The Roles and Regulation of the Redundant Phenazine Biosynthetic Operons in *Pseudomonas aeruginosa* PA14

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Abstract

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The opportunistic pathogen *Pseudomonas aeruginosa* has been well studied for its ability to cause nosocomial infections in immunocompromised patients. However, its pathogenicity is only one aspect of the biology that makes this bacterium one of the most versatile of its genus. Since its first description in 1885, *P. aeruginosa* has been known to produce colorful, small molecules called phenazines. These redox-active compounds were originally thought of as mere secondary metabolites or virulence factors that allow *P. aeruginosa* to infect plant and animal hosts. However, recently we have gained an appreciation for their diverse functions that directly benefit their producer: phenazines act as signaling molecules, regulate intracellular redox homeostasis and are implicated in iron uptake. As a result, phenazines also have dramatic effects on the structural development of multicellular communities of *P. aeruginosa*, generally referred to as biofilms. How phenazine production is regulated in response to environmental cues to allow for this functional diversity is still poorly understood.

Pseudomonas aeruginosa produces at least five different phenazines, each of which have distinct chemical properties. The genes encoding the core phenazine biosynthetic enzymes are found in two redundant 7-gene operons. These operons, *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*), encode two sets of proteins that catalyze the synthesis of phenazine-1-carboxylic acid (PCA), the precursor for all other phenazine derivatives. Although the *phz1* and *phz2* operons are nearly identical (~98% similarity), they are differentially regulated. *phz1* is regulated by quorum

sensing (QS), while the factors controlling *phz2* expression have not yet been identified. Furthermore, the contribution of *phz2* to phenazine production is not fully understood. The *phz2* operon is conserved among all *P. aeruginosa* species and we hypothesize that it may be vital to their ability to adapt to diverse environments.

In this work, we have investigated the regulation of the *phz2* operon and its contribution to colony biofilm development in *P. aeruginosa* PA14 (Chapter 2). We found that (1) phenazine production in biofilms is mediated exclusively through the *phz2* operon, (2) *phz2* expression is required for biofilm development and host colonization and (3) *phz2* is regulated by quinolones, which are prominent signaling molecules in *P. aeruginosa*'s QS system. We then investigated the roles of individual phenazines in colony development (Chapter 3) and the specificity of SoxR activation by redox active molecules (Chapter 4). We found that the effects of individual phenazines are not redundant and may be used in combination to modulate colony development. SoxR is a transcription factor that is activated by redox-active molecules including phenazines. Investigations into SoxR specificity showed that SoxR activation in non-enteric bacteria is tuned to specific redox potentials. Together, the findings presented in this thesis have expanded our knowledge about the role of phenazine production in biofilms and pathogenicity.

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Dedication

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So in the words of Rocky Balboa, I say this to you Reka: “Yo Reka, I did it!!”

CHAPTER 1

1. Introduction and Background

1.1. The pathogenic bacterium *Pseudomonas aeruginosa* and its genus

Microbiologists have been studying pathogenic bacteria for almost two centuries. Robert Koch and Louis Pasteur started their investigations into disease-causing bacteria in 1859 [1]. They formulated the germ theory of disease, which states that microorganisms are the cause of diseases such as cholera, tuberculosis, syphilis, and typhoid [2]. One of the most studied bacteria in the context of disease is *Pseudomonas aeruginosa.* It was first described in 1885 by Carle Gessard in his paper "On the blue and green coloration that appears in bandages" [3] . He characterized it as a rod-shaped, aerobic and very motile bacterium that secretes blue-green pigments. Based on his observations, he named it *Bacillus* (meaning "rod") *pyocyaneus* (meaning "blue pus"). It has since been renamed as *Pseudomonas aeruginosa*. The colorful pigments exuded by *P. aeruginosa* belong to a class of redox-active molecules known as phenazines. These compounds have long been known to act as antibiotics[4,5] and are required for full virulence[6,7]. In recent years we have gained an appreciation for the beneficial roles of phenazines for the producing organism in redox homeostasis, iron uptake and as signaling molecules, which may give *P. aeruginosa* a competitive advantage at the site of infection.

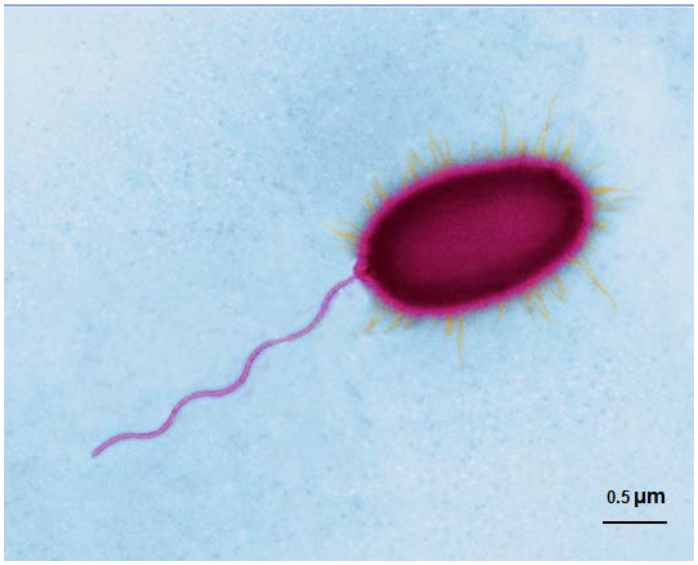


Figure 1. *Pseudomonas aeruginosa*. A gram-negative, rod-shaped bacterium that uses pili and flagella for its motility. It is ubiquitous in water, soil and host environments. Source: Kunkel Microscopy 2004

1.1.1. *Pseudomonas aeruginosa* is the most pathogenic and versatile member of its genus

Members of the genus *Pseudomonas* are gram-negative gamma-proteobacteria that are well known for their metabolic and physiological versatility and their ability to cause disease in plant and animal hosts[8-10] (Figure 2).

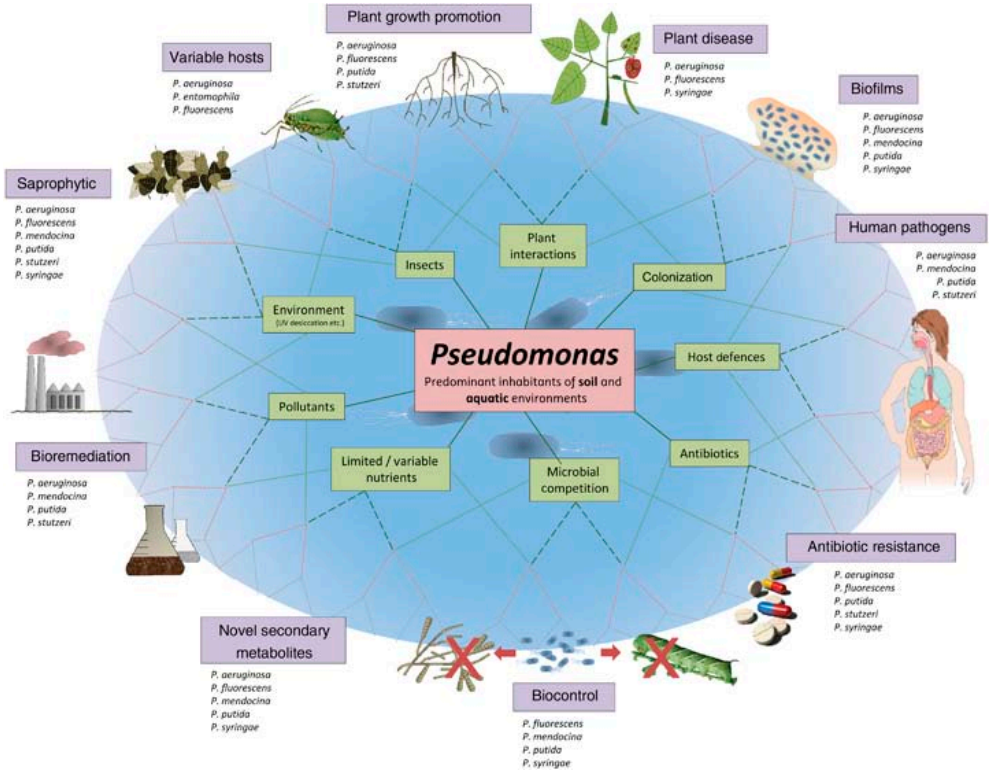


Figure 2. *Pseudomonas aeruginosa* is a versatile member of its genus. The pseudomonads inhabit diverse environments. This has led to the evolution of a wide-range of traits, many of which are shared among species. *P. aeruginosa* is one of the most versatile of the genus as it contains most of the shared traits of the genus. Source: Silby, *et al*., *FEMS Microbiol Rev*, 2011

Currently, the NCBI database lists 18 complete pseudomonad genome sequences and 72 partial sequences[11]. The complete genomes are available for strains from the plant and animal pathogen *P. aeruginosa*[12], the plant pathogens *P. syringae*[13] and *P. fluorescens*[14], as well as *P. stutzeri*[15], *P. putida*[16] and *P. entomophila*[17] (Table 1).

Table 1. General features from completed *Pseudomonas* genomes

Species/strain	Size*	% G+C*	Genes†	% coding	tRNA†	rRNA†	Released	GenBank	Reference
P. aeruginosa									
PAO1	6 264 404	66.6	5671	89.8	63	13	13/09/2000	AE004091.2	Stover *et al.* (2000)
PA7	6 588 339	66.4	6396	90.1	65	12	05/07/2007	CP000744.1	Roy *et al.* (2010)
UCBPP-PA14	6 537 648	66.3	5994	89.8	63	13	06/10/2006	CP000438.1	Lee *et al.* (2006)
LESB58	6 601 757	66.3	6026	88.9	67	13	24/12/2008	FM209186.1	Winstanley *et al.* (2009)
C3719	6 222 097	66.5	5696	86.6	40	6	04/01/2006	NZ_AAKV00000000‡	Mathee *et al.* (2008)
PA2192	6 905 121	66.2	6203	85.5	44	2	04/01/2006	NZ_AAKW00000000‡	Mathee *et al.* (2008)
P. entomophila									
L48	5 888 780	64.2	5293	89.8	78	22	10/05/2006	CT573326.1	Vodovar *et al.* (2006)
P. fluorescens									
Pf0-1	6 438 405	60.6	5741	90.0	73	19	07/10/2005	CP000094.2	Silby *et al.* (2009)
Pf-5	7 074 893	63.3	6144	88.7	71	16	30/06/2005	CP000076.1	Paulsen *et al.* (2005)
SBW25	6 722 539	60.5	6009	88.3	66	16	09/01/2008	AM181176.4	Silby *et al.* (2009)
P. mendocina									
ymp	5 072 807	64.7	4730	90.7	67	12	20/04/2007	CP000680.1	Unpublished
P. putida									
F1	5 959 964	61.9	5423	89.9	76	20	31/05/2007	CP000712.1	Unpublished
GB-1	6 078 430	61.9	5515	90.2	74	22	05/02/2008	CP000926.1	Unpublished
KT2440	6 181 863	61.5	5481	87.5	74	22	22/01/2003	AE015451.1	Nelson *et al.* (2002)
W619	5 774 330	61.4	5292	89.8	75	22	11/03/2008	CP000949.1	Unpublished
P. stutzeri									
A1501	4 567 418	63.9	4237	90.3	61	13	20/04/2007	CP000304.1	Yan *et al.* (2008)
P. syringae									
pv. *phaseolicola* 1448A	5 928 787	57.9	5436§	86.8§	64	16	01/08/2005	CP000058.1	Joardar *et al.* (2005a, b)
pv. *syringae* B728a	6 093 698	59.2	5245	88.7	64	16	12/05/2005	CP000075.1	Feil *et al.* (2005)
pv. *tomato* DC3000	6 397 126	58.3	5721§	85.6§	64	16	21/08/2003	AE016853.1	Buell *et al.* (2003)

*Size, %G+C from http://www.Pseudomonas.com, apart from PA2192 and C3719 from Mathee *et al.* (2008).
†Genes, % coding, and rRNA and tRNA content from IMG, apart from *Pseudomonas fluorescens* Pf0-1, SBW-25 and Pf-5 (Silby *et al.*, 2009).
‡The genomes of these strains have not been properly deposited in GenBank.
§Coding sequences from plasmids included in gene count and calculation of % coding.

