with gas-permeable film and incubated overnight at 37 °C with shaking at 250 rpm. After approximately 16 hours of incubation, the plate was moved into an anaerobic chamber containing a 95% nitrogen/5% hydrogen atmosphere. One hundred microliters of ferric citrate solution (80 mM citrate, 40 mM Fe(III), and 100 mM MOPS, pH~6.0) were added to each well, and the plate was incubated in the dark at room temperature in the chamber. After one hour, 100 μl of culture-ferric citrate mix were transferred to a microtiter plate containing 100 μl of FerroZine reagent (50% ammonium acetate, 0.1% FerroZine (SIGMA# P9762)) per well. FerroZine selectively binds ferrous iron, produced in our system by the abiotic reaction of reduced phenazine with ferric citrate. This plate was incubated in the chamber for 10 minutes, then removed and transferred to a plate reader for measurement of absorbance at 570 nm, the absorbance maximum for the Fe(II)-FerroZine complex.

We found that some plates exhibited a high degree of variability in growth and ferric citrate reduction when inoculated directly from frozen stocks. Cultures from these plates were grown overnight to stationary phase, then used to inoculate fresh deep-well plates that were also incubated for approximately 16 hours. These cultures had very high ferric citrate reduction activity, probably because they had grown to a higher density and produced more pyocyanin due to inoculation from a stationary-phase culture. For these

plates, the ferric citrate incubation time was adjusted to two minutes to allow FerroZine assay measurements.

4.3.3. Secondary Screen for Ferric Citrate Reduction, Growth, and Pyocyanin Production in Selected Mutants

Each of the mutants selected for the secondary screen were picked from glycerol stocks and streaked onto LB miniplates containing 60 μg/mL gentamicin sulfate and incubated overnight at 37 °C. Three individual colonies were picked from each plate and used to inoculate wells of a deep-well plate containing 500 μl LB. Each deep-well plate was also inoculated in triplicate with wild-type and Δ*phz* mutant colonies. Plates were incubated as described for the primary screen and used to generate glycerol stocks for storage. These stocks were later used to inoculate deep-well plates and incubated as described for the primary screen. Stationary-phase overnight cultures were used to inoculate fresh cultures. The next day, 10 μl from each culture were transferred to a microtiter plate containing 90 μl LB per well for a measurement of optical density at

500 nm (representing growth). Cultures were tested using the FerroZine assay after 5 minutes of incubation with ferric citrate. The deep-well plates were then covered with an impermeable seal and centrifuged for 10 minutes at 8000 × g. Two hundred microliters of supernatant were transferred to a microtiter plate and absorbance spectra from 200 to 800 nm were read for each culture. The absorbance spectra taken for our culture supernatants were affected by the presence of LB and ferric citrate, which contribute absorbance at 690 nm. Therefore, our pyocyanin absorbance values represent relative rather than

absolute amounts. The background contributed by the ferric citrate and LB at 690 nm can be observed in the absorbance value for the Δ*phz* mutant (figure 4.2).

4.3.4. Complementation with *gpsA* and *fbcC* Genes in trans

To clone *gpsA*, primers were designed using the *P. aeruginosa* PA14 genome sequence to anneal 645 base pairs upstream of *gpsA* and to the last 30 bases of PA14_43620, yielding a PCR product including the putative operon containing *gpsA* and a putative promoter region. The amplified DNA was digested using the restriction sites EcoRI, which was engineered within primer gpsA 1, and Acc65I, which was internal to the operon (in the putative ORF PA14_43630). The EcoRI/Acc65I fragment was ligated into plasmid pUCP18 digested with the same restriction enzymes and treated with calf intestinal phosphatase (Sigma). The resulting plasmid, pAPW3, contains the ~1 kb *gpsA* gene, a 645 base pair upstream "promoter" region, and 215 base pairs of ORF PA14_43630.

A DNA fragment containing the gene *fbcC* was amplified from *P. aeruginosa* PA14 genomic DNA using primers PA4429 1 and PA4429 2, which anneal 94 base pairs upstream of *fbcC* and 100 base pairs downstream from the end of *fbcC* and contain restriction sites for SmaI and XbaI, respectively. The PCR product was digested using these enzymes and ligated into plasmid pUCP18. The resulting plasmid, pAPW5, contains the ~0.8 kb *fbcC* gene.

For plasmids pAPW3 and pAPW5, cloning was carried out in *E. coli* UQ950 by standard procedures (Ausubel et al. 1992) and constructs were electroporated into *P. aeruginosa* PA14 as described previously (Bloemberg et al. 1997).

4.3.5. Preparation of Pyocyanin for Reduction Assays

Concentrated pyocyanin stocks were prepared as described previously (Price-Whelan et al. 2007). Briefly, supernatants from LB-grown cultures of the pyocyanin overproducing mutant DKN370 were extracted with chloroform. The chloroform was then extracted against 0.01 M HCl, which protonates the pyocyanin, turning it a shade of fuchsia. One molar NaOH was added drop wise to the aqueous fraction until it turned blue, and the pyocyanin was extracted back into chloroform. Water was removed from the hydrophobic phase by addition of sodium sulfate. The liquid was transferred to a new flask and dried using a rotary evaporator. The resulting blue solid was redissolved in MOPS buffer (MOPS defined medium without the glucose, $FeSO_4$, or $MgSO_4$ added) and filtered (0.2 μm).

4.3.6. Cell Suspension Assay for Pyocyanin Reduction

Cultures of wild-type PA14 and various mutants were grown to stationary phase. Cell density was measured by optical density (OD) at 500 nm, and cultures were diluted in filtered supernatant from wild-type cultures to normalize the cell density to an OD_{500} of 0.6. In cases where pyocyanin concentrations were affected by this dilution, concentrated pyocyanin prepared as in section 4.3.5 was added to normalize. Cell

suspensions were moved into an anaerobic chamber, transferred to anaerobic cuvettes, and stoppered to ensure an oxygen-free headspace. Absorbance spectra (400-800 nm) were followed over time for each cell suspension, and disappearance of a broad peak at 690 nm, representing pyocyanin reduction, was observed.

4.3.7. Construction of *gpsA* and *fbcC* Unmarked Deletions

Unmarked deletions of *gpsA* (PA14_43640) and *fbcC* (PA14_57540) were generated according to the method described in Dietrich et al. (2006). For example, for the *gpsA* deletion, ~1-kb regions flanking the 5' and 3' ends of the gene were amplified using primer pairs 1 (gpsA 1 5'b/ gpsA 1 3') and 2 (gpsA 2 5'/ gpsA 2 3'), respectively. These flanking DNA fragments were mixed as the template for overlap extension PCR, resulting in the *gpsA* deletion construct. This product was cloned into the SpeI site of the mobilizable plasmid pSMV10, which contains (1) an *oriR6K* origin of replication, allowing propagation in *E. coli* λ*pir* strains, (2) a gentamicin resistance gene (*aacC1*), and (3) the *sacB* gene, which allows for counterselection on 10% sucrose plates. The resulting plasmid, pAPW6, was transformed into *E. coli* WM3064 and mobilized into *P. aeruginosa* PA14 by biparental conjugation. Selection on gentamicin gave rise to merodiploids (containing the intact *gpsA* gene and the deletion construct) that were picked into LB liquid cultures and grown to early exponential phase, then plated on 10% sucrose plates. Colonies on sucrose plates were tested for the presence or absence of the wild-type allele by PCR.

4.3.8. Extraction and Quantification of NADH and NAD^+ from *P. aeruginosa* Cultures

Extraction and quantification of NADH and NAD^+ was carried out as described in Price-Whelan et al. (2007) , according to the method of San et al. (2002). Cultures of wild-type PA14 and various mutants were grown to stationary phase. Two × 1 mL of culture were sampled into two separate microcentrifuge tubes and centrifuged at 16,000 rcf for 1 minute. Supernatant was removed and pellets were resuspended in 300 μl of 0.2 M NaOH (for NADH extraction) or 0.2 M HCl (for NAD^+ extraction). These lysates were incubated for 10 minutes at 50 °C, then for 10 minutes on ice. While vortexing, 300 μl of 0.1 M HCl (for NADH) or 0.1 M NaOH (for NAD^+) were added dropwise to neutralize the solutions. They were then centrifuged for 3 minutes at 16,000 rcf. Supernatants were removed to fresh tubes and stored at −80 °C until quantification.

Relative NADH and NAD^+ were quantified using a modification (San et al. 2002) of the enzyme cycling assay developed by Bernofsky and Swan (1973). A master reagent mix was prepared with 1× Bicine buffer (2.0 M, pH 8.0), 8× water, 1× 80 mM EDTA, 2× 100% EtOH, 2× 4.2 mM thiazolyl blue (MTT), and 4× 16.6 mM phenazine ethosulfate. The reagent mix was warmed to 30 °C, then 90-μl aliquots were dispensed into individual wells of a 96-well microtiter plate. Five microliters of standard or sample were added to each well, then the cycling reaction was started by the addition of 5 μl of alcohol dehydrogenase (Sigma #A-3263) prepared at 347 units/mL in 0.1 M Bicine (pH 8.0). The microtiter plate was incubated at 30 °C, mixed by brief shaking, and read every 30-60 seconds for absorbance at 570 nm, which is the spectral peak of MTT that increases upon

reduction. Slopes arising from plots of absorbance at 570 nm over time were generated for NADH and NAD^+ standards as well as all samples.

4.4. Results

4.4.1. Identification of *P. aeruginosa* Mutants Defective in Pyocyanin Dependent Reduction of Ferric Citrate

Using ferric citrate reduction as a proxy for phenazine reduction (figure 4.1.A), we designed a genetic screen to identify *P. aeruginosa* enzymes involved in this process. We performed a pilot experiment to develop the screen protocol and assess the specificity of our ferric citrate reduction assay for *P. aeruginosa* phenazines. We grew cultures of wild-type *P. aeruginosa* PA14 and mutants with defects in various steps of the phenazine biosynthetic pathway. These included the Δ*phz* mutant (Dietrich et al. 2006), lacking genes required for biosynthesis of phenazine-1-carboxylate (PCA), the precursor for all pseudomonad phenazines; as well as transposon insertion mutants in the genes *phzM* and *phzS* from the *P. aeruginosa* PA14 nonredundant library (Liberati et al. 2006). The *phzM* and *phzS* mutants cannot produce the blue phenazine pyocyanin but can still make other phenazine derivatives through modification of PCA (Mavrodi et al. 2001). We found that pyocyanin was the only phenazine that contributed to ferric citrate reduction under our conditions (figure 4.1.B).

Using the conditions described above, we screened the entire *P. aeruginosa* PA14 transposon insertion mutant library (Liberati et al. 2006) for pyocyanin reduction. For experiments with cultures from the library, we measured ferrous iron at only one time

point, after an hour of incubation with ferric citrate. We predicted that our screen would reveal defects in pyocyanin-dependent ferric citrate reduction for mutants with transposon insertions in several classes of genes, including: (1) those involved in pyocyanin biosynthesis or the regulation thereof; (2) genes required for pyocyanin transport across the cell membrane; and (3) those encoding enzymes that directly catalyze pyocyanin reduction. We identified 215 mutants with defects in pyocyanin-dependent ferric citrate reduction. These mutants are listed according to functional category in Table 4.3, with mutants further characterized through a secondary screen segregated into separate sections. Sections 1 and 2 in the table contain mutants tested in the secondary screen and are discussed below (section 4.4.2 of this chapter).

We hit 16 loci representing genes involved in the regulation of pyocyanin biosynthesis, e.g., quorum sensing-related genes, and pyocyanin biosynthesis. These hits validated our method and are listed in Section 3 (table 4.3). Section 4 contains 12 mutants with transposon insertions disrupting genes putatively involved in transport. These gene products may be directly involved in phenazine transport, or in the maintenance of a solute gradient that favors phenazine transport. We identified 17 new genes putatively involved in the regulation of phenazine biosynthesis or other aspects of pyocyanin reduction—these are listed in Section 5. Thirty-five of the mutants identified in the primary screen represent uncharacterized genes with hypothetical protein products (section 6). We hit 11 genes that are involved in pilus or flagellum biosynthesis or chemotaxis and we suspect that these mutants appeared defective due to an inability to access substrate (section 7). Section 8 contains 34 mutants with disruptions in genes that play roles in cell division, DNA maintenance, RNA turnover, or protein turnover,

processes that may lead to growth defects in many cases. Finally, section 9 contains 23 uncategorized mutants with transposon insertions in gene products with no obvious role in pyocyanin reduction, either because the product already serves a known, dedicated function, or because its putative function appears unrelated to pyocyanin redox cycling. This section also includes 10 mutants that represent uncharacterized intergenic regions of the PA14 genome.

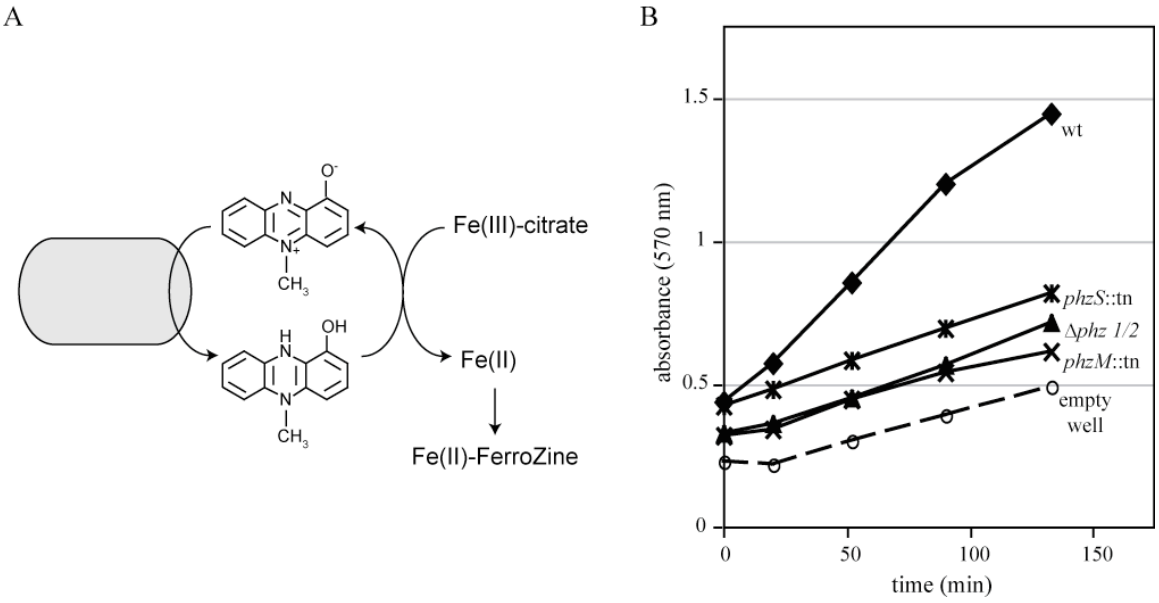

Figure 4.1. Ferric citrate reduction as a proxy for pyocyanin reduction in *P. aeruginosa*. **A)** Scheme illustrating pyocyanin-dependent ferric citrate reduction in *P. aeruginosa* and detection by the FerroZine reagent. **B)** *P. aeruginosa* phenazines other than pyocyanin, which are present in cultures of the *phzM* and *phzS* transposon insertion mutants, do not contribute to ferric citrate reduction under the conditions utilized for the screen. This experiment was performed with biological triplicates; representative data are shown.

Table 4.3. Tranposon insertion mutants defective in pyocyanin-dependent ferric citrate reduction

References relevant to annotation or phenotypes are listed for sections 1-3 (Chugani et al. 2001; Clark et al. 1980; Conolly and Winkler 1991; Daldal et al. 1989; Deziel et al. 2004; Evans and Dennis 1985; Gallagher et al. 2002; Ismail et al. 2003; Nielsen et al. 1981; Potter et al. 1987; Romero and Karp 2003; Vlamis-Gardikas 2008; Whiteley et al. 1999; Williams et al. 2007; Wood and Ohman 2006). FC, ferric citrate; GD, growth defect; PD, pyocyanin defect.

PA14 ORF	PAO1 homo-logue	gene name	annotation	mutant ID	phenotype/ functional role	Reference
Section 1: Mutants identified in secondary screen specifically defective in ferric citrate reduction (4)						
PA14_43640	PA1614	*gpsA*	glycerol-3-phosphate dehydrogenase, biosynthetic	26405	FC reduction defect	Clark 1980
PA14_57540	PA4429	*fbcC*	putative cytochrome c1 precursor	35524	FC reduction defect	Daldal 1989
PA14_57560	PA4430	*fbcB*	putative cytochrome b	30412	FC reduction defect	Daldal 1989
PA14_57570	PA4431	*fbcF*	putative cytochrome c reductase, iron-sulfur subunit	54834	FC reduction defect	Daldal 1989
Section 2: Mutants tested in secondary screen with other defects (63)						
PA14_22910	PA3194	*edd*	6-phosphogluconate dehydratase	55842	GD; central metabolism	Romero 2003
PA14_23090	PA3181	*eda*	2-keto-3-deoxy-6-phosphogluconate aldolase	41014	GD; central metabolism	Romero 2003
PA14_44070	PA1580	*gltA*	citrate synthase	34537	GD; central metabolism	Romero 2003
PA14_66290	PA5015	*aceA*	pyruvate dehydrogenase, E1 component	39618	GD; central metabolism	Romero 2003
PA14_62830	PA4748	*tpiA*	triosephosphate isomerase	55256	PD; central metabolism	Romero 2003
PA14_61400	PA4640	*mqoB*	malate:quinone oxidoreductase	39630	slight PD; central metabolism	Romero 2003
PA14_70040	PA5304	*dadA*	D-amino acid dehydrogenase, small subunit	38569	PD; energy metabolism	Williams 2007
PA14_54170	PA0782	*putA*	proline dehydrogenase PutA	46410	slight PD; energy metabolism	Williams 2007
PA14_13040	PA3929	*cioB*	cyanide insensitive terminal oxidase CioB	48456	oxygen reduction	Williams 2007

					defect; energy metabolism	
PA14_73310	PA5560	*atpB*	ATP synthase A chain	38520	slight PD; oxidative phosphory-lation	Nielsen 1981
PA14_05310	PA0407	*gshB*	glutathione synthetase	42799	GD; redox homeostasis	Vlamis-Gardikas 2008
PA14_68730	PA5203	*gshA*	glutamate--cysteine ligase	42600	slight PD; redox homeostasis	Vlamis-Gardikas 2008
PA14_53290	PA0849	*trxB2*	thioredoxin reductase 2	56384	GD; redox homeostasis	Vlamis-Gardikas 2008
PA14_72450	PA5489	*dsbA*	thiol:disulfide interchange protein DsbA	36207	GD; redox homeostasis	Vlamis-Gardikas 2008
PA14_51240	PA1013	*purC*	phosphoribosylaminoimid-azole-succinocarboxamide synthase	34993	GD; purine biosynthesis	Romero 2003
PA14_64220	PA4855	*purD*	phosphoribosylamine--glycine ligase	29794	GD; purine biosynthesis	Romero 2003
PA14_71620	PA5426	*purE*	phosphoribosylaminoimid-azole carboxylase, catalytic subunit	22699	GD; purine biosynthesis	Romero 2003
PA14_64200	PA4854	*purH*	phosphoribosylaminoimid-azolecarboxamide transferase	46986	GD; purine biosynthesis	Romero 2003
PA14_71600	PA5425	*purK*	phosphoribosylaminoimid-azole carboxylase	570	GD; purine biosynthesis	Romero 2003
PA14_15740	PA3763	*purL*	phosphoribosylformylgly-cinamidine synthase	29716	GD; purine biosynthesis	Romero 2003
PA14_70370	PA5331	*pyrE*	orotate phosphoribosyltransferase	28409	GD; pyrimidine biosynthesis	Romero 2003
PA14_70370	PA5331	*pyrE*	orotate phosphoribosyltransferase	46326	replicate	Romero 2003
PA14_62910	PA4756	*carB*	carbamoylphosphate synthetase large subunit	32277	GD; pyrimidine biosynthesis	Romero 2003
PA14_54290	PA0773	*pdxJ*	pyridoxal phosphate biosynthetic protein PdxJ	53798	GD; pyridoxal phosphate biosynthesis	Romero 2003
PA14_07740	PA0593	*pdxA*	pyridoxal phosphate	29841	GD; pyridoxal	Romero

			biosynthetic protein PdxA		phosphate biosynthesis	2003
PA14_07740	PA0593	*pdxA*	pyridoxal phosphate biosynthetic protein PdxA	40435	replicate	
PA14_52580	PA0904	*lysC*	aspartate kinase alpha and beta chain	40436	GD; amino acid biosynthesis	Romero 2003
PA14_23290	PA3165	*hisC2*	histidinol-phosphate aminotransferase	56832	PD; amino acid biosynthesis	Romero 2003
PA14_42230	PA1726	*bglX*	periplasmic beta-glucosidase	5084	GD; catabolism	Romero 2003
PA14_05620	PA0432	*sahH*	S-adenosyl-L-homocysteine hydrolase	54793	PD; amino acid metabolism	Romero 2003
PA14_05620	PA0432	*sahH*	S-adenosyl-L-homocysteine hydrolase	56708	replicate	
PA14_62710	PA4740	*pnp*	polyribonucleotide nucleotidyltransferase	53333	PD	Evans 1985
PA14_62710	PA4740	*pnp*	polyribonucleotide nucleotidyltransferase	31610	replicate	
PA14_65320	PA4945	*miaA*	delta 2-isopentenylpyrophosphate transferase	46697	PD	Connolly 1991
PA14_27960	PA2796	*tal*	transaldolase	31467	slight PD; central metabolism	
PA14_27960	PA2796	*tal*	transaldolase	33464	replicate	
PA14_66600	PA5038	*aroB*	3-dehydroquinate synthase	42535	PD	Romero 2003
PA14_66600	PA5038	*aroB*	3-dehydroquinate synthase	38358	replicate	
PA14_54390	PA0766	*mucD*	serine protease MucD precursor	35930	GD; alginate production	Wood 2006
PA14_54390	PA0766	*mucD*	serine protease MucD precursor	41549	replicate	
PA14_51440	PA0995	*ogt*	methylated-DNA-protein-cysteine methyltransferase	52740	PD	Potter 1987
PA14_68580	PA5192	*pckA*	phosphoenolpyruvate carboxykinase	41904	PD; central metabolism	Romero 2003
PA14_68580	PA5192	*pckA*	phosphoenolpyruvate carboxykinase	52736	replicate	
PA14_07700	PA0590	*apaH*	bis(5'-nucleosyl)-tetraphosphatase	36226	GD; dinucleoside	Ismail 2003

					polyphosphate metabolism	
PA14_04390	PA0336	*ygdP*	dinucleoside polyphosphate hydrolase	25921	slight PD; dinucleoside polyphosphate metabolism	Ismail 2003
PA14_50980	PA1032		probable penicillin amidase	6114	PD	
PA14_61220	PA4627		putative ribosomal RNA small subunit methyltransferase C	42280	PD	
PA14_61680	PA4664		putative methyltransferase	38864	PD	
PA14_65670	PA4968		putative esterase	25955	PD	
PA14_67970	PA5145		putative dehydrogenase	25699	PD	
PA14_29290	PA2693		putative long-chain acyl-CoA thioester hydrolase	55151	slight PD	
PA14_11250	PA4069		putative dTDP-4-rhamnose reductase-related protein	40221	GD	
PA14_07600	PA0583		putative 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	32431	GD	
PA14_49280	PA1171		probable transglycolase	31338	GD	
PA14_10260	PA4150		putative acetoin dehydrogenase E1 component	29150	GD	
PA14_20960	PA3332		putative isomerase	29854	GD	
PA14_05250	PA0401		noncatalytic dihydroorotase-like protein	41761	GD	
PA14_44420	PA1551		putative ferredoxin	38975	slight GD	
PA14_48610	PA1214		putative asparagine synthase	31864	slight GD	
PA14_68670	PA5198		putative carboxypeptidase	6442	GD	
PA14_12400	PA3976		thiamin-phosphate pyrophosphorylase	41228	PD	
PA14_23310	PA3164		prephenate dehydrogenase	54211	PD	
PA14_28250			putative secreted acid phosphatase	33463	PD	
Section 3: Mutants with defects in quorum sensing and phenazine biosynthesis (16)						
PA14_09400	PA4217	*phzS*	flavin-containing monooxygenase PhzS	44099	pyocyanin biosynthesis	Mavrodi 2001