Table 1. General features of the completed *Pseudomonas* genomes. The *Pseudomonas* genome is one of the largest in the bacterial domain (~6 Mb). Of note, the smallest genome of the pseudomonads belongs to the non-fluorescent, saprophyte *P. stutzeri.* Source: Silby, *et al.*, *FEMS Microbiol Rev*, 2011

The best-studied *P. aeruginosa* strains are PAO1 and PA14. PAO1 was isolated from a patient's wound in Melbourne, Australia in 1954[18]. It became the standard for investigations of *P. aeruginosa's* metabolism and physiology. PA14 was isolated from a burn wound and generally shows more virulent characteristics compared to PAO1[19]. PA14 is the preferred strain for the study of *P. aeruginosa* virulence and pathogenicity. The major virulence-related genomic differences between PAO1 and PA14 are found in two large pathogenicity islands with PA14

containing 322 more mobile coding sequences than PAO1[20]. These sequences are grouped into 58 PA14-specific gene clusters, of which about half are of unknown function.

1.1.2. *P. aeruginosa* is an opportunistic pathogen that adapts to different environments

P. aeruginosa thrives in diverse environments, such as water, air, soil, animal and plant hosts. It can infect a range of organisms including nematodes[21], fruit flies[22], waxmoths[23], zebrafish[24] and mammals[25,26]. As an opportunistic pathogen it is capable of causing serious infections in a variety of tissues and organs, predominantly in immunocompromised patients[27]. For example, it has been associated with many hospital-acquired infections including burn wound infections, chronic lung infections, pneumonia, respiratory tract and even infections of the eye associated with contact lens use[28 29]. *P. aeruginosa* is also the major pathogen contributing to the morbidity and mortality of patients with the genetic disorder cystic fibrosis (CF)[30]. One of the hallmarks of *P. aeruginosa* infections in CF patients is the colonization of the lungs as sessile, antibiotic-resistant biofilms (multicellular, structured communities)[31].

Transcriptomic and genetic studies revealed the importance of virulence factors in establishing chronic *P. aeruginosa* infections. Many virulence genes are located in 'conserved' regions of the genome and are required for the production of rhamnolipids, phenazines, exotoxins, and proteases[32]. Mobile DNA elements, or the 'accessory' genome, have also been suggested to be determinants of environmental adaptability in *P. aeruginosa*[33]. These include phage and plasmid elements, genomic islands, transposons and repetitive extragenic palindromic elements[11,17,34].

Accessory genes have been shown to contribute to increased virulence or competitiveness of particular strains of *P. aeruginosa*[32].

1.1.3. Physiological changes in response to the host environment

During chronic infections, *P. aeruginosa* populations change and diversify genetically. The properties characterizing the bacterial population during the initial infection period (acute) are different from those in later stages. *P. aeruginosa* isolates from acute infections are non-mucoid, motile, and susceptible to antibiotics[35]. As the infection progresses, changes in colony morphology, hypermutability, antibiotic resistance and loss of virulence traits manifest themselves[36]. In fact, genomic analysis of sequential isolates has suggested that loss of virulence may be beneficial for the persistence of infection[37]. However, conflicting studies have shown that some members of the infecting population maintain their virulence capabilities even after many years of infection[38]. The diversity in *P. aeruginosa* populations within chronic infections is a striking feature that highlights its versatility in adapting to host environments at the population level.

Acclimation to the CF lung by *P. aeruginosa* can also be accelerated by environmental factors including host immune response, nutrient limitation, oxidative stress and iron availability [39]. Despite investigations into *P. aeruginosa* gene expression during infection, the molecular basis for infection is currently unknown. Proteomic analyses have attempted to address infection by comparing the proteome of AES-1 (an acute, transmissible CF strain) to that of proteomes from common laboratory strains, such as PAO1 and the more virulent PA14 [40]. Hare *et al*. found that

of 1700 proteins identified, 183 were significantly altered between the strains. Many of these proteins are involved in virulence and metabolism but demonstrated different expression patterns between the strains. This suggests that *P. aeruginosa* alters its protein expression pattern depending on its environment.

1.1.4. Environmental effects on *P.* aeruginosa's transcriptome: planktonic vs. biofilms

Bacteria can take on dramatically different lifestyles: as free-living cells or as part of multicellular communities (biofilms). The physical and chemical properties of biofilms significantly alter gene expression patterns[41]. Transcriptional studies in PA14 have found key differences in bacterial cells grown in planktonic cultures compared to cells grown in biofilms[42,43]. Genes involved in the type III secretion system (T3SS), adaptation to anaerobic growth, and production of the extracellular matrix were highly upregulated in biofilms [43]. The T3SS is utilized by many bacterial species to deliver over 100 effector proteins into the host [44]. These effector proteins are often multifunctional proteins that help coordinate bacterial responses to the host. However, a subset of genes were similarly expressed in stationary phase planktonic culture and biofilms. These included genes involved in metabolism, translation and motility (pili- and flagella-mediated motility) and are likely linked to the nutrient depletion and slower growth rate bacterial cells experience in both stationary phase planktonic cultures and biofilms[45].

1.1.5. The metabolic versatility of *P. aeruginosa*: Use of different carbon sources and electron acceptors

P. aeruginosa can utilize a variety of carbon sources and electron acceptors for energy generation. This metabolic versatility is another characteristic that allows for its ability to survive in diverse environmental niches. Unlike *E. coli*, *P. aeruginosa* does not use glucose as its preferred carbon source[46]. Rather, it consumes organic acids and amino acids prior to glucose[47,48]. The sequential metabolism of carbon sources is regulated by catabolite repression, allowing for the utilization of preferred substrates in an ordered fashion[49,50]. Once preferred substrates are depleted, sugars are degraded through the Entner-Doudoroff pathway instead of Embden-Meyerhof glycolysis as *P. aeruginosa* lacks a key enzyme required for the latter[39 51].

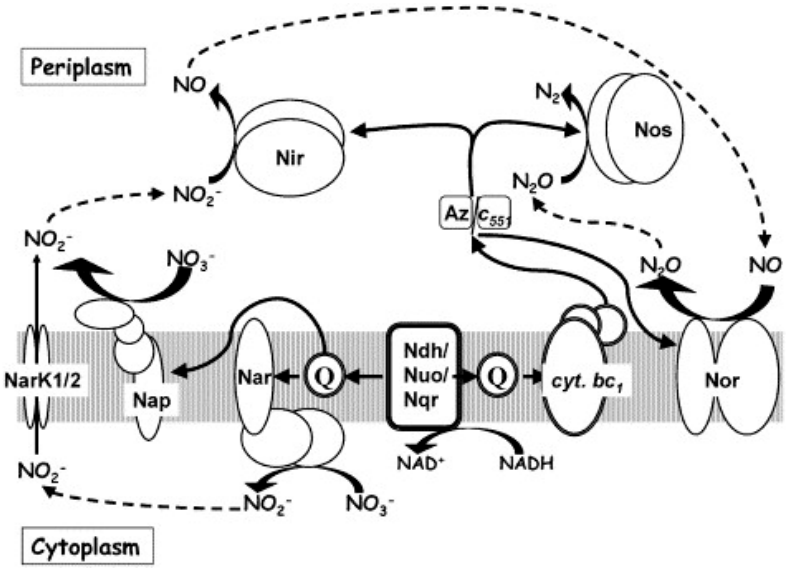


Figure 3. Denitrification in *P. aeruginosa*. *P. aeruginosa* is a denitrifying bacterium that can use nitrate as an electron acceptor to carry out anaerobic respiration. This is catalyzed by four enzyme complexes: nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR). Source: Williams *et al.*, *Adv. Micro. Phys.*, 2007

Energy production in *P. aeruginosa* is mainly based on aerobic respiration. Its metabolic versatility is highlighted by its ability to use a variety of electron acceptors. In low oxygen environments, it can flourish by using the alternative external electron acceptors nitrate and nitrite in a multi-step process called denitrification[52] (Figure 3). The membrane-bound enzyme NAR reduces nitrate to nitrite, which is further reduced to nitrite by NIR. Both reduction steps are coupled to the generation of a proton-motive force[53]. The metabolic differences between *P. aeruginosa* and other bacteria highlight different strategies to compete in various environments. *P. aeruginosa* can thrive in any soil and host environments where it can take advantage of the flux of organic acids, amino acids, sugars and nitrogenated bases.

1.2. Bacterial Communication

Members of all three domains of life use various modes of intercellular communication. Historically, research into cell-cell signaling has centered on eukaryotes. However, discoveries over the past 30 years have demonstrated that bacteria have an arsenal of signals that rival the most complex eukaryotes. It is now known that bacteria engage in cooperative and social behavior in order to perform a wide range of activities and developmental processes[54]. This research has revealed a previously unimagined complexity of bacterial communication that opens the door for further exploration into this exciting new realm.

Studies of several bacterial species known to form multicellular communities have found that they are capable of concerted actions and use extracellular signals for cell-cell communication. It is also clear that these extracellular signals can not only be detected by other bacterial cells, but

that the receiving cells can respond to these signals in a variety of ways. One such response is referred to as “quorum sensing” (QS), which involves the regulation of gene expression dependent on cell proximity and density[55]. There are many signaling pathways regulated by QS and these have a myriad of different functions within the cell and the bacterial community. These collections of signals within the community are used in a coordinated manner to benefit the population as a whole and imbue them with characteristics for survival. This is evident in chronic infections of mucoid bacterial communities and their resistance to antibiotics[56]. The QS system allows bacterial communities as a whole to respond to extracellular signals and is a prominent feature of bacterial survival mechanisms.

1.2.1. Quorum sensing in *P. aeruginosa*

Quorum sensing (QS) signaling is the best-studied communication system in bacteria. It is a complex and extensive array of molecules that can detect and react to endogenous and environmental signals. These signals trigger a response characterized by massive changes in gene expression[57]. This happens in a cell-dependent manner as gene expression is only triggered at a certain threshold concentration[55]. One of the first models used to study QS was the luminescent bacterium *Vibrio fischeri*[58]. Genes involved in the regulation of light production encode the canonical QS signaling pathway: LuxI synthesizes homoserine lactones (signal) which activate the transcriptional regulator LuxR. (Figure 4). This QS system is conserved across gram-negative bacteria.

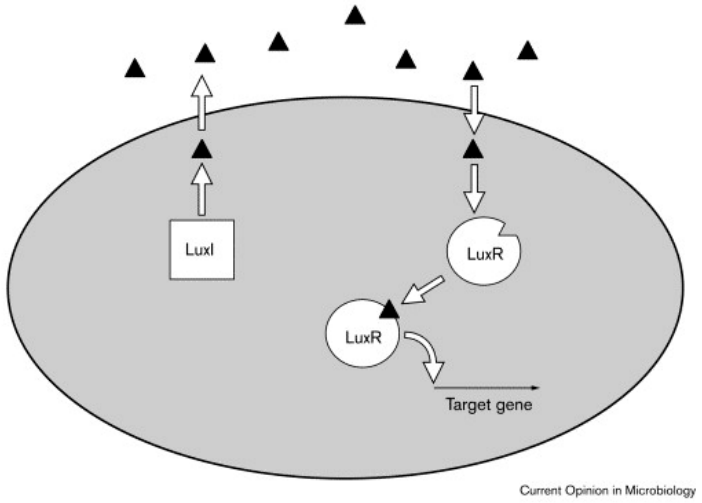


Figure 4. Simplified model of bacterial quorum sensing (QS). The QS system allows for a large-scale response to environmental factors. Bacteria exude signaling molecules that alter gene expression in a growth-dependent manner. Source: Bassler *et al.*, *Curr. Opin. Bio.*, 2000

1.2.1.1. The Las/Rhl system

In *P. aeruginosa* the two Lux-homologs LasI and RhlI catalyze the production of the N-homoserine lactones (HSL) 3-oxo-C12-HSL and 3-oxo-C4-HSL, respectively[59,60]. These HSLs activate two transcriptional regulators, LasR and RhlR, which bind to specific binding sites, "lux-boxes", in the promoter regions of their target genes[61-63]. The LasR and RhlR regulons show significant overlap, both regulating dozens of virulence genes such as the ones responsible for the production of rhamnolipids, elastases, exotoxins and proteases[64-66].

1.2.1.2. Quinolones as signaling molecules

P. aeruginosa's QS system is extended by another class of compounds, the alkyl quinolones (AQ). *P. aeruginosa* produces over 50 AQs[67,68] which vary in the lengths of their saturated or unsaturated alkyl side chains. The main AQs produced by *P. aeruginosa* are the *Pseudomonas*

quinolone signal (PQS), its precursor 2-heptyl-4-quinolone (HHQ), and N-oxide 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) (Figure 5). Despite the great number of AQs produced, their synthesis and transport are closely regulated[69-71]. PQS and its precursor HHQ are the best-studied AQs. Together they control the production of many virulence factors produced by *P. aeruginosa* including phenazines[72,73].

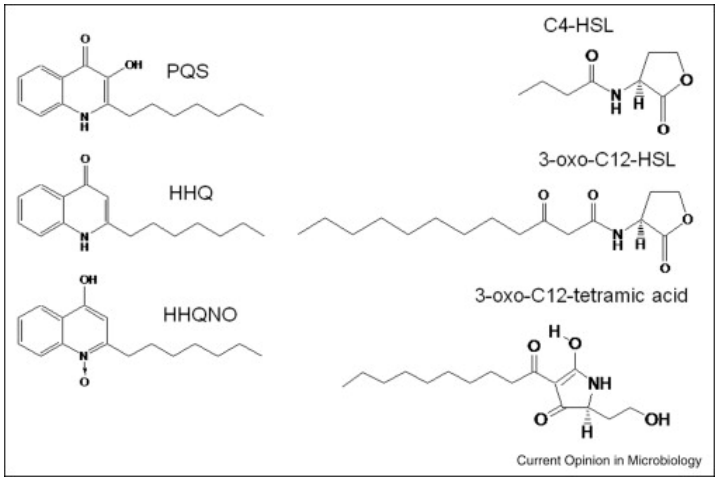


Figure 5. Structures of some common quorum sensing signals in *P. aeruginosa*. The three most abundant quinolones are the *Pseudomonas* quinolone signal (PQS), 2-heptyl-4-quinolone (HHQ) and N-oxide 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO). C4-HSL and 3-oxo-C12-HSL are products of the Rhl and Las systems, respectively. Source: Williams *et al.*, *Curr. Opin. Micro.*, 2009

Quinolones are the only known QS signals that are not members of the acylated-HSL family. The quinolone HHQ is synthesized by the enzymes encoded within the five gene operon *pqsABCDE*[74]. HHQ is then converted to PQS by the distally located monooxygenase PqsH (Figure 6). PQS production starts in late exponential phase, reaching its maximum during early stationary phase, and decreases subsequently[75]. The presence of PQS-producing *P. aeruginosa* strains in the lungs of cystic fibrosis patients suggests PQS is important for infection[76]. The PQS receptor PqsR (also known as MvfR, for multiple virulence factor regulator), is a membrane-associated protein that induces synthesis of elastase, phospholipase, 3-oxo-C12-HSL, and

phenazines[77-79]. Like PQS production, *pqsR* expression reaches maximum levels at late exponential phase. Although PqsR's relevance in the pathogenesis of *P. aeruginosa* is well-established, its precise binding motif and complete transcriptome have yet to be elucidated.

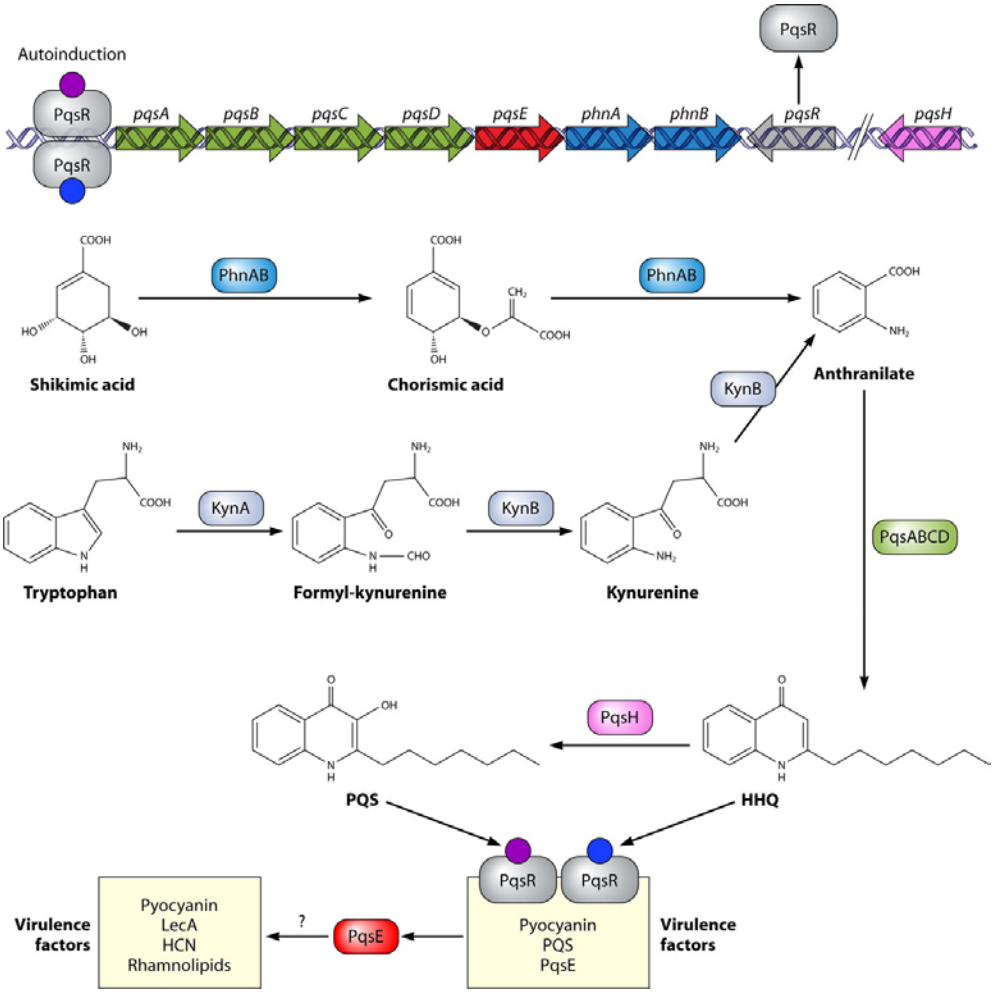


Figure 6. Synthesis, regulation and autoinduction of the quinolone signaling system in *P. aeruginosa*. Anthranilate is the substrate for quinolone biosynthesis. The quinolone PQS binds the transcription factor PqsR for its own autoinduction as well as the control of several virulence genes. PqsE is known as the "PQS response" protein, as it is needed for many PQS-dependent downstream effects. Its exact mechanism of action is unknown. Source: Jimenez *et al*., *Micro. Mol. Bio. Rev.*, 2012.

1.2.2. The formation of multicellular communities

Most bacteria are able to aggregate into multicellular communities (biofilms)[80-82]. The formation of biofilms is an active, concerted process that involves the coordinated action of billions of bacterial cells. This mode of growth is a strategy that is employed by bacteria in response to challenging environmental stimuli such as nutrient depletion and protects them from antibiotics, detergents, and other potentially harmful foreign molecules[83], allowing them to thrive in hostile environments. Although the mechanisms that govern biofilm formation can differ between species and even between strains of the same species, it seems to be an adaptation common to most bacterial species[84].

Biofilms can form on any type of surface (even on an air-liquid interface) in a wide variety of environments. Of medical concern are biofilms that form in the host (in lungs, wounds, skin, teeth, and the urinary tracts)[25,85,86] or on equipment, such as catheters, medical implants and inside water pipes[84,87]. This form of adaptation seems to be an evolutionarily conserved process to insure species survival in environments rife with competing organisms. However, in nature, biofilms tend not to consist of just one species but represent communities between multiple microbial species. In some instances, biofilms can be beneficial to their eukaryotic hosts: biofilms of *Actinobacteria* on the backs of ants provide protection from fungal and protozoan pathogens[88,89], while *P. chlororaphis* biofilms on roots protect plants from invaders[90].

Biofilms are architecturally and chemically complex structures. They are composed of a matrix made up of polysaccharides, proteins, and extracellular DNA[91] that harbors a metabolically heterogeneous population of cells. These give rise to chemical gradients across the biofilms[92] consisting of metabolic products and signaling molecules that create unique environmental

niches[92]. The best-studied gradient is that of oxygen, characterized by high levels at the top of the biofilm (which is exposed to oxygen) and low concentrations at the bottom of the biofilm (where no oxygen can penetrate)[93]. The oxygen gradient in turn affects gene expression, metabolism and redox balancing[94].

In *P. aeruginosa*, the extracellular polymeric substance (EPS) is made up of three main polysaccharides: alginate, Psl and Pel. Alginate is associated with a subset of *P. aeruginosa* variants that form mucoid colonies on agar plates[38]. It is a high molecular weight acetylated polymer composed of non-repetitive monomers of L-glucoronic and D-mannuronic acids. Alginate production confers a selective advantage within the CF lung[95], protecting *P. aeruginosa* from the consequences of inflammation and phagocytic clearance. However, despite its protective roles, alginate is not necessary for biofilm formation[96]. The common laboratory strains PAO1 and PA14 do not produce much alginate, relying on PSL and PEL for matrix construction[97]. PSL is necessary for biofilm formation in PAO1 and is also involved in cell-surface and cell-cell interactions[98]. It is composed mainly of mannose and galactose but its structure has yet to be solved[99]. In PA14, PEL is the main contributor to biofilm development and morphology. This may be in part because PA14 has a partial deletion in the *psl* gene locus [100]. The structure of PEL forms a glucose-rich polysaccharide polymer but its exact structure remains to be elucidated.