PA14_09460	PA4212	*phzC1*	phenazine biosynthesis protein PhzC	34606	pyocyanin biosynthesis	Mavrodi 2001
PA14_09490	PA4209	*phzM*	probable phenazine-specific methyltransferase PhzM	40343	pyocyanin biosynthesis	Mavrodi 2001
PA14_39960	PA1900	*phzB2*	probable phenazine biosynthesis protein PhzB	48282	pyocyanin biosynthesis	Mavrodi 2001
PA14_19120	PA3477	*rhlR*	acylhomoserine lactone dependent transcriptional regulator RhlR	37943	QS signaling network	Whiteley 1999
PA14_19130	PA3476	*rhlI*	autoinducer synthesis protein RhlI	33961	QS signaling network	Whiteley 1999
PA14_51430	PA0996	*pqsA*	probable coenzyme A ligase	23621	QS signaling network	Deziel 2004
PA14_51410	PA0998	*pqsC*	homologous to beta-keto-acyl-acyl-carrier protein synthase	32423	QS signaling network	Deziel 2004
PA14_51380	PA1000	*pqsE*	quinolone signal response protein	45262	QS signaling network	Deziel 2004
PA14_30630	PA2587	*pqsH*	putative FAD-dependent monooxygenase PqsH	47950	QS signaling network	Deziel 2004
PA14_39980	PA1898	*qscR*	probable transcriptional regulator	42798	QS signaling network	Chugani 2001
PA14_51350	PA1002	*phnB*	anthranilate synthase component II	35669	QS signaling network	Deziel 2004
PA14_30650	PA2586	*gacA*	response regulator GacA	34781	QS signaling network	Gallagher 2002
PA14_62490	PA4723	*dksA*	suppressor protein DksA	41617	regulation; PD expected	Gallagher 2002
PA14_72560	PA5499	*np20*	transcriptional regulator np20	42601	regulation; PD expected	Gallagher 2002
PA14_62530	PA4725	*cbrA*	two-component sensor CbrA	57211	regulation; PD expected	Gallagher 2002
Section 4: Mutants representing genes potentially involved in phenazine transport (12)						
PA14_41570	PA1777	*oprF*	major porin and structural outer membrane porin OprF precursor	23102	transport	
PA14_12300	PA3983		Putative Mg^{2+} and Co^{2+} transporter CorC	25649	transport	
PA14_16890	PA3670		putative auxiliary component of ABC transporter	54565	transport	
PA14_22350	PA3234		putative	45582	transport	

			sodium/proline:solute symporter			
PA14_34770	PA2309		putative ABC transporter, periplasmic binding protein	36007	transport	
PA14_36230	PA2202		putative amino acid transport system permease	56596	transport	
PA14_55770	PA4292		probable phosphate transporter	26038	transport	
PA14_56470	PA4343		putative MFS transporter	57139	transport	
PA14_56890	PA4375		putative RND efflux transporter	43547	transport	
PA14_58420	PA4502		putative binding protein component of ABC-type dipeptide trasnport system	44163	transport	
PA14_67630	PA5121		putative small-conductance mechanosensitive channel	30568	transport	
PA14_59860			candidate type III effector Hop protein	23298	transport	
Section 5: Mutants with transposon insertions in regulatory and putative regulatory genes (17)						
PA14_72970	PA5531	*tonB*	periplasmic protein tonB	32482	regulation	
PA14_61850	PA4675		putative TonB-dependent receptor	38150	regulation	
PA14_70390	PA5332	*crc*	catabolite repression control protein	44185	regulation	
PA14_17900	PA3587	*metR*	transcriptional regulator MetR	46982	regulation	
PA14_59770		*rcsB*	two-component response regulator	42212	regulation	
PA14_67560	PA5117	*typA*	GTP-binding protein TypA/BipA	37710	regulation	
PA14_69810	PA5288	*glnK*	nitrogen regulatory protein PII	33817	regulation	
PA14_03580	PA0275		putative transcriptional regulator	54298	regulation	
PA14_46850	PA1347		putative transcriptional regulator	30276	regulation	
PA14_52260	PA0928		sensor/response regulator hybrid	42741	regulation	
PA14_27400	PA2838		putative ranscriptional regulator, LysR family	30582	regulation	
PA14_27950	PA2797		putative anti-anti-sigma	44818	regulation	

			factor			
PA14_41260	PA1799		putative two-component response regulator	55775	regulation	
PA14_63210	PA4781		putative two-component response regulator	55086	regulation	
PA14_70530	PA5342		putative AraC-family transcriptional regulator	29156	regulation	
PA14_70560	PA5344		putative transcriptional regulator, LysR family	54029	regulation	
PA14_72390	PA5484		putative two-component sensor	35639	regulation	
Section 6: Mutants with transposon insertions in hypothetical proteins (35)						
PA14_04430	PA0339		conserved hypothetical protein	4850	hypothetical protein	
PA14_07500	PA0575		conserved hypothetical protein	38814	hypothetical protein	
PA14_07660	PA0587		conserved hypothetical protein	36345	hypothetical protein	
PA14_12030	PA4005		conserved hypothetical protein	23465	hypothetical protein	
PA14_12350	PA3980		conserved hypothetical protein	56619	hypothetical protein	
PA14_13350	PA3908		hypothetical protein	42104	hypothetical protein	
PA14_21210	PA3310		conserved hypothetical protein	56991	hypothetical protein	
PA14_25050	PA3016		conserved hypothetical protein	33019	hypothetical protein	
PA14_25100	PA3012		conserved hypothetical protein	41384	hypothetical protein	
PA14_25620	PA2971		conserved hypothetical protein	34098	hypothetical protein	
PA14_32440	PA2490		conserved hypothetical protein	42833	hypothetical protein	
PA14_33290	PA2423		hypothetical protein	25793	hypothetical protein	
PA14_40630	PA1847		conserved hypothetical protein	30791	hypothetical protein	
PA14_43310	PA1639		conserved hypothetical protein	31482	hypothetical protein	
PA14_45710	PA1450		conserved hypothetical protein	37135	hypothetical protein	

PA14_46080	PA1420		conserved hypothetical protein	53031	hypothetical protein	
PA14_46720	PA1358		conserved hypothetical protein	43400	hypothetical protein	
PA14_46840	PA1348		conserved hypothetical protein	47948	hypothetical protein	
PA14_46900	PA1343		hypothetical protein	44264	hypothetical protein	
PA14_49320	PA1167		hypothetical protein	30058	hypothetical protein	
PA14_49930	PA1116		conserved hypothetical protein	27355	hypothetical protein	
PA14_50250	PA1095		hypothetical protein	15779	hypothetical protein	
PA14_53980	PA0793		conserved hypothetical protein	38302	hypothetical protein	
PA14_54340	PA0769		hypothetical protein	39775	hypothetical protein	
PA14_56130	PA4320		hypothetical protein	41647	hypothetical protein	
PA14_56180	PA4324		conserved hypothetical protein	27409	hypothetical protein	
PA14_57690	PA4441		conserved hypothetical protein	23324	hypothetical protein	
PA14_60870	PA4601		conserved hypothetical protein	26307	hypothetical protein	
PA14_64170	PA4851		conserved hypothetical protein	45918	hypothetical protein	
PA14_67540	PA5115		conserved hypothetical protein	45365	hypothetical protein	
PA14_72370	PA5482		conserved hypothetical protein	27725	hypothetical protein	
PA14_03370			conserved hypothetical protein	33623	hypothetical protein	
PA14_15600			conserved hypothetical protein	29758	hypothetical protein	
PA14_59870			conserved hypothetical protein	54405	hypothetical protein	
PA14_59930			conserved hypothetical protein	41833	hypothetical protein	
Section 7: Mutants representing genes involved in motility (11)						
PA14_05320	PA0408	*pilG*	type 4 pili response	26986	pilus/flagellum	

			regulator PilG		biosynthesis	
PA14_14850	PA3805	*pilF*	type 4 fimbrial biogenesis protein PilF	26873	pilus/flagellum biosynthesis	
PA14_45720	PA1449	*flhB*	flagellar biosynthetic protein FlhB	28316	pilus/flagellum biosynthesis	
PA14_50080	PA1105	*fliJ*	flagellar protein FliJ	55510	pilus/flagellum biosynthesis	
PA14_50140	PA1101	*fliF*	flagella M-ring outer membrane protein precursor FliF	37699	pilus/flagellum biosynthesis	
PA14_50470	PA1078	*flgC*	flagellar basal-body rod protein FlgC	28337	pilus/flagellum biosynthesis	
PA14_66620	PA5040	*pilQ*	type 4 fimbrial biogenesis outer membrane protein PilQ precursor	41699	pilus/flagellum biosynthesis	
PA14_45810	PA1442		putative flagellar protein FliL	25537	pilus/flagellum biosynthesis	
PA14_50110	PA1103		probable flagellar assembly protein	25963	pilus/flagellum biosynthesis	
PA14_45610	PA1457	*cheZ*	chemotaxis protein CheZ	42358	chemotaxis	
PA14_65450	PA4954	*motA*	chemotaxis protein MotA	27981	chemotaxis	
Section 8: Mutants with insertions in genes involved in cell division, DNA maintainence, and RNA or protein turnover (34)						
PA14_22040	PA3243	*minC*	cell division inhibitor MinC	39233	cell division	
PA14_62870	PA4752	*rrmJ*	cell division protein FtsJ	33950	cell division	
PA14_73370	PA5565	*gidA*	glucose-inhibited division protein A	34284	cell division	
PA14_00030	PA0003	*recF*	DNA replication and repair protein RecF	25777	DNA/RNA/ protein	
PA14_07530	PA0577	*dnaG*	putative DNA primase	104	DNA/RNA/ protein	
PA14_07620	PA0584	*cca*	tRNA nucleotidyl transferase	43633	DNA/RNA/ protein	
PA14_08780	PA4269	*rpoC*	DNA-directed RNA polymerase beta* chain	42316	DNA/RNA/ protein	
PA14_09100	PA4239	*rpsD*	30S ribosomal protein S4	47143	DNA/RNA/ protein	
PA14_25110	PA3011	*topA*	DNA topoisomerase I	25847	DNA/RNA/ protein	

PA14_25230	PA3002	*mfd*	transcription-repair coupling factor	27969	DNA/RNA/ protein	
PA14_30660	PA2585	*uvrC*	excinuclease ABC subunit C	27887	DNA/RNA/ protein	
PA14_42280	PA1722	*pscI*	type III export protein PscI	383	DNA/RNA/ protein	
PA14_51780	PA0967	*ruvB*	Holliday junction DNA helicase RuvB	48244	DNA/RNA/ protein	
PA14_51790	PA0966	*ruvA*	Holliday junction DNA helicase RuvA	30714	DNA/RNA/ protein	
PA14_55670	PA4284	*recB*	exodeoxyribonuclease V beta chain	31955	DNA/RNA/ protein	
PA14_62730	PA4742	*truB*	tRNA pseudouridine 55 synthase	57118	DNA/RNA/ protein	
PA14_62900	PA4755	*greA*	transcription elongation factor GreA	48477	DNA/RNA/ protein	
PA14_65170	PA4934	*rpsR*	30S ribosomal protein S18	31176	DNA/RNA/ protein	
PA14_65410	PA4951	*orn*	oligoribonuclease	36369	DNA/RNA/ protein	
PA14_66710	PA5049	*rpmE*	50S ribosomal protein L31	32561	DNA/RNA/ protein	
PA14_66980	PA5070	*tatC*	sec-independent protein translocase TatC	54153	DNA/RNA/ protein	
PA14_67720	PA5128	*secB*	secretion protein SecB	46670	DNA/RNA/ protein	
PA14_68610	PA5193	*hslO*	putative chaperonin, 33 kDa	29990	DNA/RNA/ protein	
PA14_69190	PA5239	*rho*	transcription termination factor Rho	34208	DNA/RNA/ protein	
PA14_69710	PA5280	*sss*	site-specific recombinase	35643	DNA/RNA/ protein	
PA14_71870	PA5443	*uvrD*	DNA helicase II	41306	DNA/RNA/ protein	
PA14_01100	PA0090		putative ClpA/B-type chaperone	56461	DNA/RNA/ protein	
PA14_04890	PA0372		putative zinc protease	26579	DNA/RNA/ protein	

PA14_05560	PA0428		putative ATP-dependent RNA helicase, DEAD box family	42207	DNA/RNA/ protein	
PA14_05960	PA0456		putative major cold shock protein	36116	DNA/RNA/ protein	
PA14_28840			putative helicase	54413	DNA/RNA/ protein	
PA14_36760	PA2150		putative KU domain protein	29005	DNA/RNA/ protein	
PA14_64180	PA4852		putative tRNA-dihydrouridine synthase	35993	DNA/RNA/ protein	
PA14_22270			possible recombinase	47583	DNA/RNA/ protein	
Section 9: Mutants with no category (23)						
PA14_21410	PA3296	*phoA*	alkaline phosphatase	36027		
PA14_33650	PA2399	*pvdD*	pyocyaninverdine synthetase D	5205		
PA14_71940	PA5450	*wzt*	ABC subunit of A-band LPS efflux transporter	45670		
PA14_62770	PA4745	*nusA*	N utilization substance protein A	55834		
PA14_73320	PA5561	*atpI*	ATP synthase protein I	45399		
PA14_48970	PA0720		helix destabilizing protein of bacteriophage Pf1	25542		
PA14_61840	PA4674		putative virulence-associated protein	26343		
PA14_31680	PA2543		putative outer membrane protein	42253		
PA14_67530	PA5114		putative membrane protein	42570		
PA14_71670	PA5430		putative membrane protein	42169		
PA14_19170	PA3472		putative lipoprotein	47128		
PA14_44440	PA1549		putative cation-transporting P-type ATPase	31290		
PA14_49010				43220		
			intergenic region: PA14_05940	32084		
			intergenic region: PA14_07490	55726		
			intergenic region:	38436		

			PA14_07770			
			intergenic region: PA14_09190	34969		
			intergenic region: PA14_14350	27318		
			intergenic region: PA14_18560	41885		
			intergenic region: PA14_39950	40456		
			intergenic region: PA14_51360	37586		
			intergenic region: PA14_51400	41463		
			intergenic region: PA14_62950	46072		

4.4.2. Selection of Mutants Representing Candidate Pyocyanin Reductases

During our primary screen for ferric citrate reduction, we noticed that many of the wells in our plates contained less pyocyanin than the average, suggesting that these mutants were defective in growth and/or pyocyanin production. Using information regarding the putative functions of their gene products (obtained from the *Pseudomonas* genome database (Winsor et al. 2008)), we selected 117 mutants representing products with the potential for direct involvement in the pyocyanin reduction reaction, focusing on enzymes involved in energy metabolism, enzymes predicted to act on small organic and cyclic substrates, and enzymes catalyzing redox reactions. We performed a secondary screen with these mutants to test for defects in growth and/or pyocyanin biosynthesis.

Fifty of the 117 mutants tested in our secondary screen did not have significant defects in ferric citrate reduction, i.e., they produced more than 70% of the wild-type level of FerroZine absorbance. Their detection in the primary screen was probably due to

uneven inoculation, and they have been omitted from the mutant list. The remaining mutants are listed in Sections 1 and 2 (table 4.3). Thirty-one of the mutants tested in our secondary screen showed general growth defects, while 25 showed defects specifically in pyocyanin biosynthesis. Eight of the mutants listed in Section 2 are replicates that came up twice in the primary screen. These mutants were not tested in the secondary screen because their corresponding replicates were shown to have growth or pyocyanin production defects.

One of the mutants, with a transposon insertion in the gene *cioB*, appeared to be specifically defective in pyocyanin reduction. However, further characterization using a cell suspension assay for pyocyanin reduction suggested that this mutant is defective in oxygen consumption. A caveat to our screen protocol was that oxygen left in the culture after transfer to the anaerobic chamber had to be consumed by the bacteria in order to ensure stability of the ferrous iron produced through the pyocyanin redox cycling. Cell suspensions of the *cioB* mutant maintain the same relative absorbance (representing oxidized pyocyanin) for the first 30 minutes of the assay, then reduce pyocyanin at the same rate as the wild type (data not shown).

We hit several genes involved in purine biosynthesis, all of which were found to have severe growth defects in our secondary screen. The mutant with a transposon insertion into the *purH* gene piqued our interest. This mutant appeared to overproduce pyocyanin despite its inability to reach a cell density level that was more than 60% that of the wild type. After normalization for growth, the *purH* transposon insertion mutant

produced over two times as much pyocyanin per cell as the wild type. However, this mutant was not pursued further due to its severe growth defect

The secondary screen characterization left 4 mutants that represent defects that may be specific to the pyocyanin reduction reaction. These mutants displayed moderate growth defects, but despite this, they appeared to produce pyocyanin at or near wild-type levels (figure 4.2). These mutants are shaded in Table 4.1, Section 1 and contain transposon insertions in (1) PA14_43640, encoding the biosynthetic glycerol-3-phosphate dehydrogenase; and (2) PA14_57540, PA14_57560, and PA14_57570, encoding the cytochrome bc_1 complex of the respiratory chain. Interestingly, orthologues of the glycerol-3-phosphate dehydrogenase in eukaryotes have been implicated in maintenance of redox homeostasis (Bakker et al. 2001; Shen et al. 2006). The identification of cytochrome bc_1 complex mutants in a pyocyanin reduction screen also piqued our interest, as the potential for pyocyanin to facilitate respiration is part of a long-standing hypothesis regarding the physiological role of these compounds (Friedheim 1931).

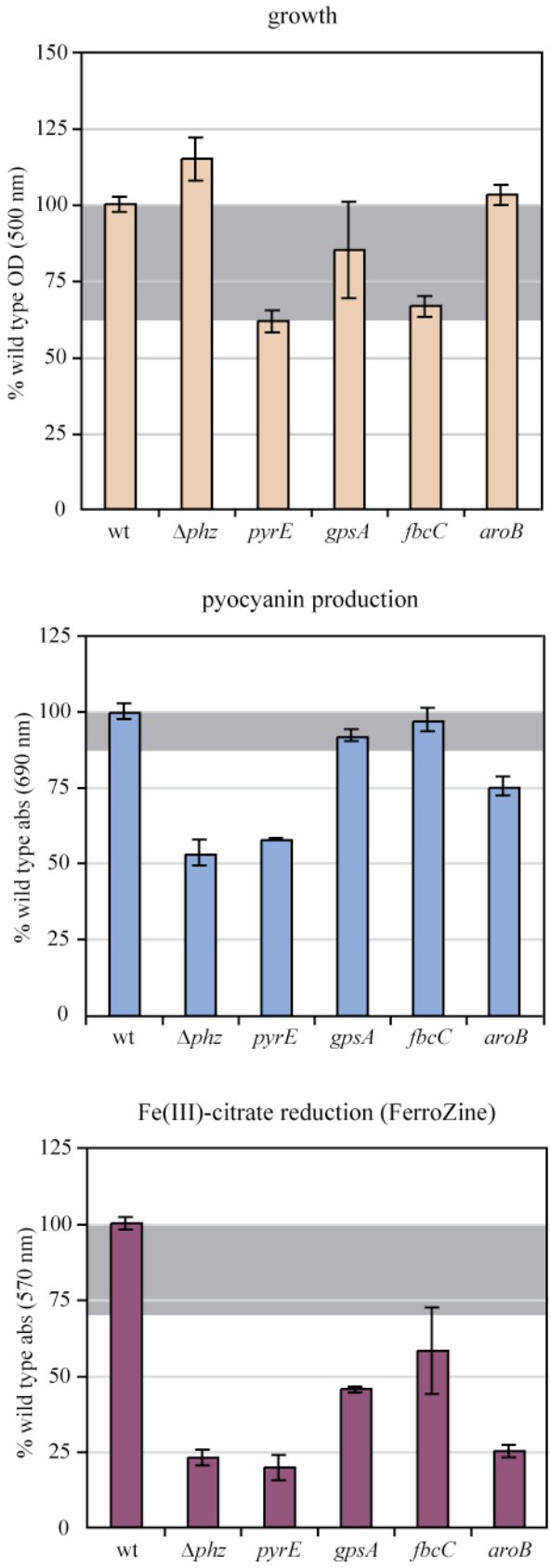
growth
% wild type OD (500 nm)
150
125
100
75
50
25
0
wt
Δphz
pyrE
gpsA
fbcC
aroB
pyocyanin production
% wild type abs (690 nm)
125
100
75
50
25
0
wt
Δphz
pyrE
gpsA
fbcC
aroB
Fe(III)-citrate reduction (FerroZine)
% wild type abs (570 nm)
125
100
75
50
25
0
wt
Δphz
pyrE
gpsA
fbcC
aroB

Figure 4.2. Representative data from the secondary screen for growth, pyocyanin biosynthesis, and ferric citrate reduction.
Absorbance at 690 nm measured for the Δ*phz* mutant represents background contributed by the ferric citrate solution. Dark gray shading indicates the data range over which samples were considered within a wild-type range for each measurement. Error bars represent the standard deviations of biological triplicates. OD, optical density. abs, absorbance.

4.4.3. The Biosynthetic Glycerol-3-Phosphate Dehydrogenase Contributes to Maintenance of Redox Homeostasis in *P. aeruginosa*

Two types of glycerol-3-phosphate dehydrogenase enzymes have been described for a diversity of organisms, ranging from *E. coli* to humans. One is a soluble protein with an NADH-binding domain, whose primary function is thought to be the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate, a precursor for phospholipid biosynthesis. This enzyme, referred to in bacteria as GpsA, is required for the growth of *E. coli* in defined media (Clark et al. 1980). We observed this phenotype for *P. aeruginosa* PA14 (figure 4.3.A), as well as a stationary-phase survival defect for this mutant in LB cultures (figure 4.3.B). The second glycerol-3-phophate dehydrogenase, called GlpD in bacteria, is a membrane-associated flavoprotein that couples the oxidation of glycerol-3-phosphate to the reduction of ubiquinone. This enzyme is required for growth of *E. coli* with glycerol as the sole carbon source (Austin and Larson 1991). The eukaryotic orthologues of these enzymes have been suggested to function together to balance the intracellular redox state (Bakker et al. 2001; Shen et al. 2006). The *P. aeruginosa* genome contains two *glpD* homologues that are represented in the PA14 mutant library (mutant IDs 42489 and 36707). We did not observe defects in pyocyanin reduction for these mutants, possibly due to redundant activities.

We confirmed that the disruption of *gpsA*, rather than downstream ORFs in the putative operon containing *gpsA*, was responsible for the pyocyanin reduction phenotype in this mutant by cloning the *gpsA* gene from *P. aeruginosa* PA14 into plasmid pUCP18 (creating plasmid pAPW3) and moving it into the *gpsA*::tn mutant. This complementation enhanced pyocyanin reduction activity, measured using an anaerobic cell suspension assay, to above-wild-type levels, probably due to the presence of the plasmid in multicopy (figure 4.4). Amending the growth medium with 7.5 mM glycerol also complemented the pyocyanin reduction defect in the *gpsA* transposon insertion mutant (data not shown).

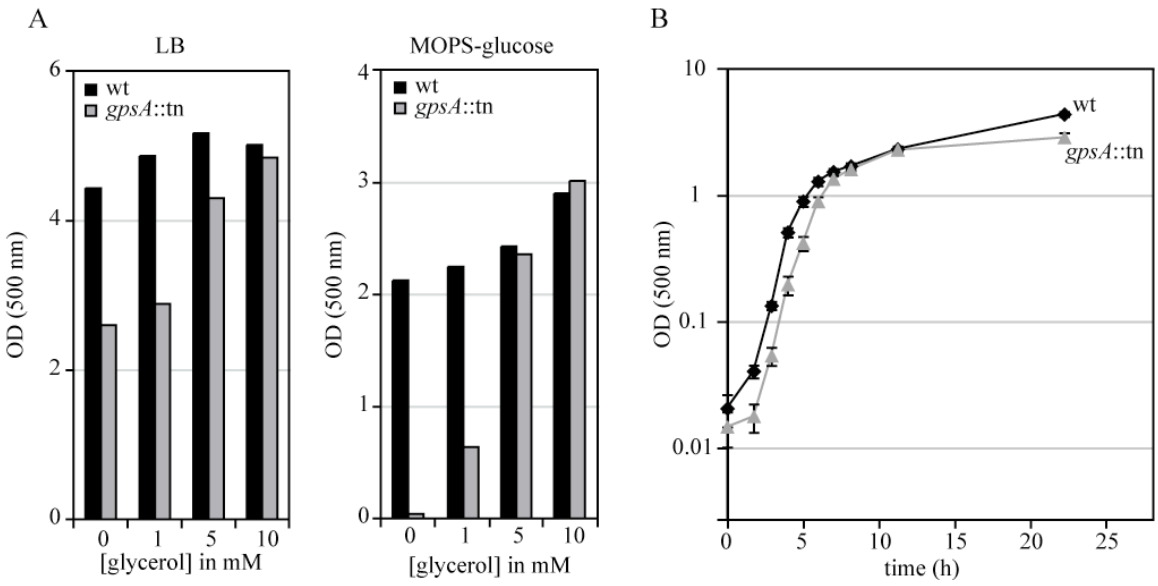

Figure 4.3. Growth of the *gpsA*::tn mutant on complex and defined media.
(A) Titration of LB-grown and MOPS-grown cultures with glycerol. Growth is not observed for *gpsA*::tn in defined medium without glycerol amendment. (B) Growth of *gpsA*::tn in LB. The increased lag phase is abolished by inoculation from exponential-phase cultures, but the cessation of growth in stationary phase is only abolished by the addition of approximately 75 mM glycerol. Error bars represent the standard deviations of biological triplicates and are obscured by the point marker in some cases.