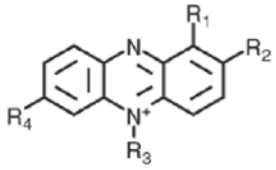
Two main laboratory models are used for the study of *P. aeruginosa* biofilms. The most prominent is the “flow cell” biofilm model: Nutritious medium with a low bacterial inoculum is streamed over the surface of a glass slide. Individual cells attach to the slide, multiply and eventually form a structured biofilm[101]. Biofilm development can be monitored by fluorescence

microscopy. The flow-cell model mimics environmental conditions found in aquatic environments. Another model for the study of biofilms is the colony biofilm assay. This assay involves spotting 10 µl of high-density cell suspension onto an agar plate. Once spotted, it is possible to follow the development of the community of cells over time. This technique is ideal for studying stages of biofilm development following initial attachment. The macroscopic colonies are particularly amenable for high-throughput screens.

1.3. Phenazines

Phenazines are redox-active, heterocyclic compounds produced by several bacterial species. Their discovery dates back to the late 19th century when doctors noticed blue-tinted pus secreted from purulent wounds in patients[102]. They were able to isolate a blue compound, "pyocyanin" that belongs to the class of phenazines. Additional phenazines were subsequently identified from culture supernatants as well as chronic *P. aeruginosa* infections[5]. Phenazine are characterized by a heterocyclic three-ring core that can be decorated with different functional groups, which change the chemical properties of phenazines (redox activity, solubility, color) (Figure 7). The colors range from the blue of pyocyanin (PYO), the lemon yellow of phenazine-1-carboxylic acid (PCA), the orange hue of 1-hydroxyphenazine (1-OH-PHZ), to the green tint of phenazine-1-carboxamide (Figure 8). *P. aeruginosa* contains a pair of redundant seven-gene operons (*phzA-G*) that encode the enzymes responsible for the biosynthesis of the phenazine PCA from chorismate[102,103] (Figure 8). The core phenazine operons are often found next to phenazine-modifying enzymes and other regulatory genes[104,105]. In *P. aeruginosa*, the *phzA1-G1* phenazine operon (*phz1*) is flanked by the methyl-transferase encoding gene *phzM* and the monooxygenase

gene *phzS*. PhzM and PhzS convert PCA to the blue phenazine pyocyanin (PYO) (Figure 8), which is unique to *P. aeruginosa*[106].



No	Name	R_1	R_2	R_3	R_4	K_{OW} ox	K_{OW} red	$E^{0\prime}$ (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 (PYO)	OH		CH_3		1.60	2.89	−172
4	2-Hydrozyphenazine-1-carboxylic acid (2-OHPCA)	COOH	OH			2.54	3.32	−34
5	Phenazine-1-carboxaminde	$CONH_2$				1.04	2.19	NA
6	1-Hydrozyphenazine (1-OHPHZ)	OH				1.81	2.35	−115

Figure 7. Some characteristics of phenazines produced by *P. aeruginosa*. The functional groups and the redox potentials at pH 7 are shown. Source: Price-Whelan *et al*., *Nat. Chem. Bio.,* 2006

Researchers and clinicians alike have delved into the physiological effects of phenazines. They found that phenazines are required for *P. aeruginosa*'s virulence and competitiveness, which is mainly due to its superoxide-generating redox activity [107].

Phenazines were originally viewed as secondary metabolites that assert their deleterious effect on other organisms via their ability to transfer electrons to oxygen. While phenazines have been observed within other bacterial species and some archaea, most of the work on the physiological role of phenazines has been done in the context of pseudomonad infections. Increased phenazine concentrations within the lung, such as during chronic *P. aeruginosa* infection of a CF patient,

can impair epithelial cell function while also attenuating immunological responses[108]. PYO reacts with oxygen to form superoxide radicals that can severely disrupt the host cells' internal redox balance [109]. These reactive oxygen species can also act as antibiotics towards other microbes competing for resources in human hosts, as well as in soil ecosystems. For example, *P. aeruginosa* biofilms that form around the roots of plants can protect the plant from pathogenic fungi via phenazine secretion. A large body of work has established the role of phenazines in physiological effects on hosts and ecological competition during *P.aeruginosa* infections. However, research within the last decade has elucidated a new role for phenazines as signaling molecules that can affect gene expression.

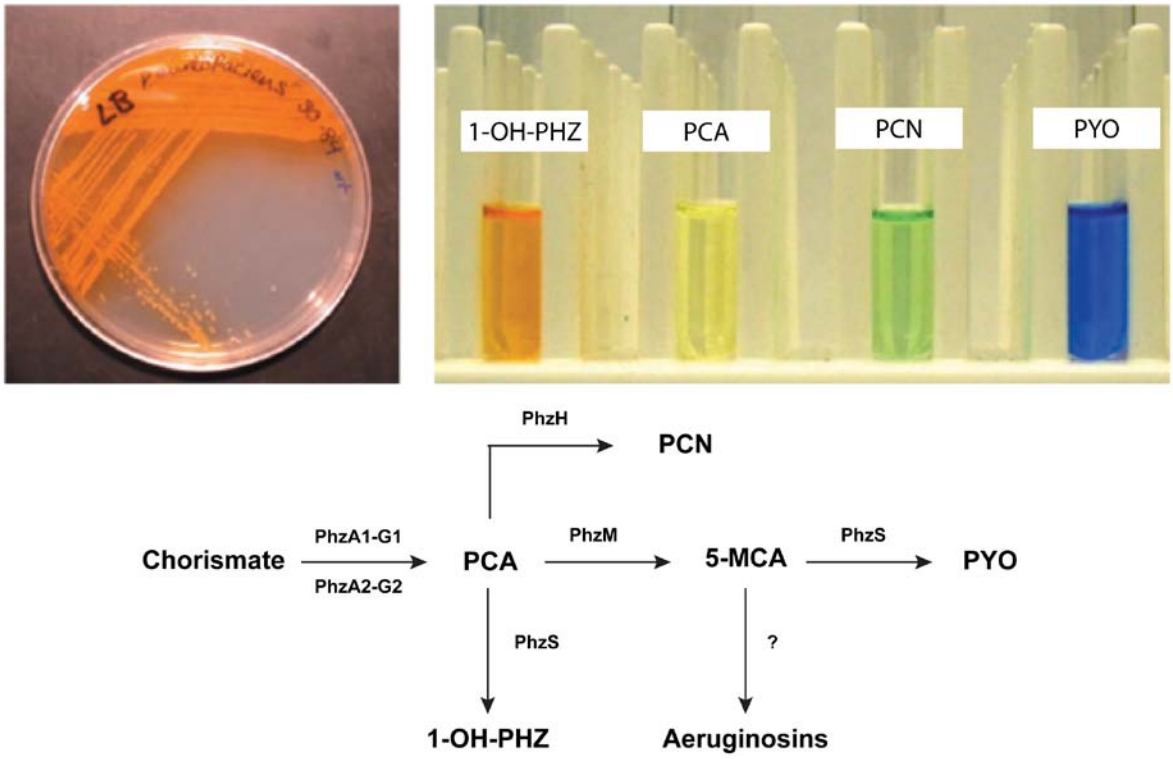


Figure 8. *P. aeruginosa* produces a variety of phenazines with colorful properties. The gene products encoded within the redundant 7-gene operons convert chorismate to phenazine-1-carboxilic acid (PCA). PCA can then be converted to several phenazines including pyocyanin (PYO), phenazine-1-carboxamide (PCN), and 1-hydroxyphenazine (1-OH-PHZ). These phenazines have different biochemical properties. Source: Adapted from Price-Whelan *et al.*, *Nat. Chem. Bio.*, 2006

1.3.1. Distribution of the phenazine operon across the bacterial domain

Phylogenetic analyses revealed that the phenazine operon (*phzA-G*) is highly conserved among phenazine-producing bacteria, such as Gram-positive actinobacteria and Gram-negative beta- and gamma-proteobacteria[105] (Figure 9). Mavrodi *et al.* have suggested that the transfer of the *phz* operon may have occurred via horizontal gene transfer in certain lineages, as the operon is

found in diverse species, such as *Streptomyces spp.* (actinobacteria) and *Pseudomonas spp.* (gamma-proteobacteria)[110,111]. The strongest evidence for horizontal gene transfer is found in *Burkholderia* species where the *phz* operon is surrounded by conserved transposon elements[105]. Additionally, in *Burkholderia* the *phz* operon has an unusually high degree of sequence conservation and it is inconsistently distributed within the genomes. Transfer of the *phz* operon between species that occupy diverse environments highlights the importance of this biosynthetic pathway.

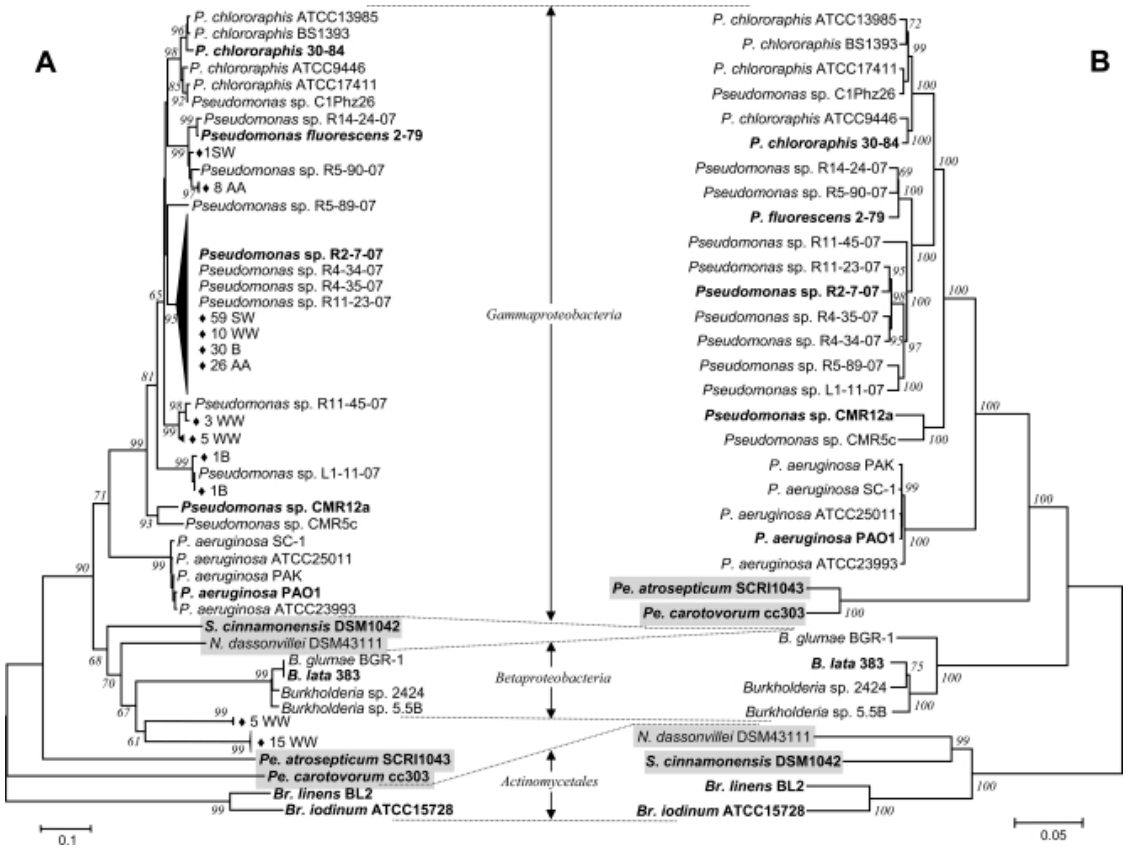


Figure 9. Distribution of phenazine producers based on *phzF* phylogeny analysis. Classification of bacterial species based on 16S sequencing (A) and *phzF* phylogeny. (B) Phenazine producing species were classified in three major clades that agree with 16S phylogeny of the taxa analyzed. Source: Mavrodi *et al*., *App. Env. Micro*., 2010

A large portion of phenazine producers are soil-dwellers and part of the rhizosphere[11,112]. Amongst those, only *P. aeruginosa* and *S. cinnamonensis* contain two redundant *phz* operons[113] (Figure 10). The fact that *P. aeruginosa* can thrive in both soil and host environments, and contains a redundant set of *phz* operons may be of importance. Does having redundant *phz* operons give *P. aeruginosa* an advantage in certain environments? Examination of the location of the operons and their flanking regions may begin to answer this question. The *phzA2-G2* operon (*phz2*) is found approximately 2 MB away from the *phz1* operon and does not have any phenazine-modifying enzymes flanking it. In addition, although the *phz1* and *phz2* operons are nearly identical (~98%), they contain distinct regulatory elements[114]. The differences between the flanking regions of *phz1* and *phz2* may point to different characteristics of each operon that were first present at the time of the duplication event. While the regulation of phenazine production through the *phz1* operon has been investigated thoroughly[6,115,116] the specific regulation of the *phz2* operon remains to be elucidated.