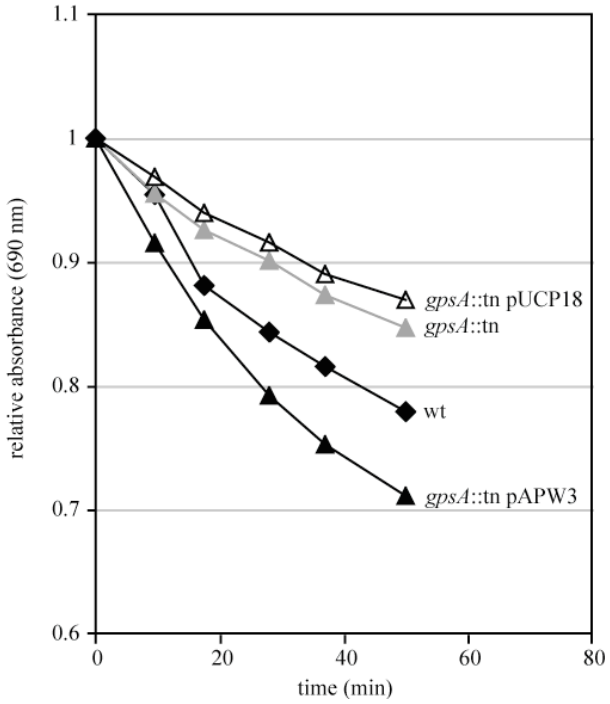

Figure 4.4. Provision of the *gpsA* gene in trans complements pyocyanin reduction activity in the *gpsA* transposon insertion mutant.
Data set shown is representative of biological duplicates; error bars were omitted for clarity.

To further characterize the effect of the *gpsA* mutation on *P. aeruginosa* physiology, we constructed in-frame deletions of this gene in the wild type and in a mutant unable to produce phenazines (*P. aeruginosa* PA14 Δ*phz* (Dietrich et al. 2006)). These mutants displayed the same defects in growth and pyocyanin reduction that we observed for the *gpsA* transposon insertion mutant (data not shown). We extracted and measured NADH and NAD^+ levels in these mutants. As described in Chapter 3, the *P. aeruginosa* PA14 Δ*phz* mutant exhibited a more reduced cytoplasm (higher NADH/NAD^+ ratio) in stationary phase than the wild type (Price-Whelan et al. 2007). Both the Δ*gpsA* mutant and the Δ*phz* Δ*gpsA* mutants showed a significant oxidation of the cytoplasm in stationary phase relative to their parent strains (figure 4.5). At first, this

result appears paradoxical: if NADH is an important electron donor for pyocyanin reduction, a mutant unable to reduce pyocyanin would be expected to accumulate NADH. In contrast, the phenotype of the Δ*phz* Δ*gpsA* mutant points to a redox-balancing role for the GpsA enzyme, which ensures sufficient NADH availability and favors the reduction of pyocyanin. The phenotype of the Δ*gpsA* mutant suggests that pyocyanin reduction reactions are draining reducing power from the cells despite NADH limitation.

We were interested to examine whether the addition of glycerol to the growth medium, which complements growth and pyocyanin reduction defects in Δ*gpsA* mutants, could also affect the intracellular redox state. We found that glycerol restores the NADH/NAD$^+$ ratio to the levels seen in the parent strains for the Δ*gpsA* and Δ*phz* Δ*gpsA* mutants (figure 4.6).

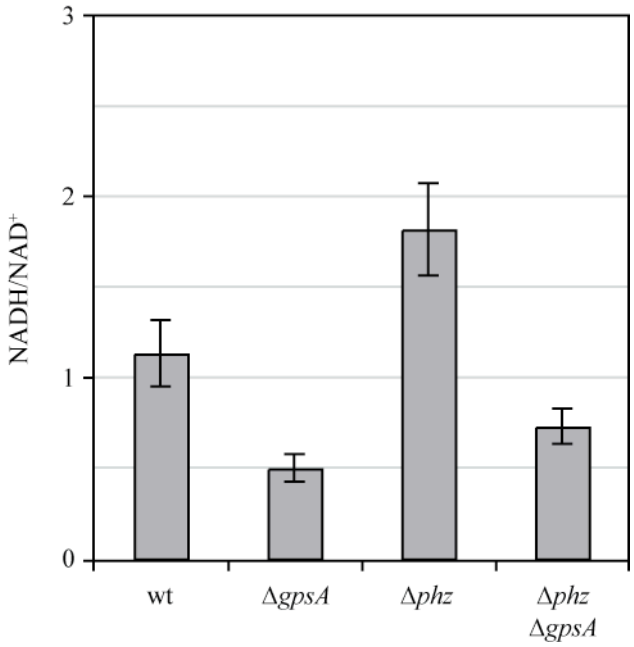

Figure 4.5. The NADH/NAD$^+$ ratio is shifted toward a more oxidized state in mutants lacking the *gpsA* gene.
Error bars represent the standard deviations of biological triplicates.

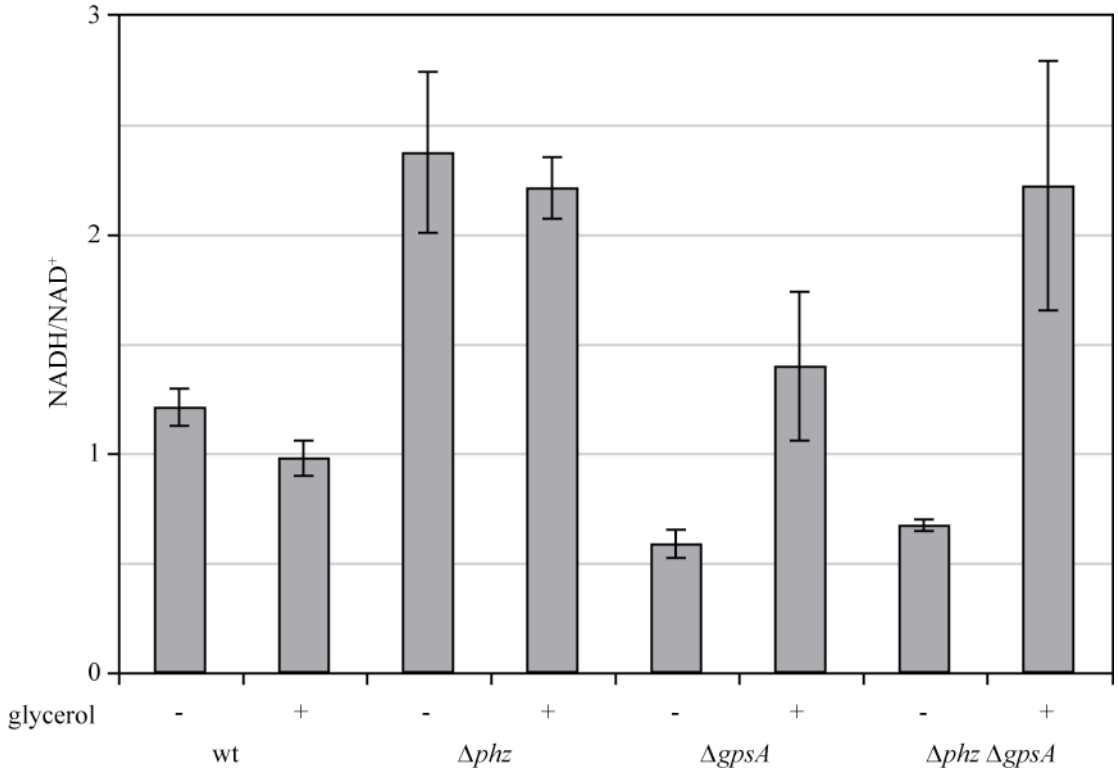

Figure 4.6. Glycerol complements the NADH/NAD$^+$ phenotypes of the Δ*gpsA* and Δ*gpsA* Δ*phz* mutants.
Error bars represent the standard deviations of biological duplicates.

4.4.4. The Cytochrome bc_1 Complex of the Respiratory Chain is Required for Full Pyocyanin Reduction Activity

Although the *P. aeruginosa* PA14 transposon insertion library generally contains one mutant to represent each nonessential gene in the genome, some genes are represented more than once by multiple insertions. The structural genes for the cytochrome bc_1 complex are represented 4 times, with one insertion each into genes PA14_57540 and PA14_57570, and two insertions into PA14_57560. Sequencing (according to the method described in Liberati et al. (2006)) revealed that one of the mutants annotated as having an insertion in PA14_57560 actually contained a transposon

insertion elsewhere in the genome. This mutant displayed wild-type phenotypes with respect to ferric citrate and pyocyanin reduction. All three of the other mutants showed similar defects with respect to pyocyanin reduction and growth (data not shown). Complementation of a representative mutant defective in formation of the cytochrome c_1 subunit of the cytochrome bc_1 complex (PA14_57540::MAR2xT7; "*fbcC*::tn" (Daldal et al. 1989; Williams et al. 2007)) with pAPW5, a plasmid containing the *fbcC* gene, confirmed that the pyocyanin reduction defect was due to the lack of a functional cytochrome bc_1 complex (data not shown). Furthermore, the kinetics of pyocyanin reduction in this mutant, unlike those observed for the *cioB* transposon insertion mutant, did not suggest a defect in oxygen consumption.

The *fbcC* transposon insertion mutant displayed a growth defect in stationary phase (figure 4.7) that may partially account for its dramatic pyocyanin reduction defect. We constructed clean deletions of the *fbcC* gene in the wild-type and Δ*phz* backgrounds and conducted cell suspension assays for pyocyanin reduction to confirm that these mutants have pyocyanin reduction defects independent of their growth defects. Cell suspension assays normalized by cell number confirmed that the Δ*fbcC* mutant had a defect in pyocyanin reduction. We were intrigued to observe that the Δ*phz* Δ*fbcC* mutant actually displayed a less severe defect in pyocyanin reduction than the *fbcC* deletion in the wild-type background. This difference was apparent in early stationary phase; by late stationary phase the Δ*phz* Δ*fbcC* mutant no longer had any defect in pyocyanin reduction relative to its parent (figure 4.8).

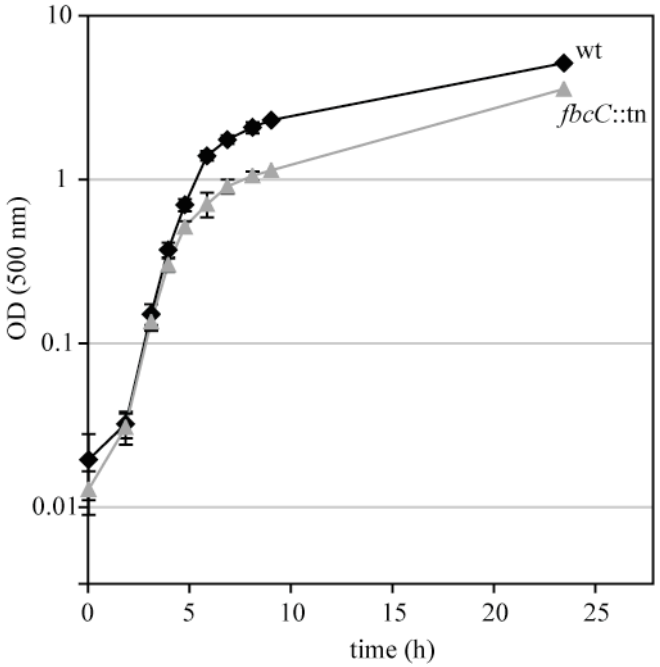

Figure 4.7. Growth of the *fbcC* transposon insertion mutant.
Error bars represent the standard deviations of biological triplicates and are obscured by the point marker in some cases.

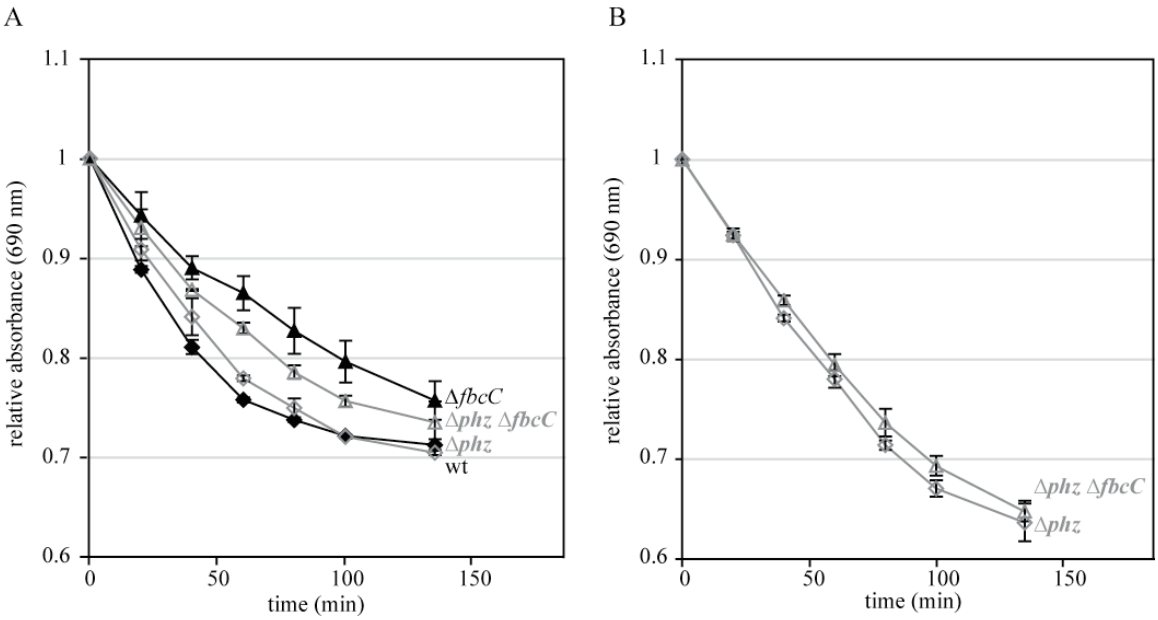

Figure 4.8. Pyocyanin reduction defects of the Δ*fbcC* and Δ*phz* Δ*fbcC* mutants.
(A) In early stationary phase, the Δ*phz* Δ*fbcC* mutant showed a less-pronounced defect in pyocyanin reduction than the Δ*fbcC* mutant. (B) In late stationary phase, the Δ*phz* Δ*fbcC* pyocyanin reduction activity was indistinguishable from that of the Δ*phz* mutant. Error bars

represent the standard deviations of biological triplicates and are obscured by the point marker in some cases.

We wondered how the *fbcC* deletion mutants would compare to their parents with respect to the cytoplasmic redox state. In contrast to the Δ*phz* Δ*gpsA* mutant, the Δ*phz* Δ*fbcC* mutant displayed a redox state that was similar to that of the Δ*phz* mutant in early stationary phase. In late stationary phase, the Δ*phz* Δ*fbcC* mutant appeared to accumulate NADH, resulting in an even more reduced cytoplasm than the parent strain (figure 4.9). This increase in NADH may represent an increase in reducing power available for residual, *fbcC*-independent pyocyanin reduction activity.

Although the Δ*fbcC* mutation leads to an accumulation of reducing power in the phenazine-null background, this mutation in the wild-type background appears to oxidize the intracellular redox state. We have observed increased production of pyocyanin in this mutant (figure 4.10); we therefore attribute the shift in the Δ*fbcC* NADH/NAD^+ pool to increased substrate availability for cytochrome bc_1-independent reactions that consume reducing power and reduce pyocyanin.

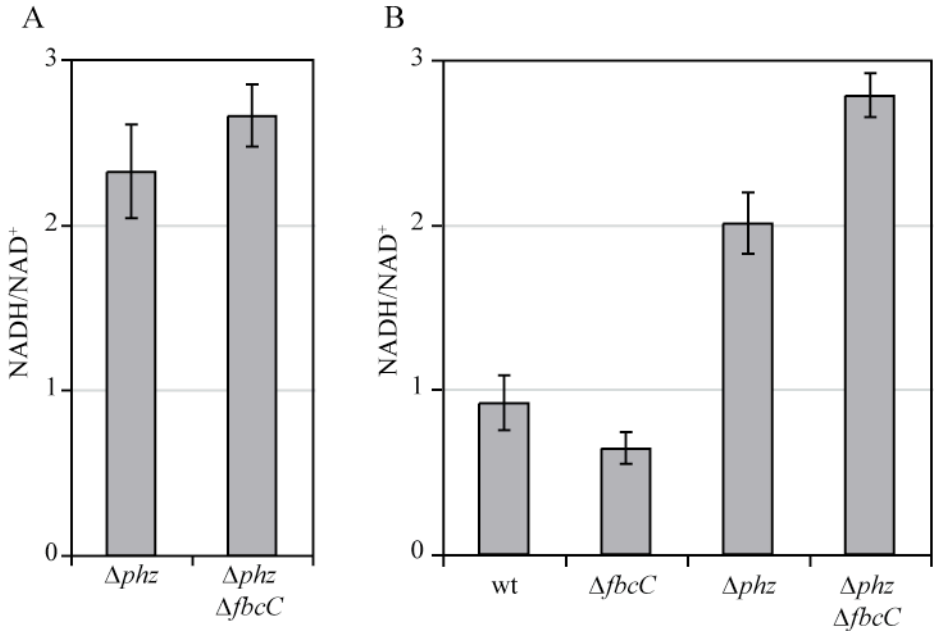

Figure 4.9. The Δ*phz* Δ*fbcC* mutant accumulates NADH in stationary phase.
(A) In early stationary phase, the intracellular NADH/NAD$^+$ ratio of the Δ*phz* Δ*fbcC* mutant resembles that of the parent. (B) In late stationary phase, the Δ*phz* Δ*fbcC* mutant is significantly shifted toward the reduced state. Error bars represent the standard deviations of biological triplicates.

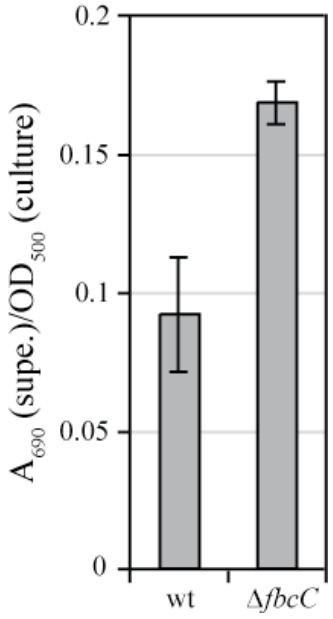

Figure 4.10. The Δ*fbcC* mutation leads to pyocyanin overproduction.
Error bars represent the standard deviations of biological triplicates.

The contrasting effects of Δ*gpsA* and Δ*fbcC* deletions on the NADH/NAD^+ ratio in the absence of pyocyanin suggest that the GpsA enzyme and the cytochrome bc_1 complex contribute to pyocyanin reduction activity via different mechanisms. To test whether their contributions are additive, we generated a double mutant, Δ*gpsA* Δ*fbcC*, and measured pyocyanin reduction activity using the anaerobic cell suspension assay. While the wild type took approximately 100 minutes to reduce all of the pyocyanin in the cell suspension, and the Δ*gpsA* and Δ*fbcC* individual mutants took about 200 minutes, the double mutant took approximately 260 minutes (figure 4.11). A synergistic effect occurs in the Δ*gpsA* Δ*fbcC* double mutant, but combining the two mutations does not have a fully additive effect as measured using the cell suspension assay.

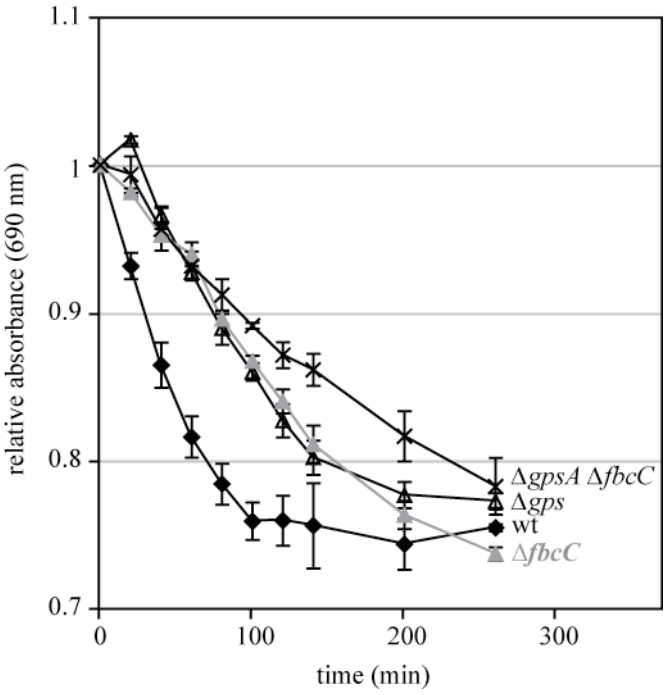

Figure 4.11. The pyocyanin reduction defect of the Δ*gpsA* Δ*fbcC* mutant is more pronounced than those of the individual *gpsA* or *fbcC* deletion mutants.
Error bars represent the standard deviations of biological triplicates.

4.5. Discussion

We set out to elucidate some of the mechanisms allowing *P. aeruginosa* to catalyze pyocyanin reduction. In screening a library of mutants for defects in pyocyanin-dependent ferric citrate reduction, we uncovered a previously unrecognized role for the bacterial biosynthetic glycerol-3-phosphate dehydrogenase in regulation of the intracellular redox state. Glycerol-3-phosphate dehydrogenases have been implicated in maintenance of redox homeostasis in eukaryotic organisms ranging from *Saccharomyces cerevisiae* to *Arabidopsis thaliana*, to humans (Bakker et al. 2001; Ben-Yoseph et al. 1993; Shen et al. 2006). In *Arabidopsis*, for example, the soluble glycerol-3-phosphate dehydrogenase (GPDHc1) is present in the cytosol, while the FAD-cofactored glycerol-3-phosphate dehydrogenase (FAD-GPDH) is associated with the mitochondrial membrane. These two enzymes form "the glycerol-3-phosphate shuttle," which allows transfer of reducing equivalents from cytosolic NADH to the quinone pool of the respiratory chain. When the soluble enzyme is not functional, the cytosolic redox state becomes more reduced, due to the accumulation of NADH.

In contrast to the oxidative role of the soluble glycerol-3-phosphate dehydrogenase in eukaryotes, we found that the *P. aeruginosa* homologue is required for a more reduced bacterial cytoplasm. Removal of the *P. aeruginosa* enzyme, GpsA, in both wild-type and phenazine-null backgrounds shifted the $NADH/NAD^+$ ratio to a more oxidized state. Adding glycerol to the growth medium for these mutants restored the redox state to that of the parent strain. The enzyme GpsA catalyzes the oxidation of NADH coupled to the reduction of DHAP, producing glycerol-3-phosphate, a precursor for phospholipid biosynthesis. While the soluble glycerol-3-phosphate dehydrogenase

purified from yeast (Albertyn et al. 1992) appears to catalyze the reverse reaction, it is much slower *in vitro*, at 3% of the rate of the DHAP reduction reaction. If GpsA catalyzes NADH oxidation, why does the cytoplasm become more oxidized when it is removed? We have formulated a simple model to describe the roles of GpsA and other important enzymes related to glycerol metabolism in phospholipid biosynthesis and the production of reducing equivalents (figure 4.12).

The critical branch point linking central metabolism to phospholipid biosynthesis is glyceraldehyde-3-phosphate, which is an intermediate in the Entner-Doudoroff pathway, the primary means whereby *P. aeruginosa* generates pyruvate as a substrate for the citric acid cycle. Glyceraldehyde-3-phosphate is converted to DHAP by triose phosphate isomerase, then to glycerol-3-phosphate by GpsA. When GpsA is absent, glycerol or glycerol-3-phosphate must be added to defined media to allow phospholipid biosynthesis to occur. GpsA, therefore, is the only enzyme that allows other carbon sources to contribute to phospholipid biosynthesis via central metabolism. Otherwise, only glycerol, which is converted to glycerol-3-phosphate by the kinase GlpK, and glycerol-3-phosphate can serve as precursors for these important anabolic products (figure 4.12).

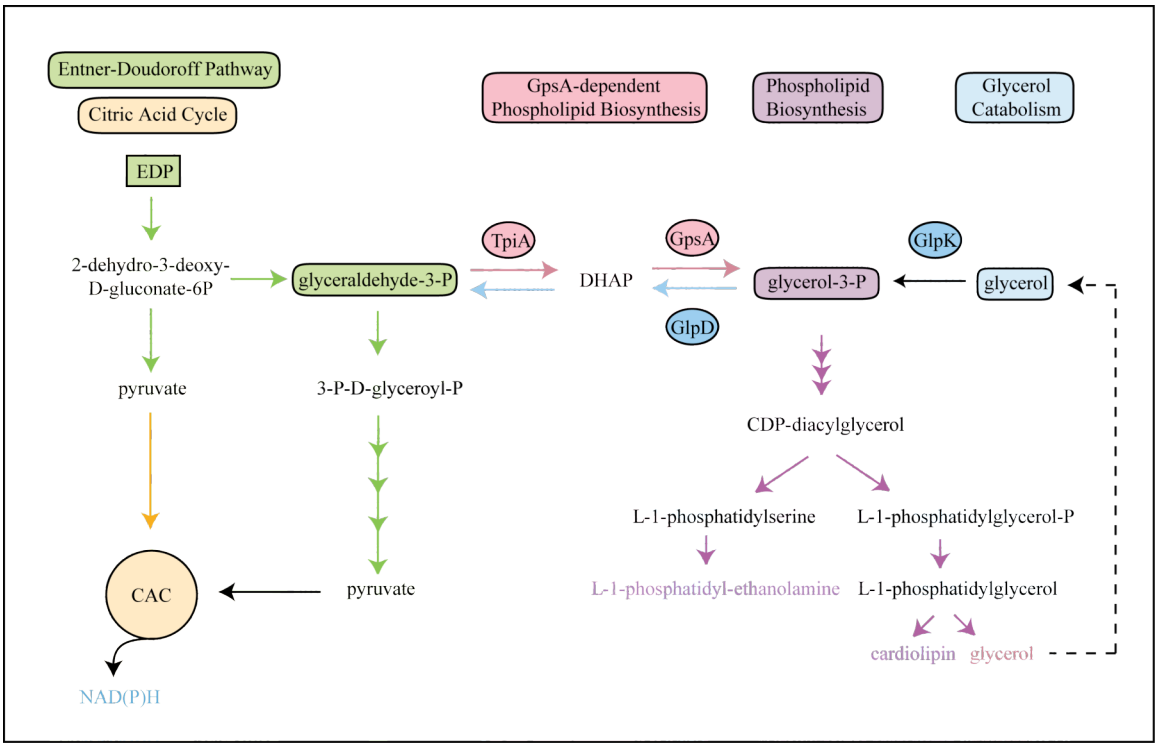

Figure 4.12. Model for flux of carbon and reducing power through central metabolism and phospholipid biosynthesis in *P. aeruginosa*.

When excess glycerol or glycerol-3-phosphate is present, the enzyme GlpD allows utilization of this metabolite as a carbon and energy source. The carbon atoms in glycerol have an average oxidation state of −0.66. It is considered a "highly reduced" substrate compared to other popular carbon sources for *P. aeruginosa* such as glucose and succinate, which have average oxidation states of 0 and +0.50, respectively. We have observed that the NADH/NAD^+ ratio is particularly high in *P. aeruginosa* cells growing on glycerol as the sole carbon and energy source (data not shown). Therefore, we hypothesize that the production of glycerol at the tail end of the phospholipid biosynthetic pathway is key to the relatively reduced intracellular redox state present in cells with a functional GpsA. These bacteria can produce glycerol and phospholipids from any carbon source fed into the Entner-Doudoroff pathway before the

glyceraldehyde-3-phosphate step. The pool of reduced carbon present as glycerol in the growth medium can contribute to NAD(P)H production via the citric acid cycle.

In bacteria lacking a functional GpsA enzyme, a larger fraction of the glycerol present in the medium or produced via phospholipid biosynthesis must remain in the glycerol-3-phosphate/phospholipid loop; as a result, less reducing power enters central metabolism (figure 4.12). This limits the amount of NAD(P)H available for pyocyanin reduction and leads to the defect in pyocyanin reduction we observe for the *gpsA* mutant. This model could be interrogated through measurements of the intracellular NADH/NAD^+ ratios in mutants lacking the other enzymes in the glyceraldehyde-3-phosphate/phospholipid pathway. We did not observe a phenotype for the *glpD* mutant tested in our screen; however, the *P. aeruginosa* genome contains another *glpD* homologue, PA14_24950, which may confer redundant activity. A double mutant lacking both of these genes would be expected to have a an oxidized intracellular NADH/NAD^+ ratio due to an inability to access electrons from the glycerol pool via central metabolism.

The identification of the cytochrome bc_1 complex as a contributor to pyocyanin reduction activity is consistent with previous reports describing the toxicity of 1-hydroxyphenazine, which differs from pyocyanin only in that it lacks the methyl group at the N5 position. This compound has been shown to act as an electron shunt from the respiratory chain at the site of the cytochrome bc_1 complex of mouse liver mitochondria (Armstrong and Stewart-Tull 1971). Recent work by Ran et al. revealed that mutations in the cytochrome bc_1 complex of the yeast mitochondrion confer resistance to pyocyanin (2003), raising the possibility that pyocyanin interacts directly with this protein.

The increasing levels of NADH present in stationary-phase cultures of the Δ*phz* Δ*fbcC* mutant suggest that the cytochrome bc_1 complex is a critical component of the respiratory chain present in the *P. aeruginosa* membrane. Based on studies pointing to the direct interaction between the ubiquinone pool and/or this complex and *P. aeruginosa* phenazines in other organisms, we hypothesize that this complex is playing a similar role in pyocyanin reduction in *P. aeruginosa*. However, it is also possible, given that this enzyme is a coupling site, that the cytochrome bc_1 complex contributes to the generation of a proton gradient, and that the proton motive force is required for pyocyanin uptake across the cytoplasmic membrane. We have observed that the *fbcC* mutant appears to excrete more pyocyanin than the wild type; whether this phenotype can be attributed to impaired uptake of pyocyanin is not known.

In conclusion, we have determined two mechanisms that contribute to pyocyanin reduction in *P. aeruginosa*. We showed that the respiratory chain, which has been proposed as an interaction site for pseudomonad phenazines in other organisms, is also involved in pyocyanin reduction in the producing organism. Further biochemical characterization will reveal whether the cytochrome bc_1 complex in *P. aeruginosa* directly reduces pyocyanin and/or whether it affects the uptake of this compound. In addition, we discovered that modulation of the NADH/NAD^+ ratio alters the availability of substrate for pyocyanin reduction, and therefore represents a second mechanism that contributes to pyocyanin reduction activity. We uncovered a potential role for the biosynthetic glycerol-3-phosphate dehydrogenase in a fundamental metabolic process: the ability for cells to maintain their intracellular redox state for optimal protein function (Bessette et al. 1999; Mossner et al. 1999). Other mutants identified through this screen

may represent proteins with previously unrecognized roles in the maintenance of redox homeostasis in bacteria. Future studies these mutants will further our understanding of the relationship between redox state and survival in *P. aeruginosa* and contribute to the overall picture of energy metabolism in this proficient pathogen.

4.6. Acknowledgements

We thank Lars Dietrich and Yun Wang for helpful discussions, and Julie Huang for excellent technical assistance.

4.7. References

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Chapter 5

The Morphological Development of *Pseudomonas aeruginosa* Biofilms Is Drastically Altered by Electron Acceptor Availability and the Ability to Reduce Pyocyanin

5.1. Abstract

Biofilm formation is recognized as an important mode of growth for *Pseudomonas aeruginosa* in a diversity of environments and is modulated in response to environmental conditions. One factor affecting *P. aeruginosa* biofilm formation is the production of pyocyanin, a redox-active small molecule that acts as an electron sink in oxygen-limited planktonic cultures. We investigated the effects of electron acceptor availability and pyocyanin redox cycling on *P. aeruginosa* biofilm development using media amended with nitrate—another substrate that can balance the intracellular redox state—and mutants defective in pyocyanin reduction. Our results indicate that wrinkled colony structures are an adaptation to electron acceptor limitation.

5.2. Introduction

Over the last few decades it has become apparent that homogeneous batch cultures are not appropriate model systems for studying the mechanisms that allow many infectious bacteria to colonize and persist in their hosts (Hall-Stoodley et al. 2004; Singh et al. 2000). One reason for this is that bacteria growing and surviving in aggregates experience steep gradients with respect to many environmental parameters, including

nutrient and electron acceptor availability, which lead to differential responses that would not be represented in a well-mixed culture. Under such conditions, bacteria may exploit strategies for energy production and conservation that are not utilized during growth in liquid culture conditions (Eschbach et al. 2004; Price-Whelan et al. 2006).

Biofilm formation and intercellular signaling are multicellular behaviors that are critical to the pathogenicity of *Pseudomonas aeruginosa* (Donlan and Costerton 2002; Kirisits and Parsek 2006). We and others have found that the production of phenazines, a class of signals produced by some pseudomonad species, has profound effects on biofilm development. In flow cell biofilms of *Pseudomonas aureofaciens* and *P. aeruginosa*, phenazine production promotes the accumulation of surface-attached biomass (Maddula et al. 2006; Wang and Newman 2009). In *P. aeruginosa* colony biofilms growing on agar surfaces, a lack of phenazine production leads to increased wrinkling and spreading relative to the wild type. Overproduction of the blue phenazine pyocyanin promotes formation of a smooth, compact colony, suggesting that rugosity is inversely proportional to pyocyanin exposure (Dietrich et al. 2008).

Phenazines are redox-active small molecules that are reduced intracellularly and excreted. Under atmospheric conditions, phenazines react with molecular oxygen and can reenter the bacterium in their oxidized forms. As discussed in Chapter 3, this redox cycling contributes to the maintenance of redox homeostasis in oxygen-limited cultures that would otherwise accumulate intracellular reductant. We have proposed that redox balancing via phenazine reduction allows fundamental cellular processes, such as the TCA cycle, to proceed under conditions in which they would likely be inhibited by the

accumulation of NADH (Price-Whelan et al. 2007). The morphologies of *P. aeruginosa* colony biofilms formed by wild-type and phenazine-null strains is consistent with the model that phenazine production enhances fitness through increased electron acceptor availability. While the wild type can utilize phenazines to dispose of reducing equivalents, the phenazine-null mutant relies on oxygen, and may benefit from maximizing colony surface area for optimal oxygen exposure (figure 5.1).

To further interrogate our model that electron acceptor availability determines colony architecture, we tested the effects of (1) treatment with nitrate, another respiratory substrate for *P. aeruginosa* (Williams et al. 1978), and (2) deletion of genes required for full pyocyanin reduction activity. Here we present morphological characterization of *P. aeruginosa* colonies grown under these conditions. It is likely that phenazines affect *P. aeruginosa* colony development in multiple ways, including modifications of gene expression, effects on flux through central metabolism, and modifications of the intracellular redox state.

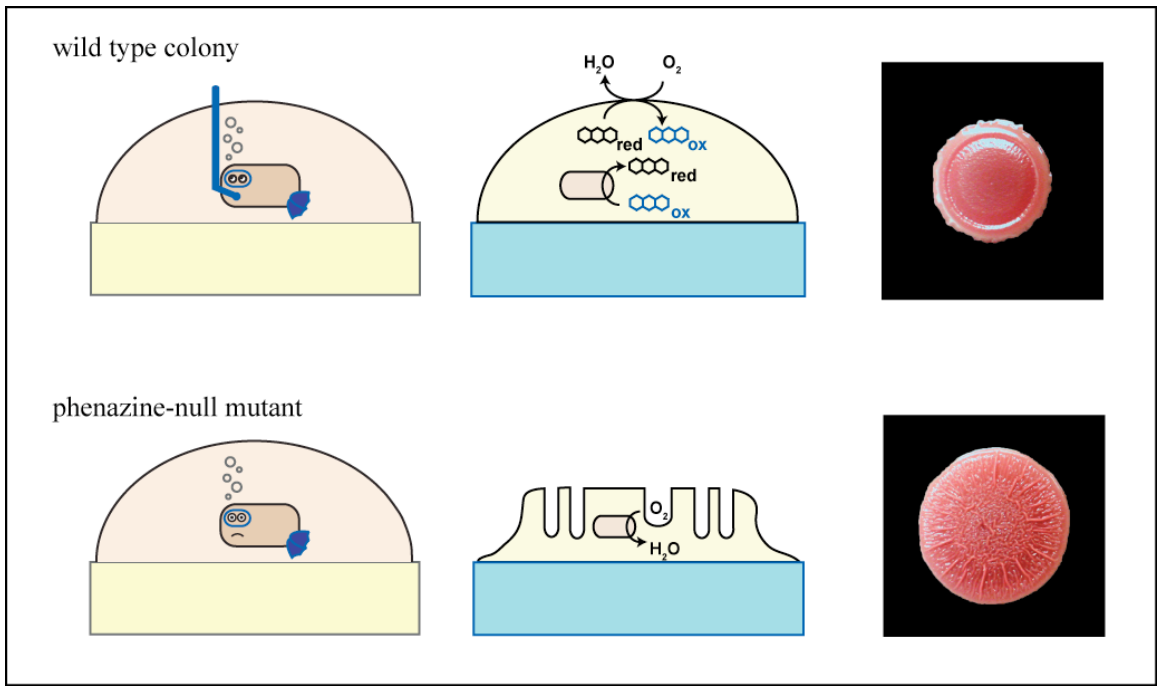

Figure 5.1. Cartoon depicting the role of phenazine cycling in colony biofilm development. Bacteria surviving at depth in a colony biofilm become limited for oxygen due to slow diffusion rates and the respiration of bacteria at the surface. Like the snorkel used by a diver, phenazines may allow these bacteria to utilize oxygen by transferring electrons from the bacteria to the oxygen at the surface. Bacteria in a colony without phenazines would have to form canyons in the biofilm structure to maximize oxygen accessibility.

5.3. Results and Discussion

5.3.1. Nitrate Promotes Smooth Colony Formation in the Phenazine-Null Mutant

We followed *P. aeruginosa* colony development using a standard assay (Friedman and Kolter 2004; Rakhimova et al. 2008). Ten microliters of overnight cultures were spotted onto agar plates containing 1% tryptone and 1% agar (TA medium). Congo Red (40 µg/mL) and Coomassie Blue (20 µg/mL) (referred to as CRCB medium) were added to aid visibility. Effects of these additions on colony morphology are noted; generally and for unknown reasons, the addition of Congo Red and Coomassie Blue exaggerates rugose phenotypes. Agar medium was poured into either 60 by 15 mm (containing 10-15 mL) or 100 by 15 mm (containing 35-40 mL) plates. A thick agar layer

is required for the elaborate wrinkling of rugose phenotypes. Plates were incubated at room temperature for 4-5 days. Potassium nitrate was added at a final concentration of 100 mM. Colony diameters were measured using Adobe Photoshop.

In light of our observations demonstrating a role for pyocyanin in modulating colony biofilm structure (Dietrich et al. 2008), we were interested to test the effects of another electron acceptor for *P. aeruginosa* energy metabolism. *P. aeruginosa* can grow anaerobically with nitrate as a terminal electron acceptor (Williams et al. 1978). We have shown that it can also utilize nitrate to balance the intracellular redox state in stationary phase, oxygen-limited "aerobic" cultures ((Price-Whelan et al. 2007) and appendix A). Figure 5.2 shows the effects of nitrate amendment on wild-type *P. aeruginosa* PA14 and the Δ*phz* mutant, which is lacking 14 genes required for the production of phenazines (Dietrich et al. 2006b), on CRCB medium after 5 days of growth. On this medium, the phenazine-null mutant formed a highly rugose structure with radial ridges up to 1 mm high. The wild type showed some dimpling but did not form these ridges. The same medium with 100 mM nitrate promoted the formation of smooth, compact colonies, with diameters approximately 17%-18% shorter than those of colonies grown without nitrate (table 5.1). This result is consistent with the idea that rugosity is an adaptation to electron acceptor limitation.

To confirm that bacteria at depth in a colony biofilm experience oxygen limitation, we used a Unisense micromanipulator fitted with a polarographic electrode to measure oxygen in wild-type and Δ*phz* colonies. We found that the oxygen concentration rose slightly as the electrode approached the biofilm surface, then rapidly declined to

near-undetectable levels at a depth of approximately 300 μm. These traces were similar for the wild-type (figure 5.3) and Δ*phz* (data not shown) colonies.

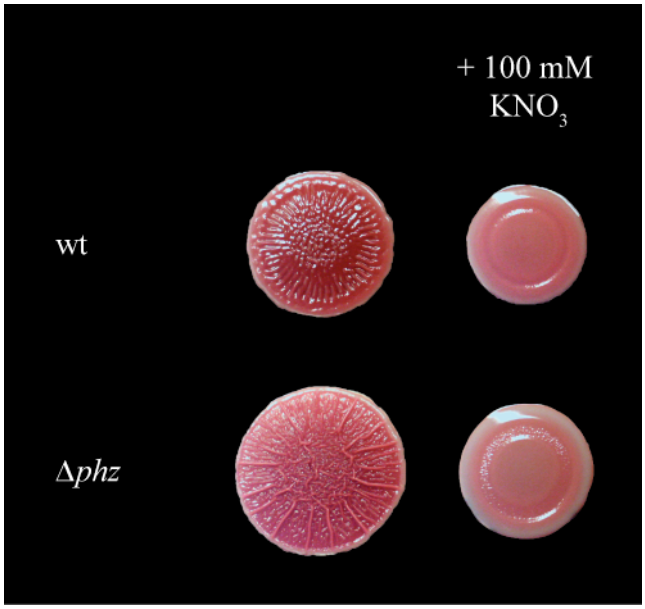

Figure 5.2. Nitrate stimulates smooth colony formation in the wild-type strain and the Δ*phz* mutant on CRCB medium.
Colonies are shown after 5 days of growth and images are representative of biological triplicates.

Table 5.1. Colony size measurements for the colonies shown in Figure 5.2
Diameters are given in centimeters.

	CRCB	CRCB + 100 mM KNO_3
wt	1.00	0.83
Δ*phz*	1.13	0.93

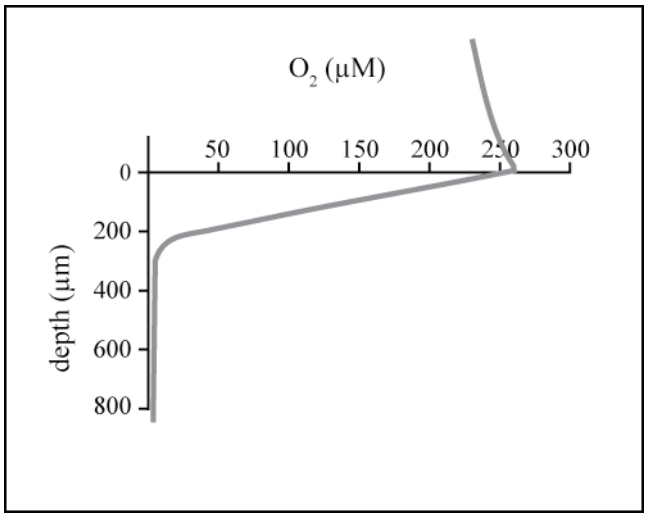

Figure 5.3. Oxygen concentration as a function of depth in a wild-type colony grown for 5 days on TA medium.
A similar profile is observed for the Δ*phz* mutant.

5.3.2. Mutations Affecting Pyocyanin Reduction Have Differing Effects on Colony Development

As described in Chapter 4, we identified two loci required for full pyocyanin reduction at the wild-type rate: (1) the gene *gpsA*, encoding the biosynthetic glycerol-3-phosphate dehydrogenase, and (2) the operon *fbcFBC,* encoding the cytochrome bc_1 complex of the respiratory chain. We constructed clean deletions of the *gpsA* and *fbcC* genes in the wild-type and Δ*phz* backgrounds and found that these mutants display different phenotypes with respect to redox balancing. The Δ*phz* Δ*gpsA* mutant has an oxidized NADH/NAD^+ pool relative to the Δ*phz* mutant, indicating that loss of a functional glycerol-3-phosphate dehydrogenase affects redox balancing, which has a secondary effect on the ability to reduce pyocyanin. The Δ*phz* Δ*fbcC* mutant has an even more reduced NADH/NAD^+ ratio than the Δ*phz* mutant, suggesting that impaired respiratory function has a more direct effect on pyocyanin reduction in this mutant.

We tested these mutants for phenotypes in the colony morphology assay. After 5 days of growth on TA medium, the Δ*gpsA* mutant formed a rugose colony, while the wild-type colony was smooth. The Δ*phz* Δ*gpsA* mutant formed an even more elaborate rugose structure, covering more surface area than the Δ*gpsA* or Δ*phz* individual mutants. On CRCB medium, the Δ*gpsA* mutant formed a dimpled colony similar in architecture to the wild type, but covering a greater surface area. The Δ*phz* Δ*gpsA* mutant on CRCB medium formed the largest colony (figure 5.4 and table 5.2).

In contrast to the Δ*gpsA* mutant, the Δ*fbcC* mutant formed relatively small, flat, and smooth colonies on CRCB and TA media. This was probably due to the severe stationary-phase growth defect that limits the production of biomass in this mutant (chapter 4, figure 4.7). This was most visible in a comparison between the Δ*fbcC* mutant and wild-type colonies grown on TA medium; the Δ*fbcC* mutant colony was thinner and more transparent. The Δ*phz* Δ*fbcC* double mutant was also thinner than the Δ*phz* mutant on TA. On CRCB medium, the Δ*phz* Δ*fbcC* mutant showed some rugosity, conferred by the deletion of the *phz* genes. The Δ*gpsA* Δ*fbcC* double mutant more closely resembled the Δ*fbcC* mutant than a combination of the two phenotypes, although the Δ*gpsA* mutation did confer some rugosity on TA medium (figure 5.4).

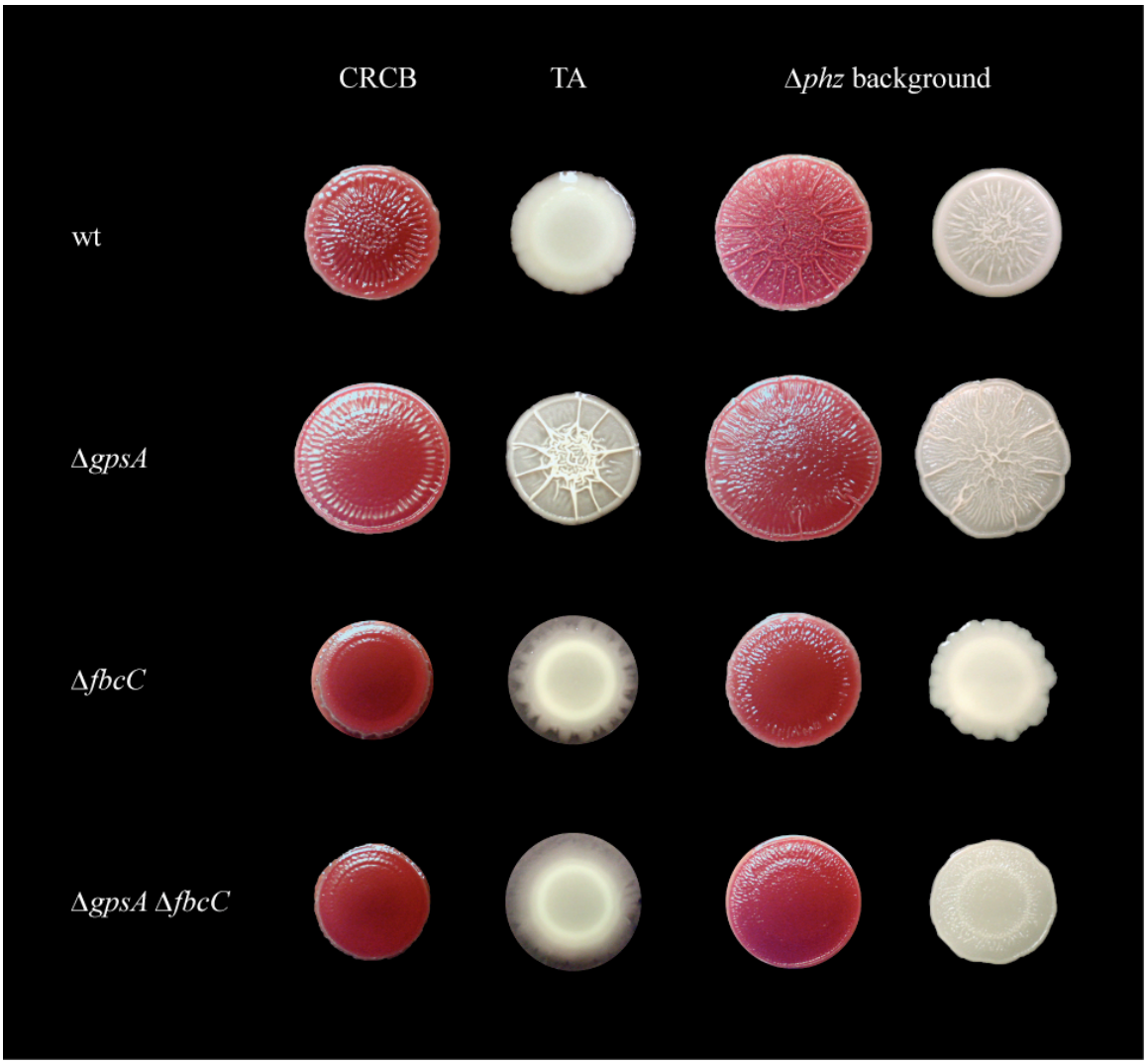

Figure 5.4. Colony morphologies of the *gpsA* and *fbcC* deletion mutants in phenazine-producing and phenazine-null backgrounds.
Colonies shown are 5 days old and images are representative of biological duplicates.

Table 5.2. Colony size measurements for the colonies shown in Figure 5.4
Diameters are given in centimeters.