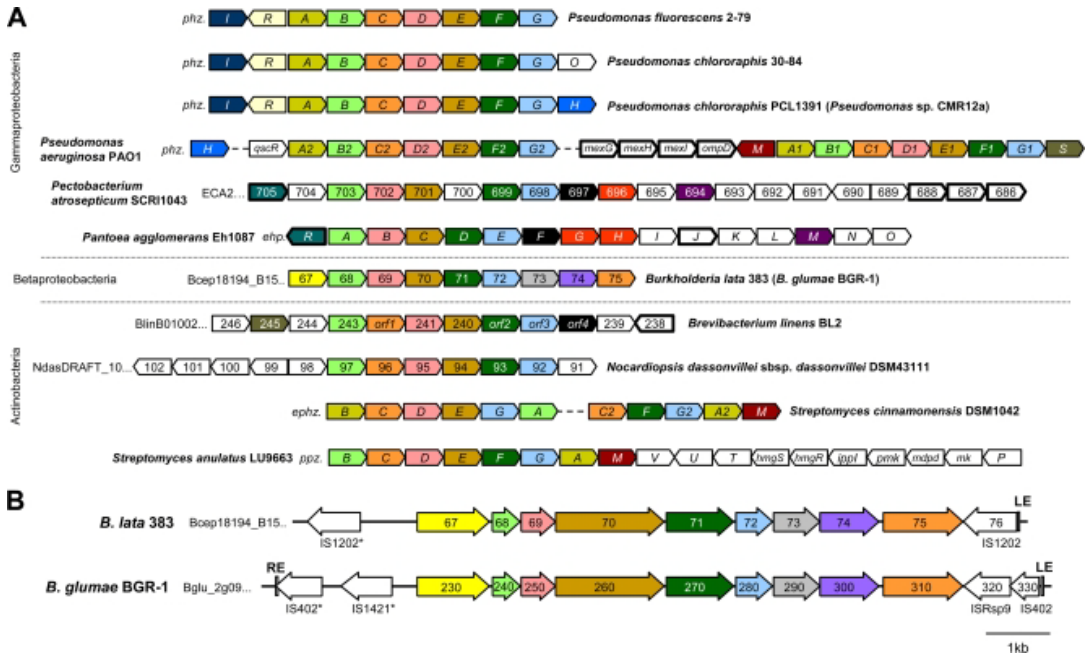


Figure 10. Organization of the phenazine biosynthetic operons in several bacterial species. A comparison of sequences of the phenazine operons across diverse bacteria. (A) The phenazine biosynthetic genes in the *Burkholderia* species. (B) In this species, they are surrounded by several mobile transposon elements. Source: Mavrodi *et al*., *App. Env. Micro*., 2010

1.3.2. Some thoughts on genetic redundancy

Evolutionarily, the perpetuation of functionally redundant genes within a genome may at first seem paradoxical. In theory, these genes should be selected against over time since at first glance they provide no obvious beneficial advantage for the organism. However, investigations into redundant genes in various organisms have found that there is a synergy between redundant genes that may be beneficial to the organism [117]. Genetic diversity through gene duplication leads to organism-specific phenotypes and adaptive characteristics. The existence of multiple gene copies in eukaryotes has been known for a long time and is considered an important

element in their molecular evolution[118,119]. However, bacteria were considered to be “simple” and were thought to carry very little, if any, redundant information in their genomes. It was surprising when the genome of *Escherichia coli* K12 showed that nearly 30% of the coding sequences could be grouped into gene families that were similar enough to be assigned similar functions[120]. They were described as 'paralog' gene families, and it was thought that their similarity reflected similar evolutionary descent, but actual or potential functional divergence. Since then, the presence of gene families typically containing between two to thirty copies has been described for nearly every prokaryotic genome sequenced. The number of paralogous genes and families appears to correlate with an increase in genome size[118,121].

Many redundant gene products are found in crucial cellular processes such as signal transduction, development and metabolism[122]. Examples of genetic redundancy include the myogenic development regulators of mammals[123], cell surface receptors in *Caenorhabditis elegans*[124] and Ser/Thr kinases in *Saccharomyces cerevisiae*[125]. The function of genetic redundancy within signaling networks has been studied thoroughly. In *S. cerevisae,* inspection of the 239 redundant genes reveals that 29% of these are found in signaling networks[125-127]. The redundant genes within signaling networks are also found to be differentially regulated compared to redundant genes in other contexts[125-128]. Differential regulation of redundant genes may facilitate signal transduction and modulate gene expression through collaboration between genes. It is this collaboration between redundant genes that may help propagate specific responses to numerous and diverse environmental stimuli.

1.3.3. Quinolone-dependent regulation of phenazine production

Quinolones, specifically PQS, have been shown to be necessary for the production of phenazines[23,129] (Figure 11). This is thought to require only one of the redundant phenazine operons, *phz1*. Expression of the *phz1* operon has been shown to be dependent upon the quinolone signaling network, as deletions of genes encoding quinolone biosynthetic enzymes (*pqsA*) or the PQS receptor (*pqsR*) correlate with reduced expression of the *phz1* operon[130,131]. However, a PqsR binding motif has not been identified upstream of *phz1*.

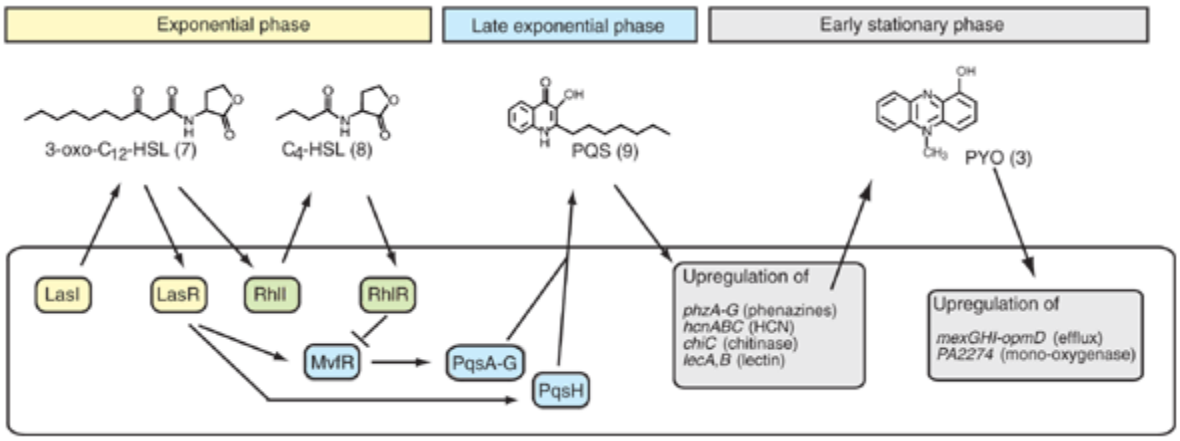


Figure 11. Model of *P. aeruginosa's* quorum sensing network. PQS is produced in late exponential phase and regulates the production of phenazines. Source: Price-Whelan *et al., Nat. Chem. Bio,* 2006

Interestingly, in *P. aeruginosa* strain PA14, deletion of the *pqsA* gene does not completely abolish PYO production[78,132]. Furthermore, deletion of *pqsH*, which is specifically required for the production of the quinolone PQS, only reduces PYO production by ~20%[133]. This is in

contrast to investigations in strain PAO1 that found that a lack of PQS production reduces PYO levels by ~90%[130]. The reason for these strain-specific differences remains to be elucidated.

1.3.4. Functions of phenazines

1.3.4.1. Phenazines as signaling molecules

Recent work has increased our understanding of the cell-cell signaling cascades present in *P. aeruginosa*. Work from the Newman lab has established that phenazines extend the QS signaling network in *P. aeruginosa*[134] and regulate a specific set of genes. Subsequent studies showed that phenazines modulate the maturation of colony biofilms[135]. Specifically, PYO and PCA altered colony formation and structure with different potencies[136], demonstrating that individual phenazines contribute differently to biofilm development. These initial investigations into the role of phenazines as signaling molecules laid the groundwork for further research into this exciting topic.

An intriguing aspect of phenazine signaling is that it can activate the redox sensor SoxR, a transcriptional activator that contains an iron sulfur cluster in its sensory domain. Redox active agents, such as phenazines, are molecules that are easily reduced and re-oxidized under physiological conditions. These molecules are secreted by bacteria, fungi and plants and can impair cell function by the generation of reactive oxygen species[7,137]. SoxR, along with another transcriptional activator, SoxS, is part of the oxidative stress response in enterobacteria, such as *E. coli* and *Salmonella enterica*[138,139]. Together, these transcription factors regulate the expression of over one hundred genes involved in the suppression of oxidative stress. However,

recent work indicates that the SoxR response pathway has different functions in non-enteric bacteria[140].

It had long been assumed that superoxide stress was the sole activator of SoxR, and that the deleterious effect of redox-cycling agents was mediated through the creation of these toxic species. However, Gu and Imlay found that the SoxR response could be activated by redox active compounds and did not necessarily require superoxide[141]. Using the natural redox-active antibiotic paraquat, they showed that SoxR could be activated under anaerobobic conditions, suggesting that SoxR is sensing redox-cycling agents directly instead of superoxide. In support of this hypothesis, studies on *Streptomyces coelicolor* and *P. aeruginosa* have found that the endogenous redox-active compounds actinorhodin and phenazines elicit a SoxR-mediated response[135,142]. Actinorhodin is a polyketide endogenously produced by *S. coelicolor* that induces the expression of several SoxR target genes[143]. Similarly, phenazines produced by *P. aeruginosa* activate several genes under the control of SoxR[135]. Interestingly, none of these genes are involved in mediating a general stress response to superoxides. Rather, the gene products may function in the export or modification of actinorhodin or phenazines in *S. coelicolor* or *P. aeruginosa*, respectively (Figure 12).

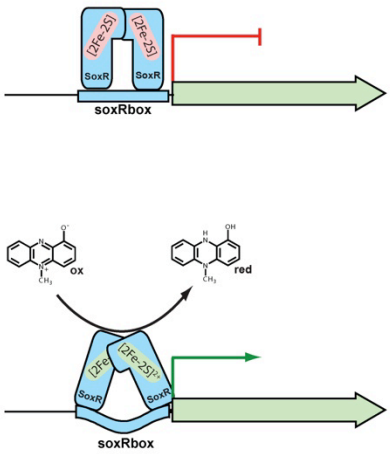


Figure 12. Activation of SoxR-dependent gene expression by pyocyanin (PYO). The transcription factor SoxR forms a homodimer that binds to the "SoxRbox" of target genes. In its reduced form, SoxR prevents transcription. PYO then oxidizes SoxR through a one electron transfer. This causes the homodimer to undergo a conformational change that allows DNA polymerase to bind the DNA, resulting in transcription of the target genes.