	CRCB	TA	CRCB	TA
wt	1.00	0.93	1.13	0.97
Δ*gpsA*	1.13	0.98	1.25	1.18
Δ*fbcC*	0.88	0.95	1.00	0.90
Δ*gpsA* Δ*fbcC*	0.88	1.00	1.00	0.93

5.3.3. Provision of the *gpsA* Gene in trans Complements the Colony Morphology Phenotype of the Δ*gpsA* Mutant

We had previously cloned the *gpsA* gene into plasmid pUCP18, creating plasmid pAPW3, and transformed this construct into the Δ*gpsA* mutant to demonstrate complementation of the pyocyanin reduction phenotype (chapter 4). Plasmid pUCP18 and derivatives were maintained by carbenicillin selection. We prepared TA and CRCB plates containing carbenicillin at a concentration of 300 μg/mL and spotted them with cultures of the wild-type strain, the Δ*phz* mutant, and the Δ*gpsA* mutant containing pUCP18, as well as the Δ*gpsA* mutant containing pAPW3. After 5 days of growth, the wild type and mutants containing pUCP18 formed colonies with structures that were similar to those formed in the absence of carbenicillin. The Δ*gpsA* mutant containing pAPW3 formed colonies with structures closely resembling those of the wild type containing the empty vector, demonstrating that loss of the *gpsA* gene alone is responsible for the colony morphology phenotype of this mutant (figure 5.5, table 5.3).

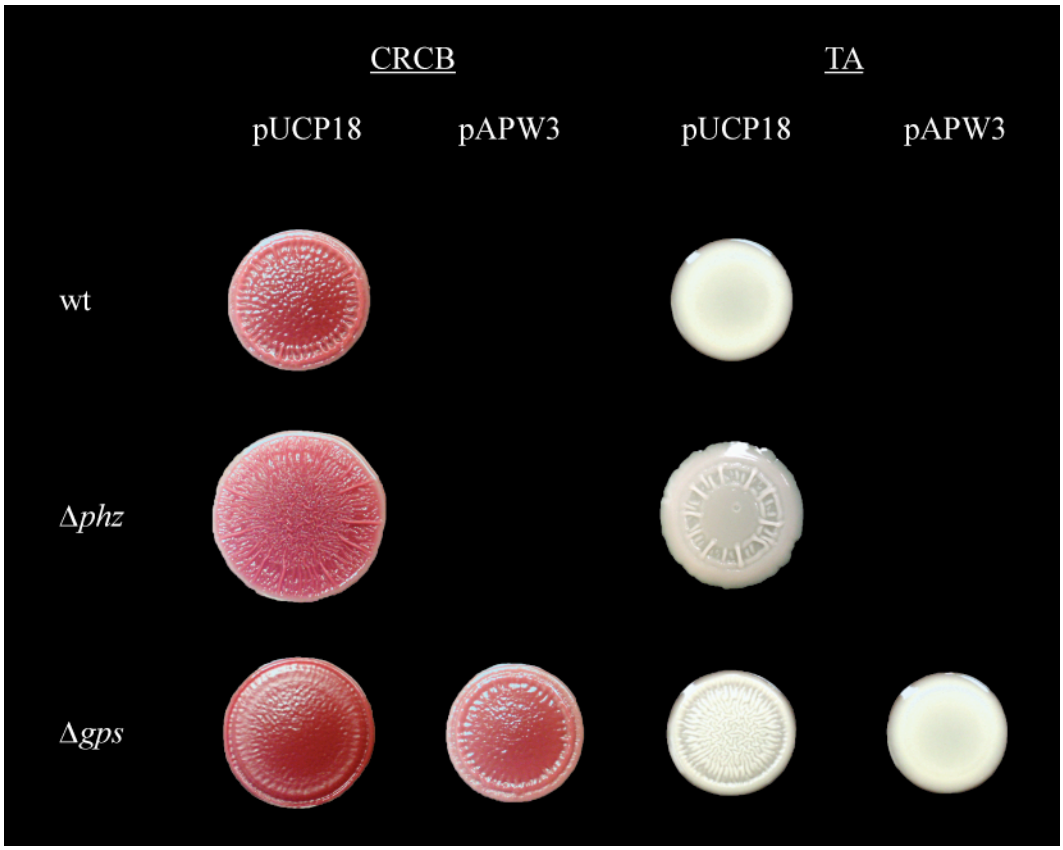

Figure 5.5. Complementation of the Δ*gpsA* mutant by pAPW3.
Colonies shown are 5 days old and images are representative of biological triplicates.

Table 5.3. Colony size measurements for the colonies shown in Figure 5.5
Diameters are given in centimeters.

	CRCB pUCP18	CRCB pAPW3	TA pUCP18	TA pAPW3
wt	1.00	-	0.85	-
Δ*phz*	1.20	-	1.00	-
Δ*gpsA*	1.05	0.98	0.90	0.85

5.3.4. Nitrate Complementation of the Δ*gpsA* Mutant Is Affected by Medium Composition

Although the Δ*phz* and Δ*gpsA* mutations both lead to increases in surface area and/or enhanced wrinkling, the morphologies were qualitatively different. We were interested to see whether the addition of nitrate could also promote the formation of smooth colonies in the Δ*gpsA* background. Colonies of the Δ*gpsA* mutant grown in the presence and absence of nitrate are shown in Figure 5.6. Nitrate amendment complemented the rugose phenotype of the Δ*gpsA* mutant grown on TA medium, and allowed radial spreading from the colony, which was also observed in the wild type under these conditions. However, nitrate addition did not appear to complement the phenotype of the Δ*gpsA* mutant on CRCB medium. Colonies of this mutant on CRCB plus nitrate covered a slightly smaller surface area, but they more closely resembled the wild type grown on CRCB without nitrate than the wild type grown on CRCB with nitrate. Finally, the nitrate addition also significantly decreased wrinkling in the Δ*phz* Δ*gpsA* background when grown on TA medium, which we would expect based on the individual phenotypes of these mutants under this condition. On CRCB medium, nitrate treatment gave rise to a colony with a smaller diameter and dimpled architecture, similar to the Δ*gpsA* mutant on nitrate (table 5.4).

Figure 5.6. Colony morphologies of the *gpsA* deletion mutant in phenazine-producing and phenazine-null backgrounds, with and without nitrate.
Colonies shown are 4 days old and images are representative of biological triplicates.

Table 5.4. Colony size measurements for the colonies shown in Figure 5.6
Diameters are given in centimeters.

	CRCB	TA	CRCB + KNO_3	TA + KNO_3
wt	0.85	0.77	0.74	0.93
Δ*gpsA*	1.00	0.77	0.93	0.88
Δ*phz* Δ*gpsA*	1.15	1.00	0.95	0.90

Our results demonstrate that the availability of electron acceptors determines the morphological development of *P. aeruginosa* colony biofilms. In Figure 5.7.A, we present a model for the reactions contributing to intracellular redox balancing for bacteria growing and surviving in a biofilm. For wild-type *P. aeruginosa* colonies on plates containing nitrate, bacteria at the surface of the colony or near the surface of the plate can

respire oxygen or nitrate, respectively (reactions 1 and 2). Bacteria limited for respiratory substrates can oxidize NADH via pyocyanin reduction (reaction 3) and this pyocyanin can be reoxidized in one of two ways. First, it can react with oxygen abiotically at the biofilm surface (reaction 4). Alternatively, as discussed in Appendix A, we have shown that *P. aeruginosa* can catalyze nitrate-dependent pyocyanin oxidation in the absence of oxygen in a reaction requiring the periplasmic nitrate reductase (Nap) system (reaction 5).

Standing liquid *P. aeruginosa* cultures form gradients where bacterial respiration renders most of the medium anoxic and pyocyanin reduction abolishes its blue color; only in the top few millimeters of the culture is pyocyanin reoxidized and visible. We propose that a similar stratification of pyocyanin redox state occurs in colony biofilms. Pyocyanin is likely produced throughout colony development so that it is evenly distributed within the biofilm, but its redox state will be dependent on the presence of either oxygen or nitrate. While it seems reasonable to suggest that there is pyocyanin turnover between the aerobic and anaerobic zones of liquid cultures, the degree of turnover in a colony may be hampered by the viscosity of the matrix. In this context, another mechanism that may allow bacteria to reduce oxygen or nitrate via phenazines is through direct electron transfer between phenazine molecules in a conducting chain. The rate of electron transfer to phenazines would be constrained by phenazine uptake and excretion rates, the reactivity of phenazines within the cell, and the kinetics of electron trnasfer between reduced and oxidized forms of pyocyanin and other phenazine derivatives.

When nitrate is absent, pyocyanin will be oxidized in the top 200-300 μm of the biofilm and reduced in the anaerobic portion of the biofilm. When nitrate is present, pyocyanin can be oxidized through the activity of the Nap enzyme. The distribution of nitrate in a colony biofilm growing on an agar plate amended with nitrate can be estimated using the equation

$$a = \left(\frac{2D_e S_o}{k_o}\right)^{1/2}$$

where D_e is the effective diffusion coefficient for nitrate in a biofilm, S_o is the concentration of solute at the biofilm-agar interface, and k_o is the volumetric reaction rate of nitrate in the biofilm (Stewart 2003). D_e for nitrate has been measured in a variety of biofilm types, including mixed-species biofilms and biofilms with and without denitrification activity. For denitrifying biofilms such as those containing *P. aeruginosa*, the D_e varies from approximately 46-68% of the diffusion coefficient for nitrate in water (1.4×10^{-5} cm^2/s at 25 °C) (Stewart 1998; Yeh and Wills 1970). We will approximate D_e as 8×10^{-4} cm^2/s. The concentration of nitrate in the agar medium for our experiment was 100 mM, and the maximum consumption rate of nitrate for denitrifying *P. aeruginosa* cultures has been reported at 140 nmol min^{-1} mg protein^{-1} (Hernandez and Rowe 1987). Finally, we have measured protein concentrations in *P. aeruginosa* biofilms that are approximately 50 mg protein per mL of colony. Using these values, we estimate that nitrate would diffuse from the agar surface through 1.2 mm of the colony. If 10 mM nitrate was provided, nitrate would diffuse only across 0.35 mm of the colony. Consistent with this, we have seen that provision of nitrate at concentrations less than 100 mM does

not fully complement rugose colony formation in the Δ*phz* and Δ*gpsA* mutants (data not shown).

Previous observations regarding the effects of pyocyanin and nitrate reduction have shown that both of these substrates contribute to maintenance of redox homeostasis in *P. aeruginosa* under oxygen-limited conditions (chapter 3 and appendix 4); furthermore, both of these factors stimulate smooth colony formation in *P. aeruginosa*. While the rugose-smooth colony transition may represent an adaptation to changes in electron acceptor availability, the phenotypes of the Δ*gpsA* and Δ*phz* Δ*gpsA* mutants rule out the possibility that the $NADH/NAD^+$ ratio is the only signal triggering this behavioral response (chapter 4). These mutants have oxidized cytoplasms and, in such a model, would be predicted to form smooth colonies even in the absence of pyocyanin. Therefore, we propose that reduced pyocyanin and nitrate are independent signals that lead to the formation of smooth colonies (figure 5.7.B). RNA array studies have demonstrated that the phenazines pyocyanin and phenazine-1-carboxylate affect gene expression in stationary-phase planktonic cultures (Dietrich et al. 2006a; Dietrich et al. 2006b), but whether there are differential responses to oxidized and reduced pyocyanin has not been investigated. The effect of nitrate on colony morphology may be mediated via sensors linked to the redox state of the quinone pool or other indicators of respiratory activity. Future studies are aimed at elucidating the regulatory mechanisms that allow *P. aeruginosa* to alter biofilm architecture in response to changing environmental conditions.

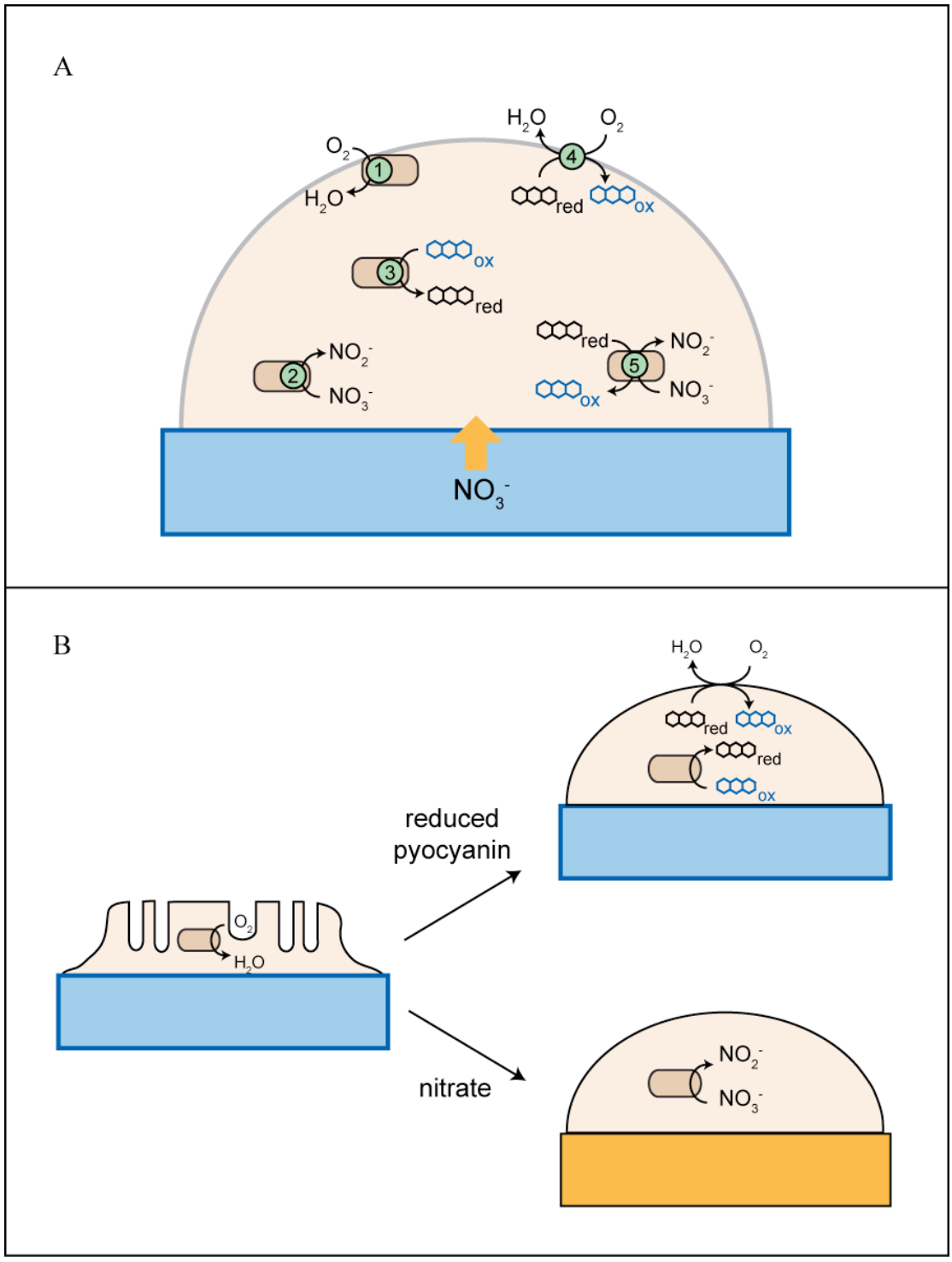

Figure 5.7. Energy metabolism and signaling in *P. aeruginosa* biofilm formation.
(A) Reactions tuning the intracellular redox state in *P. aeruginosa* biofilms. (B) Signals controlling *P. aeruginosa* biofilm architecture: in the absence of reduced pyocyanin and nitrate, *P. aeruginosa* forms a rugose colony. Either reduced pyocyanin or nitrate is sufficient to promote smooth colony formation.

5.4. Acknowledgements

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Chapter 6

Conclusions

6.1. Summary and Implications

Stationary-phase cultures of *Pseudomonas aeruginosa* form a characteristic color gradient, familiar to the many microbiologists who have worked with this proficient pathogen. Ernst Friedheim identified pyocyanin reduction as the process underlying this phenomenon in 1931; however, the work described in this thesis was the first to approach *P. aeruginosa* pyocyanin reduction at the molecular level in detail. This work has contributed to our understanding of the physiological relevance of pyocyanin reduction and provided insight into the mechanisms underlying this reaction. The physiological roles of pyocyanin have implications for *P. aeruginosa* virulence and raise broad questions about the meaning of the term "secondary metabolite." Furthermore, they draw attention to a fundamental biological problem that has received limited research attention: what is redox homeostasis for a bacterium?

6.1.1. Pyocyanin Metabolism and *P. aeruginosa* Virulence

A major contribution of this work is the finding that pyocyanin affects redox balancing in *P. aeruginosa* in a manner consistent with the effects of well-known respiratory substrates. This effect appears to translate into the formation of dense, smooth colony biofilms, where oxygen limitation normally would lead to wrinkled colony

architectures, and is particularly interesting in the context of *P. aeruginosa* infections. *P. aeruginosa* forms biofilms in the airways of patients with impaired lung function, and the densely packed bacteria in these aggregates become limited for electron acceptors such as oxygen and nitrate. While many factors probably contribute to the enhanced pathogenicity of pyocyanin-producing strains, including toxicity in other bacteria and inhibition of the host immune response, the redox balancing role of pyocyanin in this environment may represent a previously unrecognized mechanism for survival that is directly related to *P. aeruginosa* physiology.

6.1.2. What Is a "Secondary Metabolite"?

The term "secondary metabolite" generally refers to a small molecule produced during the stationary phase of the growth curve in laboratory batch cultures. That such compounds are not required for exponential growth under traditional laboratory conditions, and that their biosynthesis is often sparsely distributed throughout members of a species or genus, are often cited as reasons for their categorization as "secondary." But, when we consider that these compounds can be critical for colonization of host tissues, and directly affect substrate availability for central metabolism, they take on primary importance. This work has elaborated the role of pyocyanin, a notorious secondary metabolite produced by the pathogen *P. aeruginosa*, in altering flux through central metabolism. Pyocyanin exposure leads to the excretion of pyruvate, a compound that can later be used via a fermentative pathway in *P. aeruginosa*, or as a carbon source for other organisms present in the environment.

The recognition that stationary phase, cellular aggregation, and nutrient limitation are environmentally relevant incubation conditions has changed the way we view the physiological relevance of metabolisms that were once considered peripheral. With further investigation into the effects of other natural products on gene expression and metabolic processes, the microbiology community has begun to recognize that, rather than denoting a metabolite as "primary" or "secondary," a more useful categorization requires a description for the role of a given metabolite under discrete conditions. There is good reason to be optimistic that this insight will lead to advances in the industrial application of bacterial metabolism and the treatment of bacterial infections, by allowing us to manipulate bacterial metabolism in more predictable ways.

6.1.3 Redox Homeostasis in Bacteria

Another major contribution of this work is that it refined our thinking about mechanisms underlying *P. aeruginosa* pyocyanin reduction. I have characterized two mutants with defects in catalyzing pyocyanin reduction. One of these, lacking a functional cytochrome bc_1 complex, echoes a theme salient in research regarding the toxicity of this compound in eukaryotes—that pyocyanin and the related compound 1-hydroxyphenazine inhibit or "short-circuit" respiration at this site in the electron transport chain. The second, lacking the biosynthetic glycerol-3-phosphate dehydrogenase, lead us to develop a new model for the role of glycerol in modulating the *P. aeruginosa* intracellular redox state. Further, in combination with our previous observation that

pyocyanin alters the NADH/NAD^+ ratio in *P. aeruginosa*, it raises the bigger question of what redox homeostasis really means for this bacterium and indeed, all bacteria.

I and others have observed that the NADH/NAD^+ ratio during growth under the same conditions differs substantially in *P. aeruginosa* and *Escherichia coli*. What determines the optimal NADH/NAD^+ ratio for a bacterial strain? How do redox buffers such as NADH, NADPH and glutathione shift their reduced and oxidized pools in concert to maintain an optimal cytoplasmic E°'? What are the consequences of grossly different intracellular redox states for the regulation of redox-sensitive transcription factors and metabolic reactions in different bacterial species? Investigating these fundamental questions will contribute significantly to our comprehension of metabolic diversity and niche adaptation in the bacterial domain.

6.2. Future Directions

6.2.1. Physiological Roles for Other *P. aeruginosa* Phenazines

This thesis has focused on the phenazine pyocyanin, but a variety of other phenazine products are excreted by *P. aeruginosa*. These compounds vary widely in their hydrophobicities and redox potentials. We have proposed that their physiological roles depend on these properties and vary as a consequence. Of particular interest are the red phenazines, including aeruginosins A and B, which are relatively understudied with respect to their redox chemistry and roles in the pathogenicity of *P. aeruginosa* during persistence within the host. A more extensive characterization of these compounds at the levels of their chemical properties and physiological effects may unveil novel interacting

sites within the cell and new mechanisms contributing to *P. aeruginosa* survival during chronic lung infections.

6.2.2. Regulation of Pyocyanin Production, Transport, and Localization

Phenazine biosynthesis is known to be regulated via quorum sensing such that production does not occur until the stationary phase of growth in a batch culture. However, a series of other environmental factors influence phenazine production in the strain *Pseudomonas chlororaphis* PCL1391, and these have not been studied extensively in *P. aeruginosa*. Furthermore, *P. aeruginosa* is an intriguing anomaly among pseudomonad phenazine producers in that it possesses two differentially regulated copies of the *phzABCDEFG* operon, and at least three genes—*phzS*, *phzM*, and *phzH*—involved in the decoration of the phenazine core structure. The transcription factors and other regulatory mechanisms responsible for modulating expression of these ORFs are currently under study. The intriguing possibility that these ORFs could be induced in response to electron acceptor limitation through the activity of a redox-sensitive regulatory system has not been addressed.

6.2.3. Electron Shuttling in the Cystic Fibrosis Airway

The transition to culturing models involving nutrient limitation or surface attachment is a significant step toward approximating environmentally relevant conditions. However, researchers have recognized that additional parameters must be modified to mimic chronic lung infection. Replicating this environment is a challenge

due to deficiencies in our characterization as well as the innate heterogeneity of the lung environment. With these caveats in mind, microbiologists are establishing synthetic media that resemble the nutrient sources utilized by *P. aeruginosa* during infection of patients with the inherited disease cystic fibrosis. The work presented in this thesis suggests that phenazine reduction may play a physiological role in the persistence of *P. aeruginosa* during chronic infections. However, the physiological relevance of this redox reaction under conditions designed to mimic the cystic fibrosis lung environment has not been explored. If pyocyanin reduction can be shown to support survival of *P. aeruginosa* utilizing this combination of nutrients, strategies should be developed to limit this metabolism, perhaps in combination with antibiotic treatment, as a therapeutic approach.

Appendix A

Characterization of Pyocyanin Reduction and Nitrate-Dependent Pyocyanin Oxidation Activities in *Pseudomonas aeruginosa* PA14

A.1. Introduction

Pseudomonas aeruginosa infections are the leading cause of morbidity and mortality in patients with the inherited disease cystic fibrosis. In cystic fibrosis, impaired function of an ion channel present in epithelial cell membranes leads to the accumulation of mucus in the lung cavity. This nutrient-rich environment favors the growth and survival of colonizing *P. aeruginosa* strains, and 70 to 80 percent of cystic fibrosis patients are chronically infected by their teen years (Lyczak et al. 2002). The characteristics of the lung environment that specifically favor *P. aeruginosa* colonization and persistence are not fully understood, and microbiologists have recently become more interested in elucidating the physiological status of *P. aeruginosa* in the cystic fibrosis lung (Alvarez-Ortega and Harwood 2007; Hassett et al. 2002a; Palmer et al. 2005).

P. aeruginosa is a heterotrophic bacterium that grows optimally by respiring oxygen or nitrate. Low levels of growth have also been reported with arginine as a fermentable substrate in complex media, and survival, but not growth, can be achieved through the partial fermentation of pyruvate (Eschbach et al. 2004; Mercenier et al. 1978); however, no other terminal electron acceptors or fermentable substrates have been identified for this bacterium. In light of this somewhat limited catabolic capacity,

researchers are currently endeavoring to better understand how exactly *P. aeruginosa* generates energy for survival during chronic airway infection. In situ measurements of oxygen concentrations in lung specimens from patients with cystic fibrosis have revealed that the mucus environment is oxygen limited, particularly in areas with densely packed populations of bacteria (Worlitzsch et al. 2002). Sputum samples from individuals with cystic fibrosis contain up to 400 μM nitrate and 300 μM arginine (Palmer et al. 2007). The slow doubling time of *P. aeruginosa* growing on arginine makes it unlikely that arginine fermentation is a major mode of growth in the cystic fibrosis lung. However, whether the lung conditions favor aerobic respiration or nitrate respiration is a subject of debate (Alvarez-Ortega and Harwood 2007; Hassett et al. 2002b; Palmer et al. 2007).

We have suggested that an alternate mechanism whereby *P. aeruginosa* might be able to sustain metabolism under conditions of oxygen limitation is through the reduction of phenazine derivatives, a class of virulence factors produced by this organism. We have shown that the phenazine pyocyanin can act as an alternative oxidant in the catabolism of *P. aeruginosa* by consuming excess reducing equivalents, which accumulate in the absence of respiratory substrates at concentrations required for growth (chapter 3). This mechanism for reoxidizing intracellular reductant allows the bacterium to maintain a balanced intracellular redox state, which is important for the functioning of many metabolic processes (Green and Paget 2004), and would be particularly relevant in the heterogeneous environments that *P. aeruginosa* is now appreciated to inhabit.

Pyocyanin reduction can be observed in standing cultures of *P. aeruginosa* as the formation of a color gradient near the air-liquid interface. Colorless, reduced pyocyanin is

stable in the lower portion of the culture, rendered anaerobic due to the rapid respiration rate of the bacteria. At the surface, pyocyanin reacts abiotically with oxygen and regains its blue color in the oxidized state. We developed an assay to directly quantify the color change that occurs during *P. aeruginosa*-catalyzed reduction. During our characterization of the kinetics of this process in anaerobic cell suspensions, we found that *P. aeruginosa* also catalyzes nitrate-dependent pyocyanin oxidation. This activity is absent in a mutant lacking the gene for the periplasmic nitrate reductase, an enzyme that has been implicated in redox balancing in *Escherichia coli* and other bacteria. Measurements of the intracellular redox state as well as quantitative real-time PCR (Q-RT-PCR) experiments support the model that periplasmic nitrate reduction is an additional tactic that *P. aeruginosa* can use to maintain redox homeostasis for survival. Furthermore, nitrate-dependent pyocyanin oxidation allows *P. aeruginosa* to reoxidize pyocyanin under anaerobic conditions, and may contribute to pyocyanin cycling in the cystic fibrosis airway.

A.2. Materials and methods

A.2.1. Bacterial Strains and Culture Conditions

Strains and mutants used in this study are listed in Table A.1. All cultures were grown in Lysogeny Broth (LB) at 37 °C, shaking at 250 rpm. Unless otherwise noted, culture volumes were 10 mL each in 18 × 150 mm tubes. Plasmids and primers used in this study are listed in Table A.2. For maintenance of plasmid pMQ72 and derivatives,

gentamicin sulfate was added to LB at a concentration of 100 μg/mL for *P. aeruginosa* and 15 μg/mL for *E. coli*.

Table A.1. Strains and mutants used in this study.

Strain or mutant	Description or genotype	Source
P. aeruginosa strains		
PA14	Wild type	Rahme et al. 1995
PA14 mutants	Δ*phzA1-G1* Δ*phzA2-G2*	Dietrich et al. 2006
napA::tn	*napA*::Tn*phoA*; Kn^R	Rahme et al. 1997
narG::tn	*narG*::*TnphoA*; Kn^R	Rahme et al. 1997
E. coli strain		
UQ950	*E. coli* DH5a λ(*pir*) host for cloning	Douglas Lies, Caltech

Table A.2. Plasmids and primers used in this study.

Plasmid	Description or sequence	Source
pMQ72	pBad shuttle vector; Gm^R	Shanks et al. 2006
pAPW1	pMQ72 with *napEFDABC* operon insert	This study
Cloning primers		
nap operon f	5'-CGGCAAGCTTCTACCAGCCCTTCACCCCG-3	
nap operon r	5'-CGGCGCTAGCGGTGTCGGAGATGTTCTCGT-3'	
Q-RT-PCR primers		
napA 5'	5'-GAATTCTCCAAGCGCTTCAC-3'	
napA 3'	5'-CAGCACGTCGTAGAGGGTCT-3'	
clpX 5'	5'-CCTGTGCAATGACATCC-3'	
clpX 3'	5'-AGGATGGTGCGGATCTCTTT-3'	
recA 5'	5'-CTGCCTGGTCATCTTCATCA-3'	
recA 3'	5'-ACCGAGGCGAGAACTTCAG-3'	

A.2.2. Whole Cell Suspension Assay for Pyocyanin Reduction

Cell culture samples were concentrated or diluted in filtrates of supernatants from the same culture to normalize optical density at 600 nm to 0.8. The samples were then transferred to anaerobic cuvettes, and an anaerobic pyocyanin solution (in MOPS buffer; 50 mM morpholinepropanesulfonic acid (MOPS, Sigma) at pH 7.2, 93 mM NH_4Cl,

43 mM NaCl, 2.2 mM KH_2PO_4) was added for a final pyocyanin concentration of about 0.1 mM unless otherwise noted. The cuvettes were stoppered in the anaerobic chamber to minimize oxygen exposure. Pyocyanin reduction was followed as a decrease in absorbance at 690 nm over time.

A.2.3. Complementation of the *napA* Transposon Insertion Mutant

Primers were designed using the *P. aeruginosa* PAO1 genome sequence to anneal 490 base pairs upstream of *napE* and to the last 19 bases of *napC*, yielding a PCR product including the *napEFDABC* operon and a putative promoter region. The amplified DNA was digested using restriction sites (HindIII and NheI) engineered within the primers. It was then ligated into plasmid pMQ72 digested with the same restriction enzymes and treated with calf intestinal phosphatase (Sigma). The resulting plasmid, pAPW1, contains the *napEFDABC* operon under the control of its native promoter. Cloning was carried out in *E. coli* UQ950 by standard procedures (Ausubel et al. 1992) and constructs were electroporated into *P. aeruginosa* PA14 as described previously (Bloemberg et al. 1997).

A.2.4. Whole Cell Suspension Assay for Pyocyanin Oxidation

Optical densities at 600 and 690 nm were measured for overnight cell cultures to verify similar amounts of pyocyanin production relative to cell density. Cell culture samples were diluted with the addition of fresh LB, yielding an optical density at 600 nm near 1.6. They were then transferred to an anaerobic chamber and incubated for approximately 15 minutes to allow for reduction of the majority of the pyocyanin present

in the culture. Eight hundred microliters were transferred to an anaerobic cuvette and amended with 12.8 μl 2.5 M potassium nitrate (to give a final concentration of 40 mM) or 12.8 μl water. The cuvettes were stoppered, and pyocyanin oxidation was followed as an increase in absorbance at 690 nm over time.

A.2.5. Quantitative Real-Time PCR (Q-RT-PCR) Analysis of Gene Expression

P. aeruginosa was grown aerobically in LB medium (50 mL in a 100 mL Erlenmeyer flask) to an OD at 600 nm of 0.4 (exponential phase) or 1.4 (stationary phase). One volume of culture was mixed with two volumes of Bacterial RNAProtect (Qiagen), incubated for 5 minutes at room temperature, and centrifuged for 10 minutes at 5000 rcf. Total RNA was extracted from the cell pellet using the RNAEasy Mini kit (Qiagen), according to the manufacturer's instructions, including the optional DNase treatment step. cDNA was generated using the extracted RNA as template for a Taqman (ABI Biosciences) random-primed reverse-transcriptase reaction following the manufacturer's protocol. The cDNA was used as template for Q-RT-PCR (Real Time 7300 PCR Machine, Applied Biosystems) using the Sybr Green detection system (Applied Biosystems). Samples were assayed in triplicate. Signal was standardized to *recA* using the following equation: Relative expression = $2^{(CTstandard\ -CTsample)}$, where CT (cycle time) was determined automatically by the Real Time 7300 PCR software (Applied Biosystems). Primers (Integrated DNA Technologies) for Q-RT PCR were designed using Primer3 software (Rozen and Skaletsky, 2000) and sequences are shown

in Table A.2. Criteria for primer design were a melting temperature of 60^{o}C, primer length of 20 nucleotides, and an amplified PCR fragment of 100 base pairs.

A.3. Results

A.3.1. *P. aeruginosa* Pyocyanin Reduction Rates Under Anaerobic Conditions Are Concentration Dependent

While the reduction of pyocyanin by stationary-phase *P. aeruginosa* cultures is a familiar phenomenon to microbiologists working with this organism, an assay had not been developed to follow this reaction directly. We tested the ability of stationary-phase cell suspensions to reduce pyocyanin across a range of concentrations representing the amounts typically produced by late stationary phase LB cultures. We found that the initial reduction of pyocyanin after the first 10 minutes of anaerobic incubation correlated linearly with the amount of pyocyanin in the suspension (figure A.1).

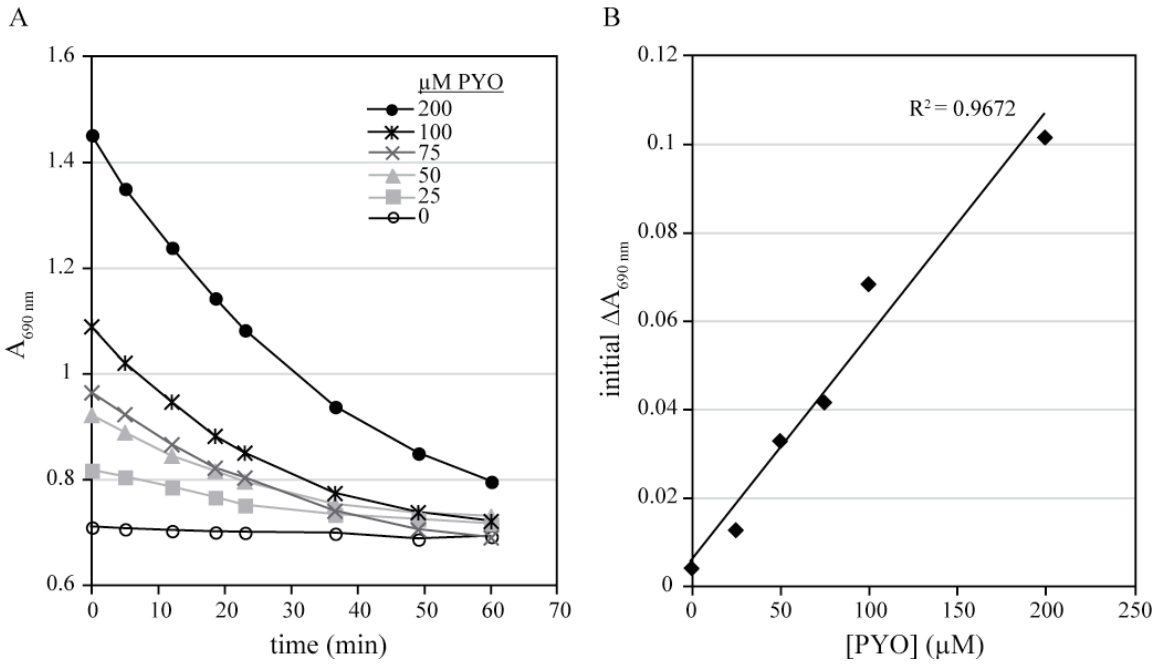

Figure A.1. *P. aeruginosa* pyocyanin reduction rates are concentration dependent. (A) Relative absorbances of anaerobic cell suspensions, containing varying concentrations of pyocyanin, over time. (B) The initial change in absorbance of pyocyanin as it is reduced by *P. aeruginosa* correlates with pyocyanin concentration. PYO, pyocyanin.

A.3.2. *P. aeruginosa* Catalyzes Nitrate-Dependent Pyocyanin Oxidation

While investigating the production of phenazines in cultures grown aerobically or anaerobically on nitrate, we pregrew cultures aerobically in the presence of nitrate to use as inocula for anaerobic cultures. We were surprised to find that, in the presence of nitrate, aerobic stationary-phase cultures appeared to be defective in their ability to reduce pyocyanin. At first, this observation was made in aerobic stationary-phase cultures that had been allowed to sit on the bench top for approximately one hour. During this time, the wild-type strain typically consumes most of the oxygen present in the medium, and reduces pyocyanin to its colorless form. Only the pyocyanin at the top of the tube, which is exposed to oxygen, remains blue because it is reoxidized abiotically by oxygen

(Friedheim 1931). In the presence of nitrate, this pyocyanin color gradient does not form (figure A.2).

The *P. aeruginosa* genome contains two operons with the potential to confer nitrate reductase activity: (1) *narGHJI*, encoding a membrane-bound respiratory nitrate reductase (Sharma et al. 2006), and (2) *napEFDABC*, encoding a putative periplasmic nitrate reductase (Potter et al. 2001; Vollack et al. 1998). We reasoned that the apparent inhibition of pyocyanin reduction arose from competition between a putative "phenazine reductase" and a nitrate reductase for electron donor (figure A.2.A). We obtained transposon insertion mutants lacking each of the Nar and Nap systems and tested them for the same phenotype observed in the wild type, i.e., nitrate-dependent inhibition of pyocyanin reduction. We found that the *narG* mutant still displayed the phenotype observed for wild-type PA14 (data not shown), whereas the *napA* mutant differed from the wild type in that it did not show inhibition by nitrate. We cloned the entire *napEFDABC* operon and its promoter into plasmid pMQ72 and transformed this construct, pAPW1, into the *napA* transposon insertion mutant (hereafter referred to as *napA*::tn). In this complemented mutant, inhibition of gradient formation by nitrate was re-established (figure A.2.B).

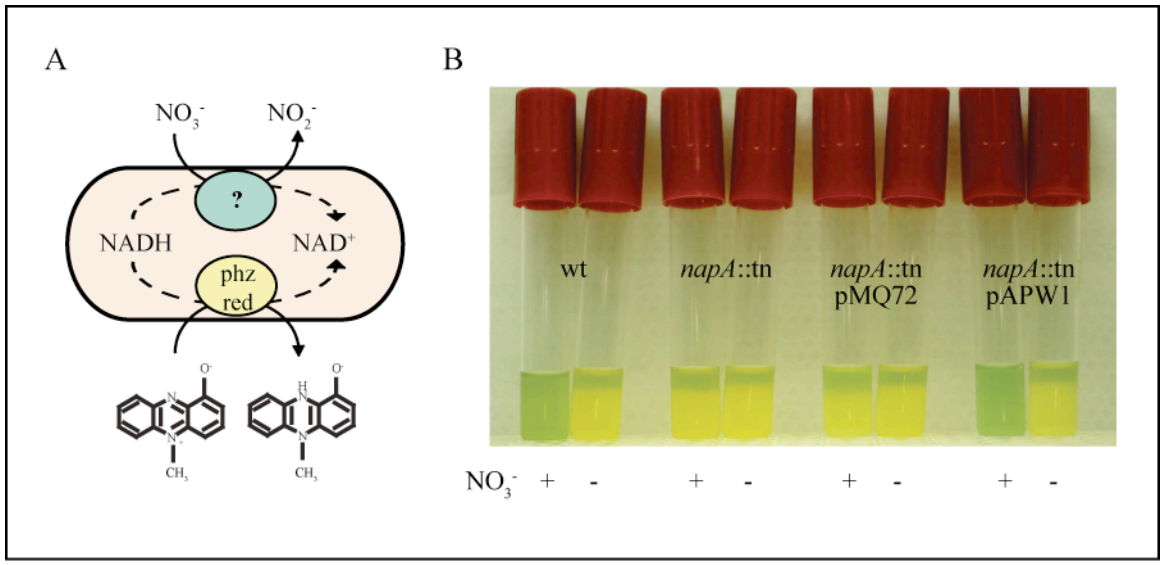

Figure A.2. Pyocyanin gradient formation in *P. aeruginosa* cultures is inhibited by nitrate in a Nap-dependent manner.
(A) Model depicting competition between putative nitrate and pyocyanin reductases for reducing power. (B) Functional NapA is required for nitrate-dependent inhibition of pyocyanin reduction. "phz red" represents a putative phenazine reductase.

The observation that a putative periplasmic nitrate reductase was responsible for the gradient formation phenotype lead us to rethink the hypothesis that this result was due to an inhibition of pyocyanin reduction. Nap enzymes in other bacteria are capable of coupling nitrate reduction to the oxidation of the redox-active, heterocyclic compound methyl viologen (Sears et al. 2000). We therefore wondered if the Nap enzyme of *P. aeruginosa* was capable of coupling nitrate reduction to oxidation of pyocyanin. This activity could also give rise to the gradient formation phenotype we observed for the *napA*::tn mutant.

A.3.3. The *P. aeruginosa* Periplasmic Nitrate Reductase (Nap) Is Involved in Nitrate-Dependent Pyocyanin Oxidation

To confirm that Nap contributes to pyocyanin oxidation, we compared the wild-type strain, the *napA*::tn mutant, and the *napA*::tn mutant containing the complementing plasmid for pyocyanin oxidation activity in the presence of nitrate. After incubating stationary-phase cultures under anaerobic conditions to allow them to reduce the pyocyanin present in the medium, we added nitrate or water and followed the oxidation of pyocyanin spectrophotometrically over time. This quantification revealed the ability of the wild-type strain to couple nitrate reduction to pyocyanin oxidation, and demonstrated that this activity was Nap dependent (figure A.3). An even higher rate of pyocyanin oxidation was observed for the complemented *napA*::tn mutant than the wild type, and we attribute this higher activity to provision of the complementing *napEFDABC* operon in multicopy. We observed a decrease in the concentration of oxidized pyocyanin for the wild-type strain with no nitrate, as well as for the *napA*::tn mutant and the *napA*::tn mutant containing the empty vector pMQ72, because residual oxidized pyocyanin remaining in these cell suspensions at the start of the assay was reduced.

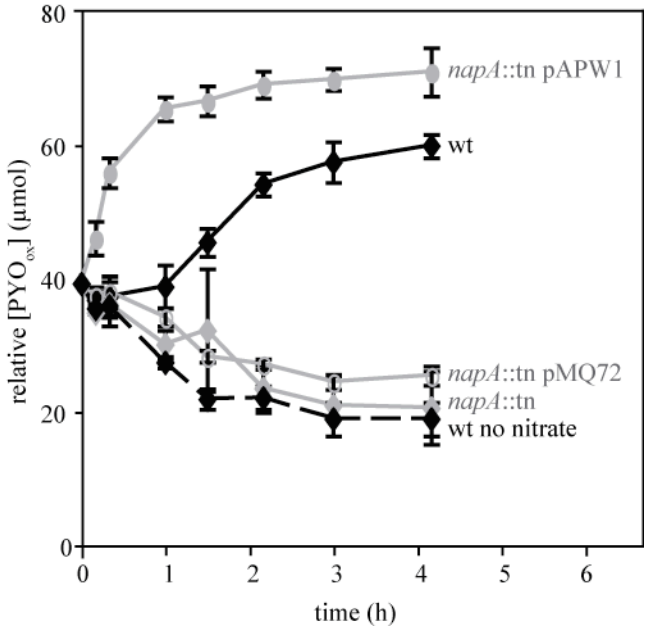

Figure A.3. Nap is involved in nitrate-dependent pyocyanin oxidation.
Error bars represent the standard deviations of biological triplicates.

A.3.4. Nitrate Reduction, via Nap, Balances the Intracellular NADH/NAD$^+$ Ratio

P. aeruginosa napA is homologous to a gene encoding the functional subunit of the periplasmic nitrate reductase complex characterized in *E. coli*, *Paracoccus pantotrophus*, *Rhodobacter sphaeroides*, and other bacteria (Potter et al. 2001). In *E. coli* and *P. pantotrophus*, it has been suggested that Nap functions to maintain redox homeostasis in stationary phase (Potter et al. 1999; Sears et al. 2000). To test the idea that Nap may play this role in *P. aeruginosa*, we incubated cultures of the wild-type strain, the *napA*::tn mutant, and the complemented *napA*::tn mutant in the presence of 40 mM nitrate for approximately 4 hours beyond entry into stationary phase and measured the intracellular NADH/NAD$^+$ ratios for each. We found that nitrate addition decreased the NADH/NAD$^+$ ratio when added to the wild type as well as the complemented Nap mutant (figure A.4). The addition of nitrate to the *napA*::tn mutant, however, had less of

an effect; these cells were unable to use nitrate to bring the intracellular NADH/NAD^+ ratio down to the wild-type level. The decrease in the intracellular NADH/NAD^+ ratio that we still observed when nitrate was added to the Nap mutant must derive from another activity that allows for the reoxidation of intracellular NADH as a consequence of nitrate reduction. We suspected that this activity might result from induction of the Nar system, but performing the same experiment with the *narG* mutant yielded results identical to those for the wild type (data not shown), indicating that this activity is not responsible for the decrease in NADH/NAD^+ ratio observed for the *napA*::tn mutant in the presence of nitrate.

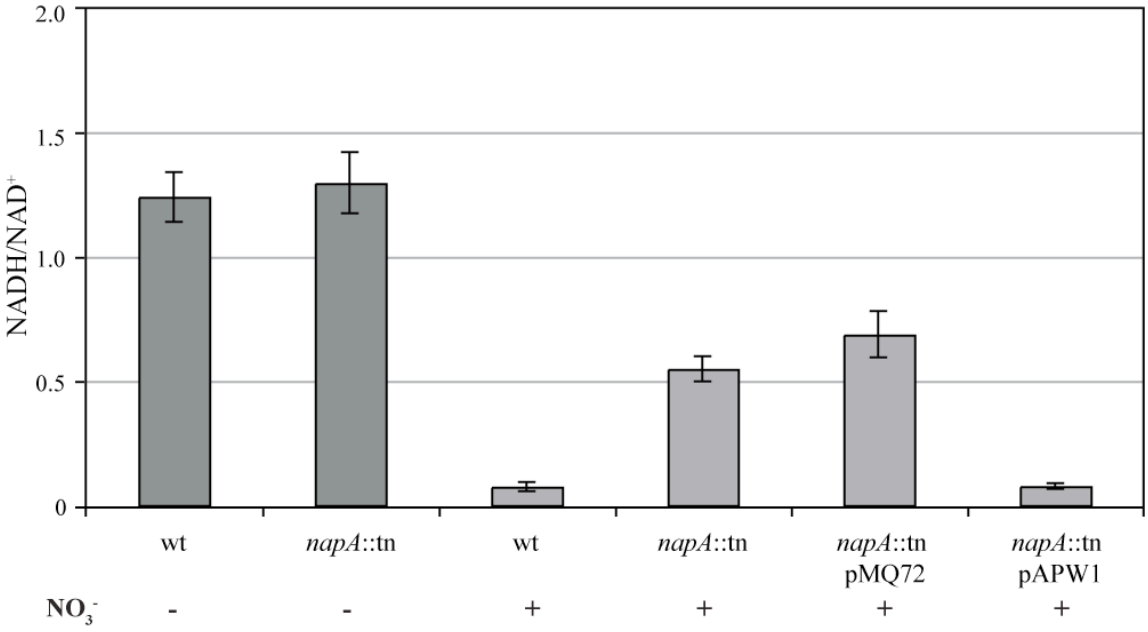

Figure A.4. Nap facilitates redox balancing in oxygen-limited, nitrate-amended cultures. Error bars represent the standard deviations of biological triplicates.

A.3.5. The Nap Operon is Upregulated in Stationary Phase

We previously described another system that balances the intracellular redox state in *P. aeruginosa*: electron transfer to the virulence factor pyocyanin (chapter 3). Based on the observations that the biosynthesis of pyocyanin is induced in stationary phase, and that the Nap system has a physiological effect similar to that of pyocyanin reduction, we wondered if the *P. aeruginosa* Nap system might also be upregulated under stationary-phase conditions. To test this, we grew the wild-type strain aerobically to an optical density (at 600 nm) of 0.4 (exponential phase) or 1.4 (stationary phase), and removed aliquots for RNA extraction. After preparation of cDNA, Q-RT PCR was performed to analyze expression of *napA* and the constitutively expressed genes *clpX* and *recA*. We found that expression of the gene *napA* was 52.5 ± 11.5–fold higher in stationary phase relative to exponential phase, while the control gene *clpX* showed only a 1.04 ± 0.06 change in expression (figure A.5). Changes in gene expression were normalized to the control gene *recA*, and the standard deviations for these values represent biological triplicates.

This experiment was done in the absence of nitrate, and we have also observed that preincubation with nitrate is not necessary for the inhibition of pyocyanin reduction by nitrate (data not shown). This suggests that the expression of the *nap* operon is not dependent on nitrate and is induced by other environmental conditions, such as oxidant limitation and/or cell density. Consistent with this, *nap* genes have been identified in the regulon of RpoS, a stationary phase-specific sigma factor (Schuster et al. 2004).

Given that pyocyanin reduction and nitrate reduction via Nap have seemingly redundant physiological effects, we might expect the production of their machineries to be cross-regulated such that only one system is active under specific conditions. In support of this idea, the stationary-phase regulator RpoS has been shown to upregulate Nap but downregulate expression of one of the operons involved in phenazine biosynthesis; hence, an RpoS mutant overproduces pyocyanin (Suh et al. 1999). Such a mutant would be defective in nitrate reduction via Nap, but might be able to complement this defect in redox balancing by channeling more reducing power to the machinery involved in pyocyanin reduction. On the other hand, we observe an additional activity for Nap that suggests that cells may benefit from the simultaneous activity of both systems: Nap reoxidizes pyocyanin in a nitrate-dependent fashion, regenerating substrate for pyocyanin-dependent NADH oxidation. Nap therefore allows pyocyanin to act as an electron shuttle between intracellular NADH and nitrate, and provides an additional route for the disposal of excess reducing equivalents. Whether this redox cycling is a spurious lab artifact or leads to synergistic NADH reoxidation (under conditions where oxidants for reduced pyocyanin are limiting) remains to be determined. Additionally, the differential expression of these activities across concentration gradients in communities of bacteria may have beneficial effects that are not apparent in well-mixed batch cultures of planktonic cells.