A closer look at the SoxR regulon in non-enteric bacteria, such as *P. aeruginosa* and *S. coelicolor*, reveals that it is very different than the SoxR regulon of enteric bacteria, such as *E. coli*. The SoxR regulon from enteric bacteria is composed of only the transcription factor SoxS, which regulates the expression of more than 100 genes gene, many of which are involved in a general stress response to superoxide (*e.g.* superoxide dismutase)[144,145]. The SoxR regulon in non-enterics differs in both number and types of genes affected. In *P. aeruginosa*, SoxR controls the expression of a Resistance-Nodulation-cell Division (RND) family efflux pump MexGHI-OpmD, a Major Facilitator Family (MFS) transporter, and a putative monooxygenase[135]. In *S. coelicolor*, SoxR is responsible for genes encoding putative reductases, a monooxygenase and an ABC transporter[143]. The differing regulons of *P. aeruginosa* and *S. coelicolor* indicate that the SoxR response may be specific to endogenously produced redox-active signals. In support of this, the growth of SoxR mutants in both of these non-enteric bacteria is unaffected by

endogenous or exogenous redox-active compounds suggesting that SoxR is not part of the detoxification response in these organisms.

1.3.4.2. The role of phenazines in iron reduction

Iron is an essential element that is required for crucial metabolic processes such as respiration (ferredoxins, cytochromes) and key enzymatic reactions (fumirase and aconitase of the TCA cycle)[146]. However, under aerobic conditions iron is not readily usable, as it is commonly found in the poorly soluble form Fe^{3+}[147,148]. As such, host defense systems employ a series of mechanisms to limit iron availability for the invading pathogens. These mechanisms include proteins that use iron such as hemoglobins, cytochromes and ferritins or chelators of extracellular iron such as glycoproteins, transferrins and lactoferrins[149,150]. The phenazine PYO may assist *P. aeruginosa* in the acquisition of iron by reducing it and freeing it from transferrin, a protein that normally sequesters iron so that it is available only to the human host[151,152]. Another pseudomonad strain, *P. chlororaphis,* has been shown to reduce iron oxides through electron transfer to the phenazine PCN, and it is thought that this ability may be important in the rhizosphere, where iron is also present predominantly in an insoluble, oxidized form[153]. In addition to phenazines, *P. aeruginosa* also uses strong extracellular iron chelators, termed siderophores, for iron uptake[154]. Interestingly, transcriptomic studies of *P. aeruginosa* have found that the biosynthetic genes that control phenazine and siderophore production are upregulated during infection[111].

1.3.4.3. The role of phenazines in redox homeostasis

Phenazines also act as substrates in intracellular redox transformations. The redox transformations of phenazines can be observed as a color change in cultures that have become limited for terminal electron acceptors. The phenazine PYO is blue in its oxidized state, but colorless upon reduction. A shaking culture is blue because oxygen is continuously introduced into the medium. If the culture is limited for oxygen the cells will rapidly reduce all phenazines and the culture will lose its blue color[155]. 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) to reduce extracellular iron oxides [153].

The redox potentials of phenazines are such that they can be easily reduced by the bacterial cell and react extracellularly with higher potential oxidants such as ferric iron and oxygen, acting as electron shuttles between the bacterium and an external substrate[156,157]. In homogeneous liquid cultures of *P. aeruginosa*, phenazines affect gene expression and oxidize the intracellular redox state[158-160]. Under conditions where no other oxidant is available, phenazine-dependent electron transfer between cells and an oxidizing electrode supports survival[9,161]. Phenazines also help maintain redox homeostasis by acting as electron acceptors for the re-oxidation of accumulating NADH. Maintaining a proper redox balance in the pyridine nucleotide pool is essential for metabolism[162]. This suggests that an important role for phenazines could be to serve as intracellular redox buffers.

The building of cellular communities such as biofilms leads to the creation of gradients due to limited diffusion and consumption of substrates by individual cells within a community. Cells within biofilms use different strategies to ensure substrate acquisition and survival, depending on

the specific microenvironment they inhabit. Mechanisms that aid in redox homeostasis at the cellular level have been characterized in diverse organisms. In mammals, redox-balancing mechanisms are involved in the development of lung and blood vessel systems, which prevent oxygen starvation of the developing embryo [163]. In such large, multicellular species, cells must cope with limited oxygen availability that leads to the formation of zones with varying concentrations of oxygen. During processes such as tumor angiogenesis, relative oxygen concentrations act as cues that determine adaptive morphological features, facilitating oxygen delivery to cells within the macroscopic structure[94].

In summary, *Pseudomonas aeruginosa* is a versatile bacterium that can inhabit diverse environments such as water, air, soil and host organisms. Phenazine production and formation of multi-cellular communities are two important aspects of its physiology that help this bacterium adapt to different environments. How phenazines modulate biofilm development is poorly understood..The second chapter will address how phenazine production is affected in biofilms. More specifically, we addressed the role of the second phenazine operon in phenazine production in the biofilm environment. In the third chapter, we investigated the roles of individual phenazines on colony development. It has been established that phenazines are necessary for colony development but exactly which phenazines are involved in this process has yet to be elucidated. Finally, in chapter four we investigated the activation of the transcription factor SoxR by phenazines. Specifically, we addressed the ability of SoxR to respond to specific redox potentials. The work described below is aimed at furthering our understanding of the intimate link between phenazines and biofilm development.

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Chapter 2. The redundant phenazine biosynthetic operons in *Pseudomonas aeruginosa* exhibit environment-dependent expression and differential roles in pathogenicity

This chapter is adapted from a manuscript that has been submitted for publication (Recinos, et al., 2012).

2.1. Introduction

Gene duplications give rise to genetic redundancy, an unstable condition that would not be expected to persist over evolutionary time. Despite this, genomes from diverse organisms maintain redundant genes[1,2]. Redundancy may be favored for a variety of reasons. For example, differential expression of redundant genes may allow an organism to thrive under varying environmental conditions[3,4]. Redundant genes are found in diverse organisms, and their products are involved in crucial cellular processes such as signal transduction, development and metabolism[5]. Examples of systems utilizing genetic redundancy include regulators of myogenic development in mammals[6], cell surface receptors in *Caenorhabditis elegans* [7] and Ser/Thr kinases in *Saccharomyces cerevisiae*[8].

An excellent example of maintained genetic redundancy is the versatile bacterium *Pseudomonas aeruginosa*, an opportunistic pathogen that can thrive in both soil and host environments. The *P. aeruginosa* genome contains a set of redundant seven-gene operons, each encoding the biosynthetic enzymes for phenazine-1-carboxylic acid (PCA)[9]. Additional genes encode

decorating enzymes that derivatize this precursor, generating other phenazines (Fig. 13A). The pseudomonad phenazines are toxic to many other organisms and cell types due to their inherent redox activity[10,11]. Studies conducted in various plant and animal models of infection have implicated phenazines in colonization and pathogenicity[12-14]. For example, the most familiar biological phenazine, pyocyanin (PYO), has been shown to be necessary for pseudomonad infection of the lungs of mice[15]. Some of the deleterious effects of pseudomonad phenazines leading to toxicity in host cells include the generation of reactive oxygen species, inhibition of ciliary beating, and reduction of the macrophage response [11,16,17].

In addition to the effects that secreted phenazines can exert on hosts, recent studies have elucidated roles for phenazines in *P. aeruginosa* physiology. Phenazines can act as intercellular signals, altering gene expression within a population of pseudomonads[18]. Additionally, *P. aeruginosa* is not negatively affected by the redox toxicity of phenazines[19]; rather, phenazines balance the intracellular redox state and may support survival when other electron acceptors are not available[20,21]. The signaling and redox balancing effects of phenazines are thought to contribute to their dramatic influences on *P. aeruginosa* colony biofilm development, where phenazine production induces a drastic morphotypic switch between wrinkled (rugose) and smooth phenotypes[22].

The various advantages conferred by phenazines may support conservation of the *phz* biosynthetic operon in the more than 57 phenazine-producing species currently identified[23]. *phz* operons have been discovered in Gram-positive Actinobacteria and Gram-negative Beta- and Gamma-proteobacteria and are often found next to phenazine-modifying enzymes and regulatory

genes[23]. However, although the *phz* operon displays a broad phylogenetic distribution, bacterial genomes containing more than one *phz* operon are rare: of the bacterial genomes sequenced to date, only those belonging to *P. aeruginosa*, the closely related *Pseudomonas* sp M18, and *Streptomyces cinnamonensis* contain a second, redundant *phz* operon[23]. In *P. aeruginosa*, the regions surrounding each of the *phz* operons are highly divergent. The *phzA1-G1* operon (*phz1*) is flanked by *phzM*, encoding a methyltransferase and *phzS*, encoding a monooxygenase, which convert PCA to the well-studied phenazine pyocyanin (PYO) (Fig. 13A). The *phzA2-G2* operon (*phz2*) is found approximately 2 MB away from the *phz1* operon and is not flanked by phenazine-modifying enzymes. A third phenazine-modifying enzyme, PhzH, is encoded at a distinct site in the genome and is responsible for the conversion of PCA to phenazine-1-carboxamide (PCN). Additional phenazine derivatives that have been detected in *P. aeruginosa* cultures are either (1) intermediates and by-products that arise from PhzM and PhzS activity and/or (2) produced by enzymes for which the coding genes are not known[24-26].

The *phz1* and *phz2* operons are nearly identical (~98% similarity at the DNA level), yet they are preceded by distinct promoter regions[9] . Although this suggests that different environmental cues may be required to activate each operon, the differential regulation of the *phz* operons has not been thoroughly explored. Studies examining *phz* gene expression and the relative contributions of the *phz* operons to phenazine production have typically conflated *phz1* and *phz2* or measured PYO as the representative product, discounting other phenazines. Despite the difficulties associated with dissecting the regulation of the two *phz* operons, some advances have been made on this front. Several lines of evidence suggest that *phz1* is regulated by quorum sensing (QS). The *phz1* promoter contains a *lux* box 390 bp upstream of the *phzA1* translational start site[27]. *lux*

box motifs are found upstream of many genes regulated by QS [28] and recruit the LuxR family transcriptional regulators LasR and/or RhlR. The upstream *lux* box motif, LasR, and RhlR have been shown to be necessary for the full induction of *phzA1*[28], indicating that expression of the *phz1* operon is strongly dependent on quorum sensing cues. It has also been shown that the *phz1* operon is regulated by quinolones. Although it has been reported that binding of the *Pseudomonas* quinolone signal (PQS) to its receptor protein PqsR (MvfR) is required for wild-type PYO production[29,30], a PqsR binding motif has not been identified in the promoter region of *phz1*[29]. On the other hand, simultaneous transcription of *phz1* and *phz2* has been observed[9]. Furthermore, the PQS-dependence of phenazine production has been observed in several RNA array studies and is often attributed to the QS control of *phz1*, but it is unlikely that microarray probes can distinguish between *phz1* and *phz2* due to their high similarity. The quinolone dependence of *phz2* expression has therefore remained an open question.

To investigate the quinolone-dependent regulation of the *phz* operons, we generated a panel of mutants lacking genes involved in the biosynthesis of phenazines and quinolones and evaluated their effects on phenazine production, colony biofilm morphogenesis and pathogenicity. Promoter-YFP fusions were used to examine the relative expression levels of *phz1* and *phz2* during growth in liquid batch cultures and during colony development. The relative contributions of *phz1* and *phz2* to *P. aeruginosa* pathogenicity were tested using a murine model of lung colonization. Our results have identified a previously underestimated role for *phz2* under planktonic growth conditions, and discovered a novel, near-exclusive dependence on *phz2*-derived phenazines during *P. aeruginosa* biofilm development and host infection.