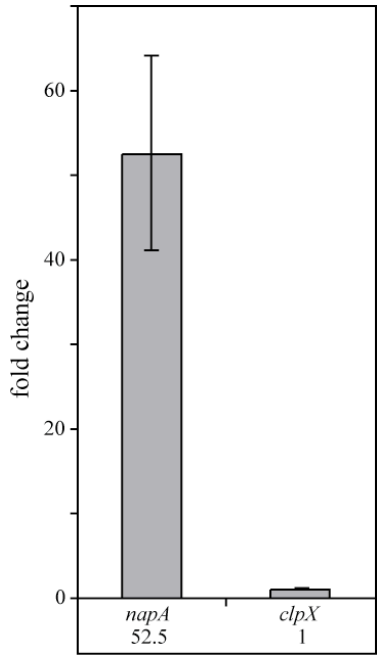

Figure A.5. Wild-type *P. aeruginosa* shows increased *nap* expression in stationary phase. Error bars represent the standard deviations of biological triplicates.

A.4. Discussion

Pseudomonads are popular model organisms for the study of biofilms, which are known for their persistence in the face of changing environmental conditions and assaults with toxic compounds (Anwar et al. 1990; Morris and Monier, 2003; O'Toole and Kolter, 1998). The respiratory activity of cells in these aggregates, as well as the production of extracellular polymers that limit diffusion, quickly leads to oxygen limitation for cells in the center of the biofilm (Worlitzsch et al. 2002). Researchers have recently become focused on the relevance of anaerobic metabolism to the ability of *P. aeruginosa* to colonize the lung (Eschbach et al. 2004; Hassett et al. 2002b; Schreiber et al. 2006; Yoon et al. 2002). We have identified two ways in which the putative *P. aeruginosa* Nap system may contribute to survival under conditions of oxygen limitation by facilitating

redox balancing. In one mechanism, Nap transfers electrons directly from the quinone pool to nitrate. In the second mechanism, Nap couples the oxidation of pyocyanin to nitrate reduction, regenerating pyocyanin as an electron acceptor for NADH oxidation.

The finding that the *nap* operon in *P. aeruginosa* encodes genes required for nitrate-dependent reoxidation of the NAD(H) pool is consistent with previous work in *Alcaligenes eutrophus* H16 (since renamed *Ralstonia eutropha*) and in *P. pantotrophus*, in which homologues of the *P. aeruginosa nap* genes have been shown to play roles in maintenance of redox homeostasis (Sears et al. 2000; Siddiqui et al. 1993). However, in organisms such as *E. coli* and *Pseudomonas* sp. strain G-179 (actually a *Rhizobium* species), Nap can or does function as the primary nitrate reductase in nitrate respiration (Bedzyk et al. 1999; Potter et al. 1999; Potter 2000; Stewart et al. 2002). In addition to this variation in metabolic capacity, differential expression of the *nap* genes in response to environmental conditions such as oxygen concentration and nitrate availability have been demonstrated in different bacteria. In *P. aeruginosa*, expression of the *nap* operon has not been observed in stationary phase during anaerobic growth on nitrate (Filiatrault et al. 2005), and we have not observed that prior incubation with nitrate is required to observe Nap activity. Therefore, we propose that in this organism Nap is expressed specifically as a mechanism to balance the intracellular redox state during stationary phase in aerobically grown cultures.

We have previously reported a role for pyocyanin reduction in the maintenance of redox homeostasis in *P. aeruginosa* (figure A.6.A). Here we report a similar role for the *P. aeruginosa* Nap system (figure A.6.B). One might suspect that, given that both

mechanisms ultimately decrease the intracellular NADH concentration, they might be competing for substrates, and that the mechanism with a higher binding affinity for NADH would inhibit the activity of the other (figure A.2.A). However, we have also observed that nitrate and pyocyanin can act synergistically to balance the intracellular redox state in a mutant unable to produce pyocyanin (chapter 3, figure 3.3.B). This suggests that nitrate-dependent pyocyanin oxidation is an alternate mechanism for pyocyanin regeneration under conditions where oxygen is unavailable (figure A.6.C).

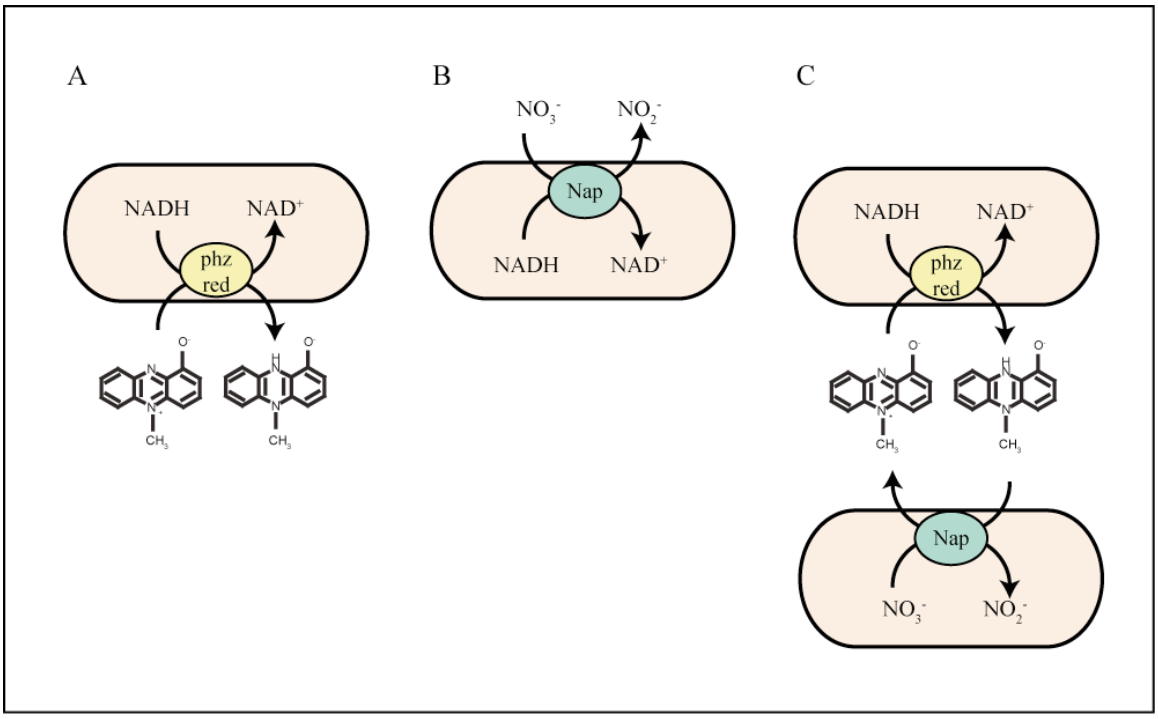

Figure A.6. Model: Pyocyanin reduction (A), Nap-dependent nitrate reduction (B), and Nap-dependent pyocyanin oxidation (C) contribute to *P. aeruginosa* redox homeostasis under oxygen-limited conditions.
"phz red" represents a putative phenazine reductase.

Our results, combined with previously reported observations, indicate that *P. aeruginosa* Nap transfers electrons from the quinone pool in the electron transport chain to nitrate (Potter et al. 2001), leading ultimately to the reoxidation of intracellular NADH.

We have used a genetic approach to identify enzymes involved in electron transfer to pyocyanin, and identified two enzymes that contribute to pyocyanin reduction activity in *P. aeruginosa*. The first, the biosynthetic glycerol-3-phosphate dehydrogenase GpsA, represents an additional mechanism for redox balancing in this organism, as mutants lacking this enzyme showed a decreased $NADH/NAD^+$ ratio compared to the parent strain, which may limit the availability of NADH for pyocyanin reduction. The second enzyme identified through our screen, the cytochrome bc_1 complex of the respiratory chain, may directly catalyze pyocyanin reduction. As we move closer toward a comprehensive list of mechanisms contributing to *P. aeruginosa* redox balancing under oxygen-limited conditions, we gain a better understanding of the metabolic potential of this pathogen. This gives us a better picture of the many ways in which *P. aeruginosa* may adapt to the conditions of the cystic fibrosis airway and will inform our thinking about therapeutic approaches.

A.5. Acknowledgements

We thank Kelli L. Palmer and Marvin Whiteley for the gifts of the *napA* and *narG* transposon insertion mutants. We would also like to thank Jared Leadbetter for helpful comments during the initial characterization of *P. aeruginosa* nitrate-dependent pyocyanin oxidation activity, and Nicky Caiazza for technical advice.

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Iglewski, B. H., McDermott, T. R., Mason, R. P., Wozniak, D. J., Hancock, R. E. W., Parsek, M. R., Noah, T. L., Boucher, R. C., and Hassett, D. J. (2002) *Pseudomonas aeruginosa* anaerobic respiration in biofilms: Relationships to cystic fibrosis pathogenesis. *Dev. Cell* **3**: 593-603.

Appendix B

The Redox State of Pyocyanin Affects Partitioning into Bacterial Cell Pellets

B.1. Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* produces virulence factors that contribute to its ability to colonize a diversity of hosts, ranging from microscopic fungi to plants and animals (Hogan and Kolter 2002; Mahajan-Miklos et al. 1999; Rahme et al. 1995). Among these factors are a class of redox-active antibiotics called phenazines. The toxicity of these compounds is usually attributed to their ability to accept electrons from intracellular redox buffers, such as NADH and glutathione, and then react with oxygen to form radical intermediates (Hassan and Fridovich 1980; O'Malley et al. 2004; Reszka et al. 2004). Given that these reductants are common metabolic substrates for most organisms, including *P. aeruginosa*, one might expect the toxicity of phenazines to be universal. However, the effects of phenazine exposure on *P. aeruginosa* growth and survival are negligible under traditional batch culture conditions (Baron and Rowe 1981; Price-Whelan et al. 2007), and phenazines have been shown to promote survival when *P. aeruginosa* is incubated anaerobically with a poised-potential electrode as the sole electron acceptor (Wang et al. 2008). It appears that *P. aeruginosa* has evolved mechanisms for tolerating, and benefiting from, the production of these reactive compounds.

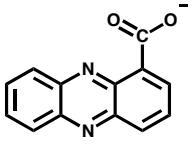
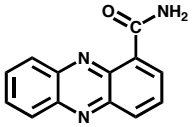
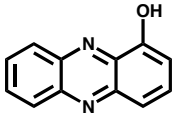
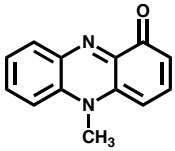
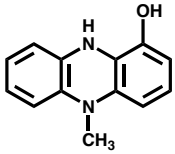
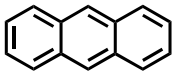
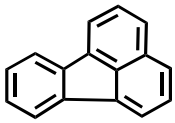
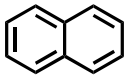
Several characteristics of *P. aeruginosa* have been cited as contributing to its tolerance of many antibiotics and phenazines specifically, including decreased outer membrane permeability and high numbers and expression levels of efflux pumps (Hassett et al. 1992; Mesaros et al. 2007). However, studies examining the effects of *P. aeruginosa* phenazines on gene expression and central metabolism suggest that phenazines do enter the cytoplasm at levels that are physiologically relevant. We have proposed that, rather than merely ensuring that all antibiotics are indiscriminately pumped out, *P. aeruginosa* executes control over the uptake, efflux, localization, and reactivity of its phenazine products that allows a coordinated physiological response (Dietrich et al. 2006; Dietrich et al. 2008).

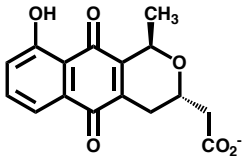
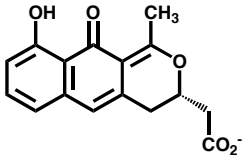
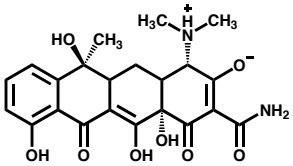
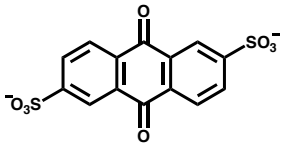
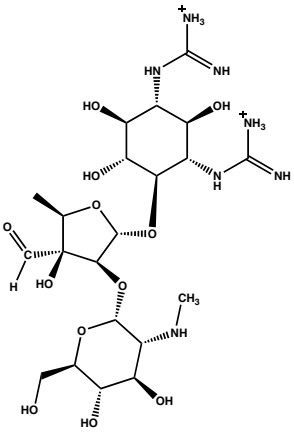
Although all of the phenazines produced by *P. aeruginosa* are roughly the same size, they vary in net charge and hydrophobicity, which are also properties that affect transport. Phenazine-1-carboxylate has a deprotonated carboxyl group and a negative charge at neutral pH, making it the least hydrophobic and most soluble *P. aeruginosa* phenazine. The other three well-characterized phenazines produced by *P. aeruginosa*—phenazine-1-carboxamide, 1-hydroxyphenazine, and pyocyanin—are more hydrophobic, with predicted logP values ranging from 1.04 to 1.81. The production of pyocyanin, which is an intense blue color, during chronic lung infections has made it the most recognizable small molecule produced by *P. aeruginosa* (Villavicencio 1998). Aside from its coloration, pyocyanin is also unique in that it is a zwitterion that can tautomerize to form an uncharged species. The diffusion rate of pyocyanin across bacterial or eukaryotic cell membranes has not been studied experimentally; the chemical structure and zwitterionic nature of this compound is cited as sufficient support for the assumption

that it can diffuse freely across biological membranes (Lau et al. 2004; Look et al. 2005; Rada et al. 2008; Schwarzer et al. 2008). Table B.1 lists predicted logP values for *P. aeruginosa* phenazines and other small molecules that have been studied with respect to their transport across biological membranes.

During an experiment testing NADH and NAD^+ levels in anaerobically incubated cultures, we uncovered a previously unacknowledged parameter—redox state—that may affect the transport of pyocyanin across the cytoplasmic membrane. Implications for putative mechanisms underlying pyocyanin uptake in *P. aeruginosa* are discussed.

Table B.1. Predicted logP values for heterocyclic and aromatic compounds of interest. LogP values were estimated using the KOWWIN software (http://www.syracuseresearch.com/esc/est_kowdemo.htm). NA, not applicable (compound has a net charge at neutral pH).

compound and structure references	structure	logP (KOWWIN prediction)	known transport mechanisms and references
phenazine-1-carboxylate (Mavrodi et al. 2001)		**NA**	
phenazine-1-carboxamide (Mavrodi et al. 2001)		**1.04**	
1-hydroxy-phenazine (Mavrodi et al. 2001)		**1.81**	
pyocyanin (oxidized) (Mavrodi et al. 2001)		**1.60**	
pyocyanin (reduced)		**2.89**	
anthracene		**4.35**	uptake by passive diffusion; active efflux (Bugg et al. 2000)
fluoranthene		**4.93**	uptake by passive diffusion; active efflux (Bugg et al. 2000)
naphthalene		**3.17**	passive uptake and release (Bugg et al. 2000)

DHK		**NA**	dedicated efflux pump (Tahlan et al. 2007)
(S)-DNPA		**NA**	dedicated efflux pump (Tahlan et al. 2007)
tetracycline (Anderson et al. 2005)		**-1.41**	uptake by passive diffusion; active efflux (Anderson et al. 2005; Sigler et al. 2000)
2,6-anthra-quinone disulfonate		**NA**	efflux pump (Shyu et al. 2002)
streptomycin (Wright and Thompson, 1999)		**NA**	active uptake and efflux (Taber et al. 1987)

B.2. Results and Discussion

Acid-base extraction, followed by measurement using an alcohol dehydrogenase-based enzyme cycling assay, constitutes a standard protocol for quantification of individual pools of NADH and NAD^+ from bacteria and yeast (Leonardo et al. 1996; Vemuri et al. 2007). In this method, cells are pelleted by a 1-minute spin at maximum

speed in a microcentrifuge, and supernatant is removed. Cells are then resuspended in either 0.2 M NaOH (for NADH extraction) or 0.2 M HCl (for NAD^+ extraction). The sample is incubated at 50 °C for 10 minutes, then cooled on ice for 10 minutes. For NADH extractions, 0.1 M HCl is added, while for NAD^+ extractions, 0.1 M NaOH is added, drop wise with gentle vortexing to partially neutralize the solutions. The extractions are then centrifuged at 16,000 rcf for 5 minutes, and supernatants are removed and assayed according to the method of Bernofsky and Swan (1973).

We have used this method to show that relative availabilities of electron donors and acceptors affect the intracellular redox balance of *P. aeruginosa*, and that pyocyanin contributes to the oxidation of the cytoplasm under conditions of electron acceptor limitation (Price-Whelan et al. 2007). One concern that arose during these experiments was the possibility that pyocyanin carryover from bacterial cell pellets could affect NADH extraction efficiency. NADH can react directly with pyocyanin producing NAD^+ (Kito et al. 1974), so samples affected by pyocyanin carryover would appear to have more oxidized NADH/NAD^+ ratios than samples without phenazine.

To test the effect of pyocyanin on the NADH/NAD^+ extraction protocol, we added increasing concentrations of pyocyanin to samples from a phenazine-null culture at the same time that we initiated the extraction by resuspension in base or acid. We found that more than 5 μM pyocyanin was required in the extraction step in order to observe an effect on the amounts of NADH, and more than 10 μM pyocyanin was required for a measurable effect on the NAD^+ value (figure B.1). Given that our samples typically contain 50-100 μM pyocyanin, this would represent a carryover greater than 10%.

During an experiment that required anaerobic culture incubation, we carried out the initial centrifugation step of the extraction under anaerobic conditions, then moved the cell pellets into an oxygen-containing atmosphere for resuspension. We found that the absence of oxygen during this initial step drastically affected our results: the NAD(H) pool appeared to be more oxidized than pools for samples prepared entirely in the presence of oxygen. To confirm that this effect was specific, we grew a wild-type *P. aeruginosa* culture aerobically to stationary phase, split it into two fractions, and placed one fraction in the anaerobic chamber. After approximately 1 hour, two 1-mL samples were taken from each fraction and centrifuged as described in either anaerobic or aerobic conditions. The supernatant was removed from the anaerobic cell pellet, and the pellet was moved into atmospheric conditions. We resuspended all pellets in 1 mL of water instead of acid or base, and measured the concentration of pyocyanin before and after extraction using a previously reported HPLC method (Price-Whelan et al. 2007). Samples centrifuged under anaerobic conditions, then extracted under aerobic conditions, retained much more of the pyocyanin in the cell pellet than samples that had been centrifuged under aerobic conditions (figure B.2).

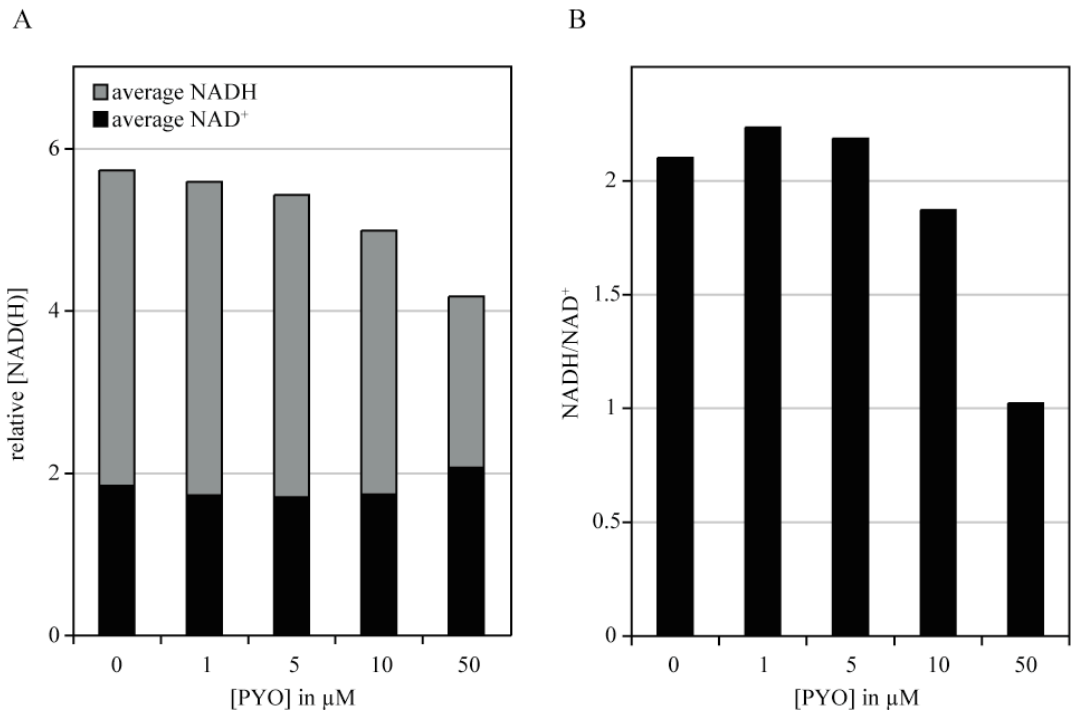


Figure B.1. Effect of pyocyanin addition on NADH assay under aerobic conditions. (A) Relative amounts of NADH and NAD^+ for each sample. (B) NADH/NAD^+ ratio calculated from the values shown in (A).

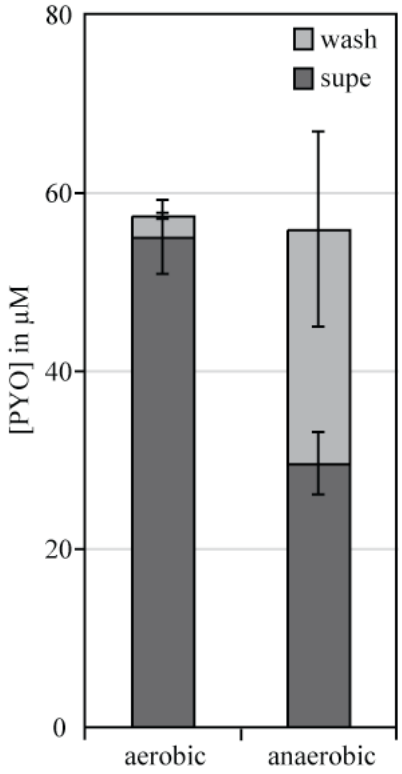


Figure B.2. Pyocyanin concentrations in supernatants and washes from the NADH/NAD$^+$ extraction method.
"Wash" refers to pyocyanin extracted from the cell pellet. Error bars represent standard deviations of triplicate samples.

To test whether the anaerobic centrifugation effect alone was sufficient to significantly alter the levels of NADH and NAD$^+$ measured in the samples, we carried out the same experiment described above, but subjected the cell pellets to base or acid extractions to isolate the NADH and NAD$^+$. Samples that were centrifuged under anaerobic conditions contained lower levels of NADH and higher levels of NAD$^+$ than samples centrifuged under aerobic conditions, resulting in a NADH/NAD$^+$ ratio that was almost 5-fold lower than that observed for the aerobically centrifuged sample (figure B.3). These results demonstrate that exposure to oxygen affects partitioning of pyocyanin into pellets of *P. aeruginosa*, and is a confounding factor in the NAD(H) extraction

protocols that must be considered when designing future experiments. This caveat did not affect the experimental results presented in Chapter 3, however, because all extractions were carried out on vortexed samples under normal atmospheric conditions.

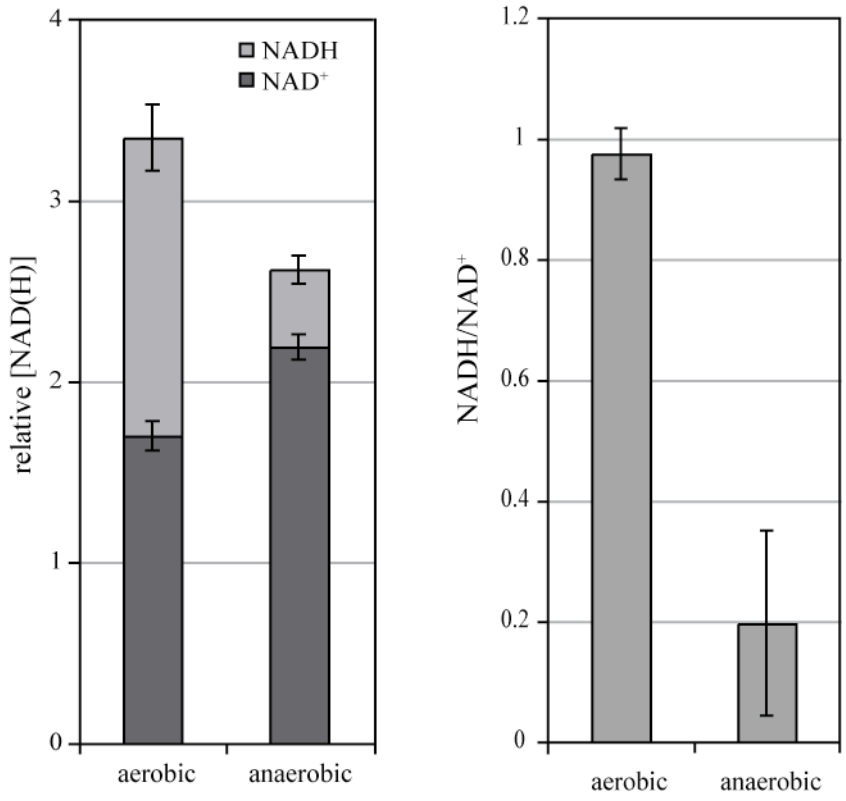


Figure B.3. Effect of pyocyanin carryover on relative NADH and NAD^+ levels under aerobic and anaerobic conditions.
Error bars represent standard deviations of triplicate samples.