2.2. Results

2.2.1. Both *phz* operons contribute to phenazine production in planktonic cultures

P. aeruginosa planktonic cultures begin to produce phenazines in early stationary phase. To investigate the relative contributions of the two *phz* operons to phenazine production in planktonic cultures, we deleted each individual operon in *P. aeruginosa* PA14. Using high-performance liquid chromatography (HPLC) analysis of culture supernatants, we compared the production of phenazines from the *phz1* deletion mutant (Δ*phz1*), the *phz2* deletion mutant (Δ*phz2*) and a mutant in which both phenazine operons had been deleted (Δ*phz*)[18] . We were able to quantify the levels of PCA and PYO; however the levels of PCN and other phenazines were below our detection limit. The PCA levels produced by Δ*phz1* and Δ*phz2* were significantly lower than that produced by the wild type (Fig. 13B, bottom). Δ*phz1* produced more PCA than Δ*phz2*, but the sum of PCA produced in the individual mutants (22 µM and 6 µM, respectively) did not reach wild type levels (95 µM). Downstream phenazine modifications may have prevented us from accurately quantifying the total PCA produced by each operon (Fig. 13A). The Δ*phz1* mutant produced PYO at a level near that of the wild type, while the Δ*phz2* mutant was severely defective in PYO production (Fig. 13B, top). The PYO production phenotypes of the individual mutants are intriguing in light of their chromosomal environments: *phz1*, which does not contribute significantly to PYO production, is the operon that is flanked by the genes for the PYO-producing enzymes PhzM and PhzS.

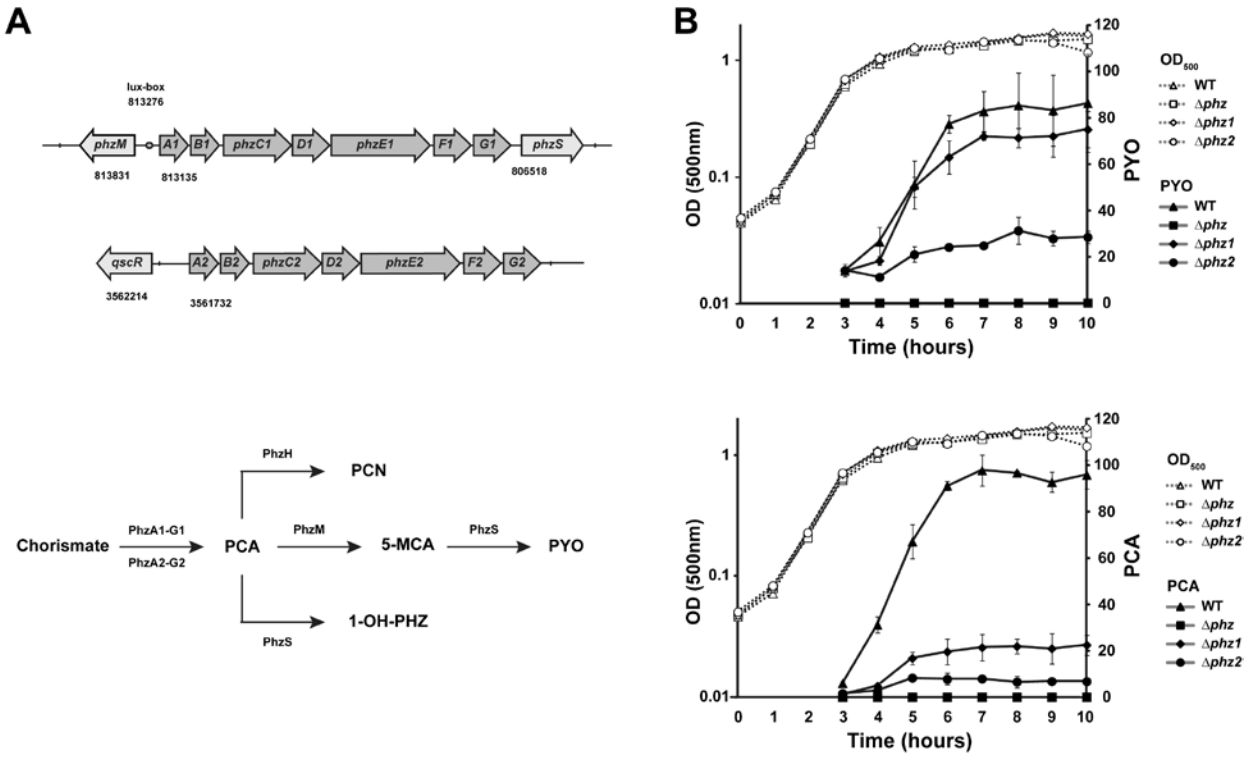

Figure 13A-B. PA14 expresses a redundant set of phenazine biosynthetic operons that contribute to phenazine production in planktonic culture. (A) Diagram showing differing flanking regions and regulatory elements of *phz1* and *phz2*. The operons are composed of 7 genes that catalyze the synthesis of phenazine-1-carboxylic acid (PCA) which is the precursor to pyocyanin (PYO), phenazine-1-carboxamide (PCN) and 1-hydroxyphenazine (1-OH-PHZ). (B) Growth curve of WT, Δ*phz*, Δ*phz1* and Δ*phz2* strains with PYO (top panel) and PCA (bottom panel) quantification. Error bars indicate standard deviation of three independent experiments.

To address the possibility that the *phz* operons are expressed at different levels, we created fluorescent reporter constructs containing the 500 bp promoter regions upstream of each operon fused to the gene encoding Venus, a YFP derivative. These reporters, P*phzA1*YFP (A1YFP) and P*phzA2*YFP (A2YFP) were integrated into the chromosome at a neutral site in the wild type and the Δ*phz* mutant. We observed that the expression of P*phzA1*YFP was higher than that of P*phzA2*YFP in the wild type and Δ*phz* backgrounds (Fig. 13C, top). This concurs with

previously published work reporting that *phz1* is expressed at higher levels than *phz2* in planktonic cultures[31-33]. It is noteworthy that expression of *phz1* and *phz2* were lower in the Δ*phz* mutant background than in the wild type (Fig.13C, bottom).

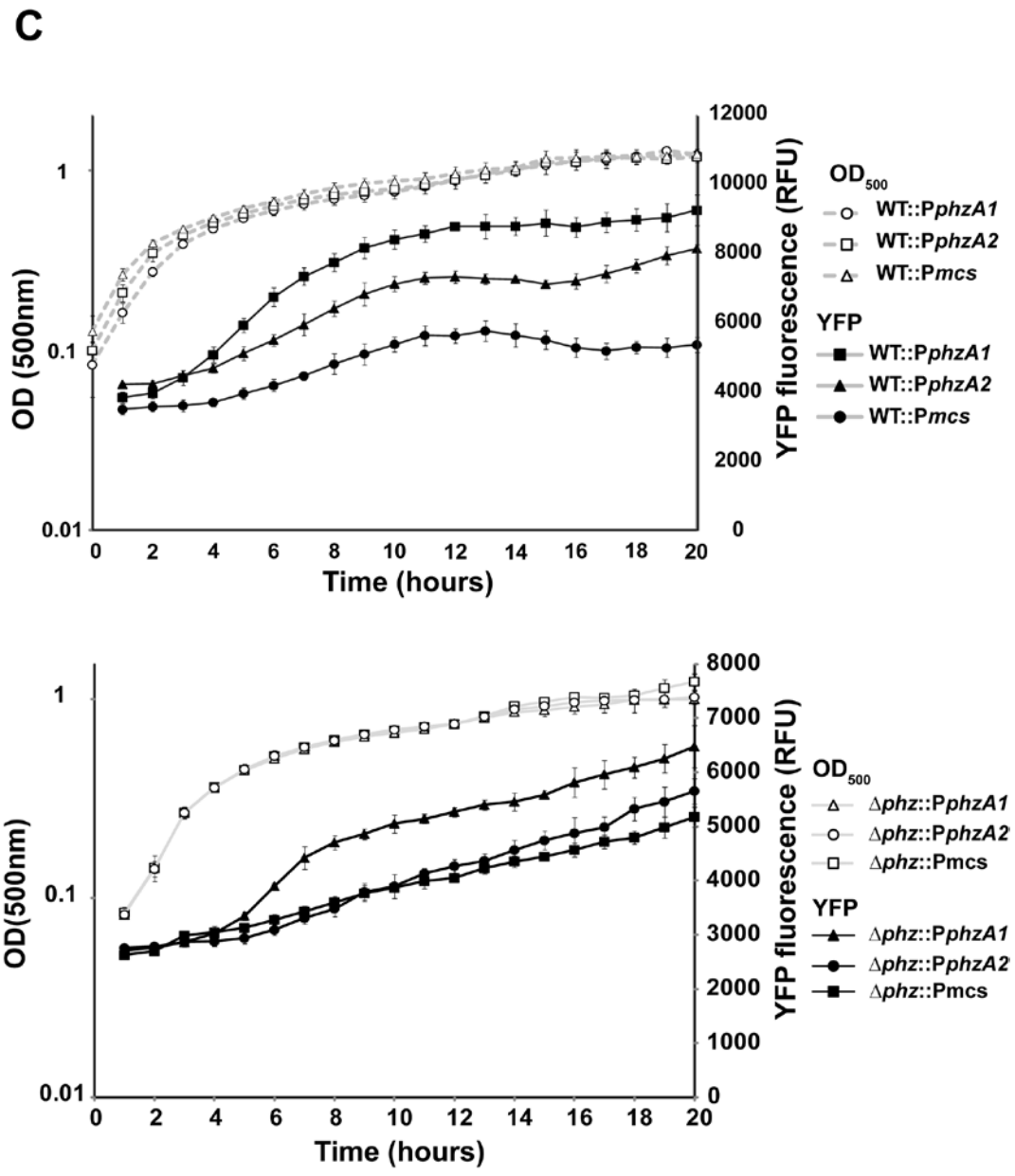

Figure 13C. The *phz1* operon is expressed at higher levels than *phz2* in planktonic culture. (C) Expression levels of YFP reporter constructs for *phz1* and *phz2* operons in WT (top panel) and Δ*phz* (bottom panel) backgrounds. Error bars indicate standard deviation of one experiment performed in biological triplicates.Experiment was repeated four times with similar results.

Overall, these data demonstrate that both *phz1* and *phz2* make significant contributions to phenazine production during growth in planktonic cultures. However, the observed difference in PCA production between the Δ*phz1* and Δ*phz2* mutants was not consistent with the expression patterns of the *phz1* and *phz2* operons: *phz1* was the higher-expressed operon, but Δ*phz2* showed more severe defects in PCA and PYO production. However, PCA measurements for the Δ*phz1* and Δ*phz2* strains may not accurately represent total PCA production due to the various phenazine-modifying enzymes (PhzH, PhzM and PhzS) that use PCA as a precursor (Fig. 13A).

2.2.2. The *phz2* operon is responsible for producing the majority of the PCA pool in planktonic cultures

We made a triple deletion strain (Δ*phzHMS*) that lacks the *phzH*, *phzM* and *phzS* genes and in this background deleted either *phz* operon in order to assess their contributions to the total PCA pool. The Δ*phzHMS* mutant produced more PCA than the wild type (Fig. 14B); this represents the total PCA production from both operons. That this amount was higher than the combined total PCA produced by the *phz1* and *phz2* mutants confirms that conversion of PCA to other phenazines prevented us from accurately quantifying the total PCA pool. Deleting *phz1* in this background (Δ*phzHMS*Δ*phz1*) decreased PCA production slightly, while deleting *phz2* drastically reduced PCA production (Δ*phzHMS*Δ*phz2*), indicating that *phz2* is responsible for the majority of the PCA production in planktonic cultures.