The propensity of pyocyanin to remain associated with *P. aeruginosa* specifically under anaerobic conditions may be a clue to the transport mechanisms determining intracellular pyocyanin concentrations. While the general principles governing the diffusion and/or transport of small organic compounds across the bacterial cytoplasmic membrane are poorly understood, microbiologists have addressed the uptake of small

molecules on an individual basis, making predictions based on the size, charge, and polarity of the compound and testing these predictions empirically. Examples of molecules for which uptake mechanisms have been examined include the antibiotics tetracycline and streptomycin as well as environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs). Tetracycline and some PAHs have been shown to passively diffuse across the membrane, but require active transport for maximal efflux rates (Sigler et al. 2000). PAHs diffuse across the membrane at rates consistent with their predicted logP values (Bugg et al. 2000). Streptomycin is thought to require active transport for uptake and efflux; therefore, the toxicity of this compound depends on the ability of the target bacterium to produce sufficient energy for import (Taber et al. 1987).

The predicted logP values for pyocyanin fall between those of the hydrophobic PAHs and the hydrophilic antibiotics streptomycin and tetracycline (table B.1). Pyocyanin is taken up and reduced by *P. aeruginosa*, then released to the extracellular space where, under normal atmospheric conditions, it can abiotically react with oxygen to become reoxidized. If oxidized and reduced pyocyanin had the same ability to cross the cytoplasmic membrane, we would expect to recover the same amount of pyocyanin after centrifugation under aerobic and anaerobic conditions. However, the higher logP value of reduced pyocyanin suggests that it would diffuse across the membrane more readily than oxidized pyocyanin. The higher concentration of pyocyanin observed in the wash fraction from cells centrifuged in the presence of reduced pyocyanin is consistent with this model (figure B.4).

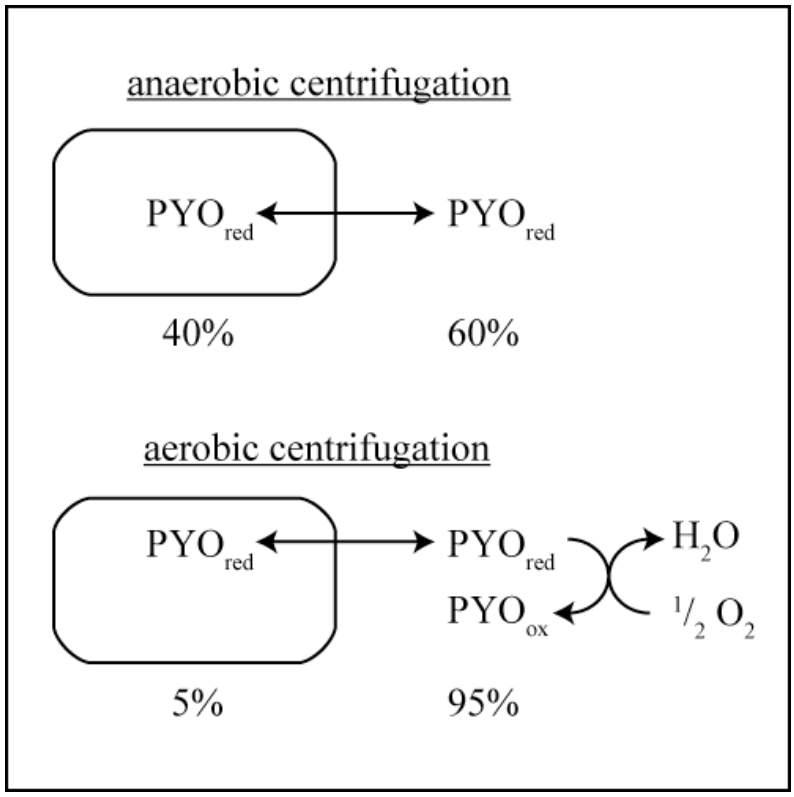


Figure B.4. Model for distribution of reduced and oxidized pyocyanin across the cell membrane under anaerobic and aerobic conditions.
Percent distribution values for pyocyanin represent approximations of values presented in Figure B.2.

Although the logP values of reduced and oxidized pyocyanin are probably high enough to allow diffusion across the membrane, preliminary evidence suggests that active transport does play a role in the localization of at least some of the *P. aeruginosa* phenazines. As we have previously reported, deletion of the *mexGHI-opmD* operon, encoding a putative efflux pump that is expressed in response to pyocyanin exposure, leads to a lag phase-specific growth defect in *P. aeruginosa* that is absent when the operon is deleted in a phenazine-null background. Furthermore, mutants lacking a functional MexGHI-OpmD pump are defective in the excretion of an uncharacterized red pigment that is probably a combination of aeruginosin A and derivatives, charged

phenazines that are produced by *P. aeruginosa* under some conditions (Dietrich et al. 2008; Herbert and Holliman 1969; Holliman 1969). Finally, we cannot rule out that the intracellular accumulation of pyocyanin under anaerobic conditions is due to an energy requirement for pyocyanin efflux that depends on oxygen as an electron acceptor. Further experiments with respiratory inhibitors and radiolabelled pyocyanin (allowing direct measurement of the intracellular fraction) are required to examine this possibility.

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Appendix C

The Transcriptional Response to Oxidation of the NADH/NAD^+ Pool Supports a Direct Activation Model for Pyocyanin and SoxR

C.1. Introduction

Bacteria experience abrupt changes in the relative availability of electron donors and acceptors, including strong oxidants such as oxygen and its derivatives. During these transitions, cells must maintain an intracellular balance of major redox buffers that allows maintenance activities, such as protein turnover and DNA repair, to proceed. For some species, transcriptional regulators that respond to the intracellular redox state have been identified, but in most cases the phylogenetic distributions as well as the prevalence of their functions have not been explored (Green and Paget 2004). The redox-sensitive transcription factor SoxR is an exception. This protein is encoded in 176 sequenced bacterial genomes, and has been shown to respond to treatment with exogenous and/or endogenous redox-active small molecules in divergent bacterial species such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptomyces coelicolor* (Dietrich et al. 2008; Gaudu et al. 1997).

SoxR is a homodimer composed of a DNA-binding domain and an activation domain with exposed iron-sulfur clusters (Watanabe et al. 2008). The protein is bound to DNA, regardless of its redox state, at a specific promoter sequence known as the soxRbox. When the iron-sulfur clusters become oxidized, a conformational change in the

homodimer contorts the DNA and enables transcription of the downstream gene. The structure and mechanism of transcriptional activation are well conserved, as demonstrated through complementation experiments in which *Pseudomonas putida* SoxR was shown to fully complement an *E. coli soxR* mutant (Park et al. 2006).

While the modes of activation for divergent SoxR orthologues are superficially similar, the function of the SoxR regulon appears to be different in enteric bacteria such as *E. coli* compared to other bacterial families. *E. coli* SoxR activates transcription of *soxS*, which encodes another transcription factor. SoxS activates transcription of many genes, including those involved in superoxide detoxification (Pomposiello and Demple, 2000). Thus, the paradigm for SoxR activation has been that xenobiotics such as methyl viologen (paraquat) trigger production of superoxide, which oxidizes SoxR and stimulates transcription of *soxS* (figure C.1.A). However, the SoxR regulons of other bacteria do not contain *soxS* or any other transcription factors. They typically contain 1-3 genes that are involved in small molecule metabolism and transport (Dietrich et al. 2008; Palma et al. 2005). Furthermore, recent work has indicated that endogenous redox-active compounds, such as the antibiotics produced by *Pseudomonas* species, can oxidize SoxR directly. In *P. aeruginosa*, SoxR-dependent transcription of *mexG*, a gene encoding part of an efflux pump, occurs under anaerobic conditions, eliminating the need for superoxide generation in the SoxR-activation model (Dietrich et al. 2006).

Two different superoxide-independent models have been proposed for SoxR activation at the molecular level. In the first model, depicted in Figure C.1.B, a reductase system is required to maintain the iron-sulfur clusters in their inactivated state, and SoxR

oxidation occurs by default when this reductase system is limited for electron donor (Kobayashi and Tagawa 1999; Koo et al. 2003). In the second model, redox-active small molecules accept electrons directly from the SoxR iron-sulfur clusters, leading to activation (figure C.1.C). The activation of the SoxR regulon in *P. aeruginosa*, which depends on exposure to redox-cycling compounds, is consistent with both of these proposed models. *P. aeruginosa* produces several phenazine derivatives, redox-active small molecules that are required for endogenous SoxR activation (Dietrich et al. 2006). As discussed in Chapter 3, *P. aeruginosa* also catalyzes the reduction of its own phenazine products, including the blue phenazine pyocyanin, and this is coupled to an oxidation of the intracellular redox state (Tsaneva and Weiss 1990). Pyocyanin and the synthetic phenazine safranine O have both been shown to allow electron transfer from SoxR to a cathode in three-electrode amperometric cells (Ding et al. 1996; Kobayashi and Tagawa 2004), demonstrating that the direct reduction of phenazines by SoxR is possible.

Here we present studies aimed at delineating the effects of redox-cycling drugs on the intracellular redox state and their direct interaction with SoxR. This work supports the hypothesis that electron transfer from SoxR to pyocyanin is required for SoxR activation in *P. aeruginosa*, and that exposure to a redox-active small molecule such as pyocyanin or methyl viologen is the major determinant of SoxR oxidation state *in vivo*.

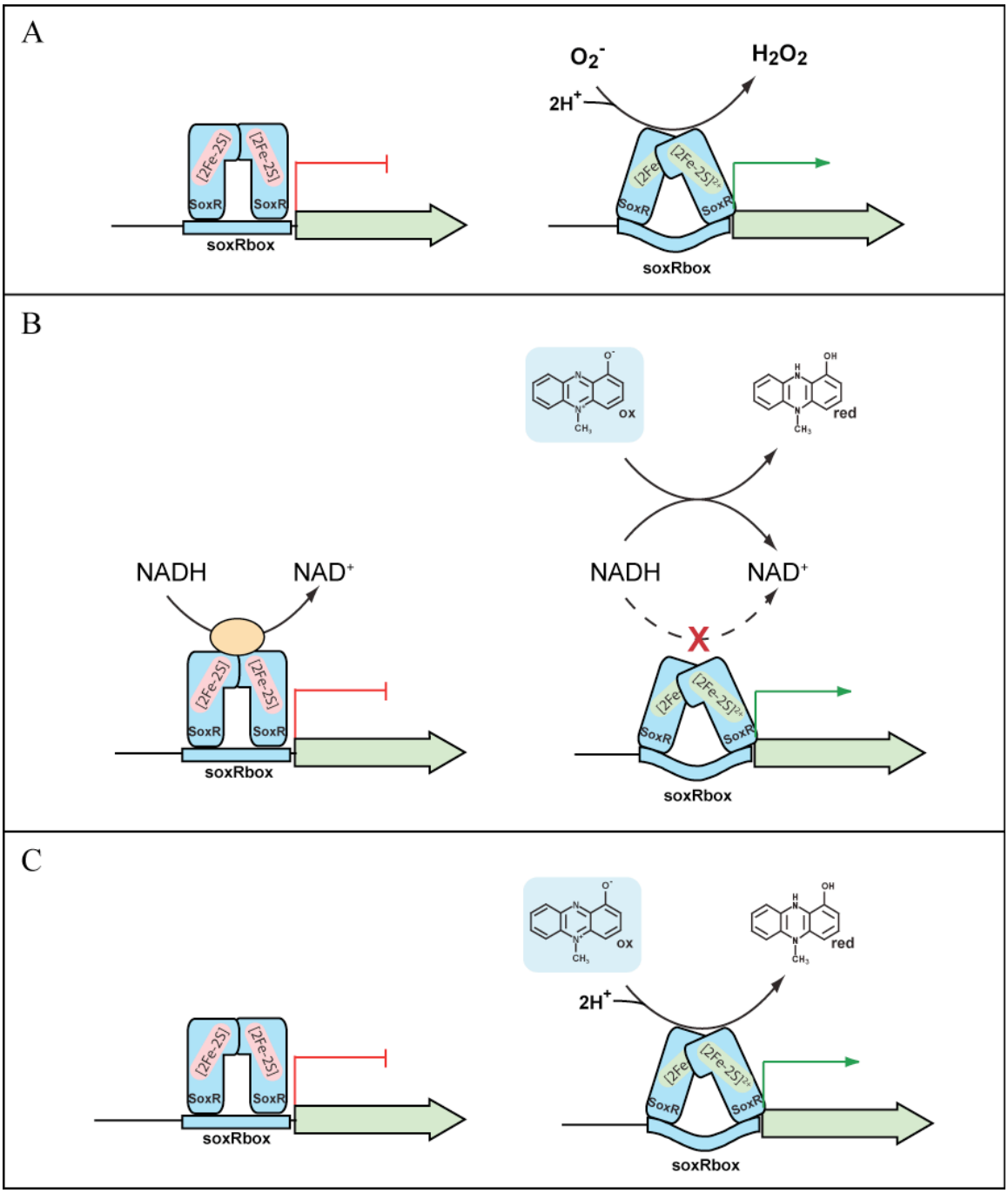

Figure C.1. Cartoon depicting models for SoxR activation.
SoxR is bound to DNA regardless of its activation state, but activates transcription only in its oxidized form. (A) Until recently, the major oxidant for SoxR was thought to be superoxide. (B) An alternate model for SoxR activation posits that the cytoplasm itself is not reduced enough to maintain SoxR in the inactive state and that an NADH-dependent reductase system is required. In the presence of a redox-cycling compound such as methyl viologen or pyocyanin, NADH is depleted and SoxR becomes oxidized by default. (C) In a third model, pyocyanin or another redox-active compound directly interacts with the iron-sulfur cluster to oxidize SoxR.

C.2. Results and Discussion

In addition to methyl viologen, the xenobiotic compounds plumbagin, menadione, and phenazine methosulfate have been shown to activate expression of the SoxRS regulon in *E. coli* (Greenberg et al. 1990; Tsaneva and Weiss 1990; Walkup and Kogoma 1989). While all of these compounds are categorized as redox-cycling drugs and are assumed to generate superoxide under the conditions used for each study, their effects on the intracellular redox state have not been explored. We inoculated two sets of cultures of *E. coli* MG1655 growing aerobically on LB, and added methyl viologen to a concentration of 200 μM to one set. Methyl viologen did not affect exponential-phase growth of *E. coli* (figure C.2A). In late exponential phase, after approximately 3.5 hours of growth, samples were taken from each culture and subjected to acid/base protocols for NADH or NAD^+ extraction. The NADH and NAD^+ samples were analyzed using an alcohol dehydrogenase-based enzyme cycling assay. Extraction and assay procedures were developed based on the methods published by Bernofsky and Swan (1973) and are described in Price-Whelan et al. (2007).

As shown in Figure C.2B, exposure to methyl viologen lead to an oxidation of the intracellular redox state in *E. coli*. This result is consistent with a role for an NADH-dependent SoxR reductase system in determining the oxidation state of SoxR, as methyl viologen, like pyocyanin in *P. aeruginosa*, would be expected to decrease the amount of reductant available for this enzyme. However, it does not rule out the possibility that the major oxidant for the SoxR enzyme itself is the redox-active compound. To test this hypothesis, a system that decouples the oxidation of the NAD(H) pool from the presence of a redox-cycling compound is required.

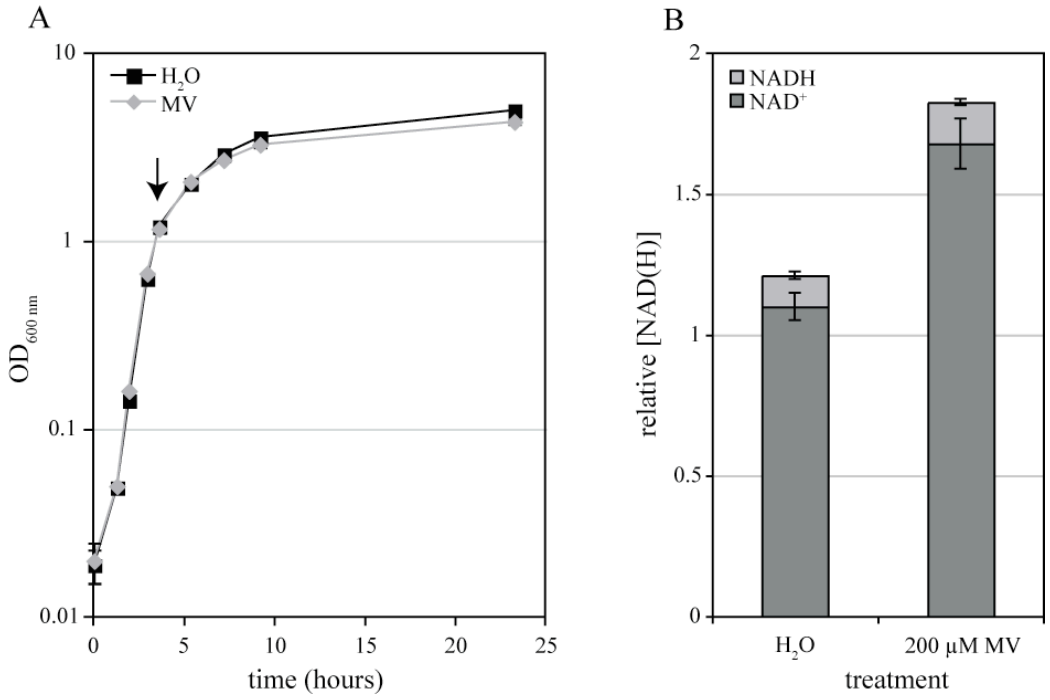

Figure C.2. Methyl viologen oxidizes the cytoplasm of *E. coli*.
E. coli cultures were grown in LB and sampled at 3.5 hours (A) for NAD(H) extraction and quantification (B). Error bars represent biological triplicates and may be obscured by the marker for some time points in (A). MV, methyl viologen.

During a screen for *P. aeruginosa* PA14 mutants defective in pyocyanin reduction, we identified a mutant with a constitutively oxidized cytoplasm. This mutant, which is lacking a functional biosynthetic glycerol-3-phosphate dehydrogenase (GpsA), is unable to produce sufficient reducing power to catalyze electron transfer to pyocyanin at the same rate as the wild type. It therefore has an oxidized intracellular redox state even when it is unable to produce pyocyanin as a redox sink (chapter 4, figure 4.5), and thus allows us to test whether an altered $NADH/NAD^+$ ratio is sufficient to activate SoxR *in vivo*.

We grew the *P. aeruginosa* PA14 wild type, a mutant that is defective in pyocyanin production (Δ*phz*), the glycerol-3-phosphate dehydrogenase mutant (Δ*gpsA*)

and a mutant lacking both pyocyanin and GpsA (Δ*phz* Δ*gpsA*) to stationary phase in LB. RNA was extracted from these samples and cDNA was prepared according to the method described in Dietrich et al. (2006). We set up quantitative real-time PCRs (Q-RT-PCRs) with primers designed to amplify the genes *mexG*, *recA*, and *clpX*. *mexG* is a SoxR target gene with an upstream soxRbox, while *recA* and *clpX* are standard control genes for Q-RT-PCR experiments in *P. aeruginosa*. The Q-RT-PCR program, primer sequences, and analytical methods have been described previously (Dietrich et al. 2006).

Analysis of the Q-RT-PCR data, normalized to *recA*, revealed that *mexG* gene expression is induced 127 (±18)– and 83 (±6)–fold in wild-type and Δ*gpsA* pyocyanin-producing cultures, respectively, compared to Δ*phz* culture. The Δ*phz* Δ*gpsA* culture showed the same expression level as the Δ*phz* culture (figure C.3). Normalization to *clpX* expression levels gave similar results. The induction of *mexG* expression in the wild type compared to the Δ*phz* mutant was as previously reported (Dietrich et al. 2006). Despite the decreased NADH/NAD^+ ratio of the Δ*phz* Δ*gpsA* mutant, SoxR does not activate *mexG* gene expression under this condition. This result strongly supports a direct-interaction model for the activation of SoxR in *P. aeruginosa*, and indicates that SoxR is present in its reduced, inactivated state even when intracellular NADH becomes limited.

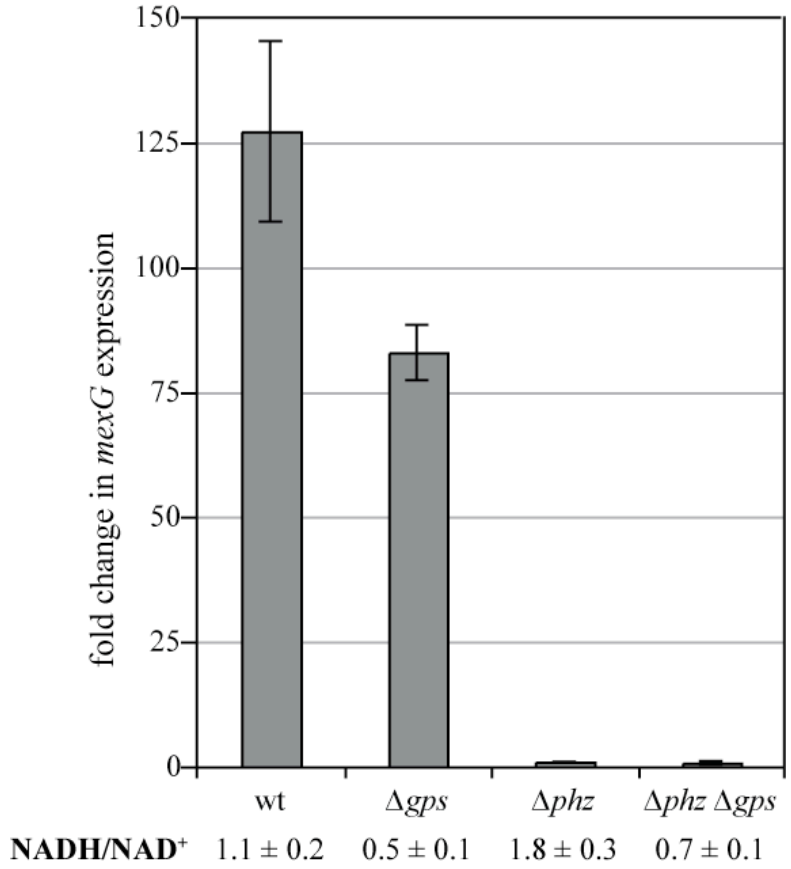

Figure C.3. Relative differences in expression of the *mexG* gene in various mutants compared to the Δ*phz* mutant.
$NADH/NAD^+$ values are taken from Figure 4.5 (chapter 4). Error bars represent the standard deviations of biological triplicates.

Although the intracellular $NADH/NAD^+$ ratio is often used as an indicator of the cytoplasmic redox state, a major caveat to our work lies in the possibility that changes in the $NADPH/NADP^+$ pool may not correlate with the overall intracellular redox potential, and NADPH is the relevant electron donor for SoxR reduction. Our assay is specific for the nonphosphorylated pyridine nucleotides, and further study of the NADPH pool is required to confirm its relevance in this context. Furthermore, while this work suggests that a reductase system is not required to maintain SoxR in a reduced state under conditions of abundant electron donor and acceptor availability, it is still possible that a

similar SoxR reductase is present in the cell that facilitates recovery of the reduced form of the enzyme after an oxidation event. Work by Koo et al. (2003) presents genetic evidence that such an enzyme exists in *E. coli*.

Our results indicate that phenazines are the major determinant of SoxR oxidation state in stationary-phase *P. aeruginosa.* Further work is required to pin down the major determinant of SoxR redox state in *E. coli*. Several studies have suggested that superoxide can directly activate SoxR in this organism, but that it is not required (Fee 1991; Liochev and Fridovich 1992; Touati 2000). Experiments similar to the anaerobic activation experiment performed in *P. aeruginosa* must be carried out in *E. coli* to test whether methyl viologen alone can oxidize *E. coli* SoxR. While depletion of NADH alone is not sufficient to oxidize the enzyme in *P. aeruginosa*, the different steady-state $NADH/NAD^+$ ratios in pseudomonads compared to the enterics (Wimpenny and Firth,1972), as well as differences in central metabolic pathways in these bacteria (Fuhrer et al. 2005), may correlate with differences in their ability to manage SoxR oxidation. Studies comparing the expression of *soxS* in a mutant with a constitutively oxidized cytoplasm to the wild type will address whether SoxR responds to the intracellular oxidation state in *E. coli*. This future work will allow us to conclude whether the diverse functionalities of SoxR in enterics versus other bacterial families correspond to differences in activation mechanisms.

C.3. References

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