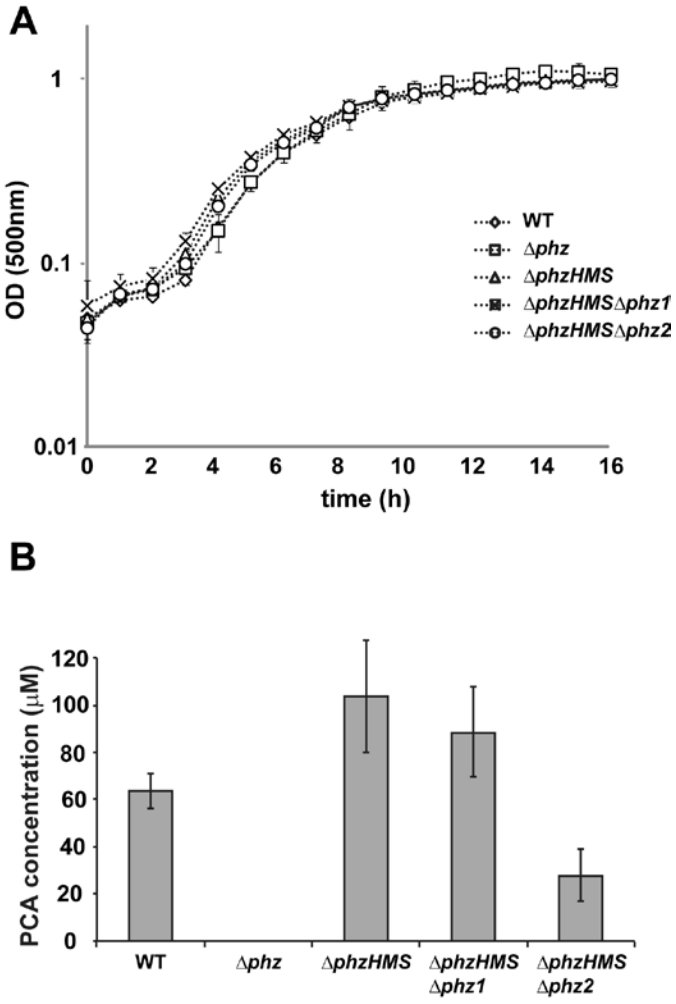

Figure 14. The *phz2* operon contributes the majority of the PCA pool in planktonic culture. (A) Growth curves and (B) PCA quantification of Δ*phzHMS*, Δ*phzHMS*Δ*phz1* and Δ*phzHMS*Δ*phz2* deletion strains. PCA quantification was performed after 16 hours of growth for B. Error bars indicate standard deviation of three independent experiments.

2.2.3. The *phz2* operon is sufficient for wild-type phenazine production in colony biofilms

Growth-dependent control of phenazine production has been characterized most extensively using planktonic cultures. However, phenazines have also been shown to affect the morphology of different types of biofilms[22,34]. We evaluated the importance of the individual *phz* operons in *P. aeruginosa* biofilm morphogenesis. Using a colony morphology assay, we compared the development of Δ*phz*, Δ*phz1* and Δ*phz2* to that of the wild type. As has been previously shown,

the phenazine-null mutant (Δ*phz*) exhibited a wrinkled morphology[18]. Strikingly, the presence of the *phz2* operon alone was sufficient for maintenance of the wild type (smooth) phenotype. In contrast, deleting the *phz2* operon led to a wrinkled morphology much like that of the Δ*phz* strain (Fig.15A). Biofilms formed by the Δ*phz* and the Δ*phz2* strains exhibited a two-fold increase in surface coverage compared to those formed by the wild type (Fig. 15B), while loss of the *phz1* operon had no effect on colony morphology. This suggests that *phz2*, but not *phz1*, is important for phenazine production in biofilms.

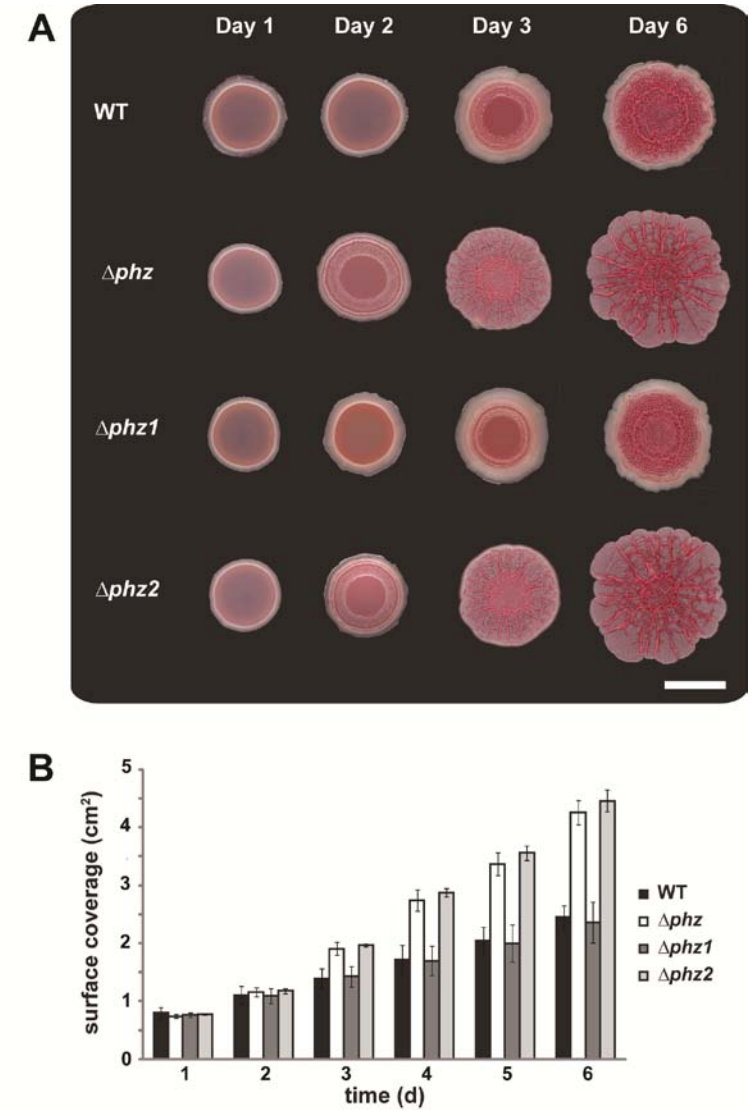

Figure 15. The *phz2* operon is necessary and sufficient for maintaining WT colony morphology. (A) Growth of WT, Δ*phz*, Δ*phz1* and Δ*phz2* colonies on agar plates and (B) surface area quantification over the course of 6 days. Error bars indicate standard deviation of three independent experiments.
Scale bar = 1cm.

To confirm that the mutant biofilm phenotypes were consistent with their phenazine production profiles, we extracted and quantified the phenazines from the agar on which the biofilms were grown. The Δ*phz1* strain produced ~60% of the amount of PCA produced by the wild type, but generated ~60% more PYO than the wild type (Fig. 16A). This shift toward PYO production is consistent with the increased PYO/PCA ratio we observed for the Δ*phz1* mutant grown planktonically. The combined total of PYO and PCA produced by the Δ*phz1* biofilm (201 µM)

was comparable to that produced by the wild type biofilm (225 μM) (Fig. S1). We were not able to detect any PCA or PYO peaks from the Δ*phz2* strain in our HPLC analysis (Fig.S2). Removal of *phzH*, *phzM*, and *phzS*, from the *phz1* and *phz2* backgrounds confirmed that all of the detectable phenazines produced by the wild-type colony could be produced by *phz2* alone (Fig. 16B). Complementation with the *phz2* operon restored phenazine production and rescued the wrinkled colony morphology (Fig. S3).

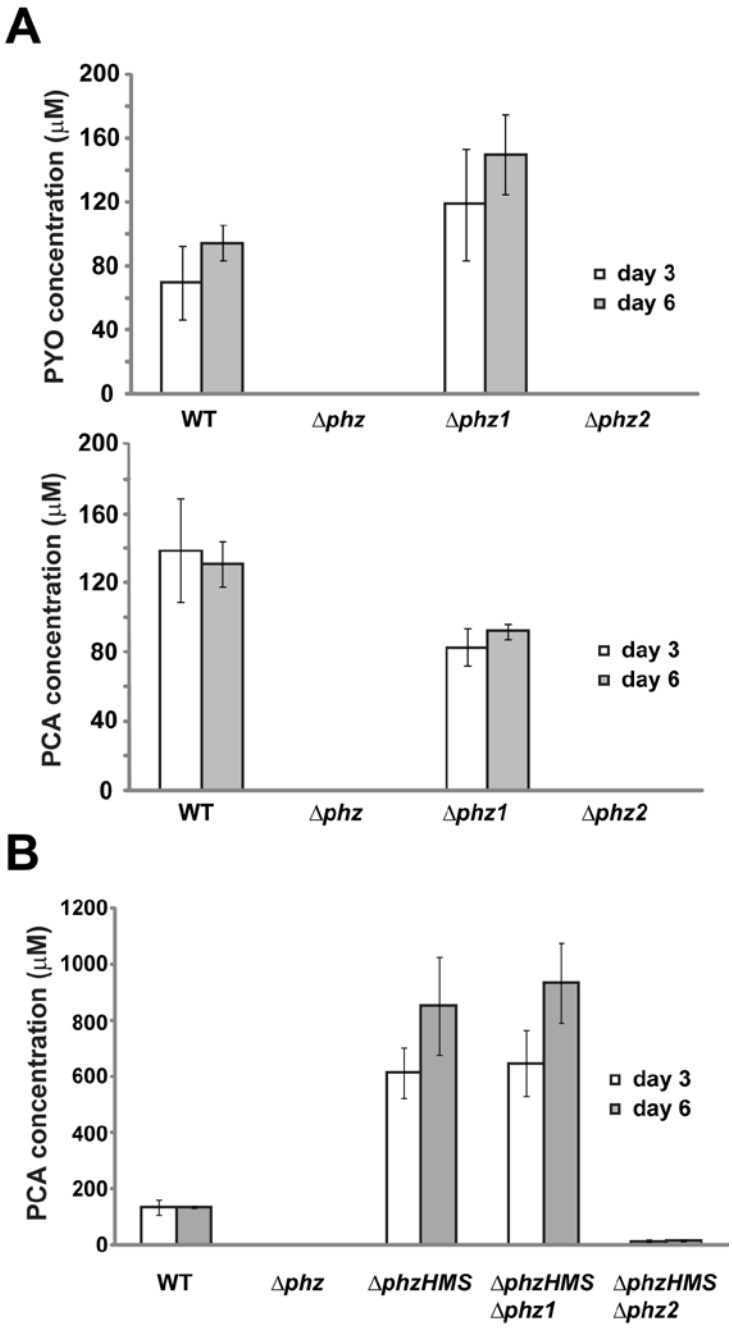

Figure 16. The total PCA pool in biofilms is produced by *phz2*. (A) Quantification of PYO and PCA produced by WT, Δ*phz*, Δ*phz1* and Δ*phz2* colonies grown on agar plates. (B) Quantification of PCA produced by Δphz*HMS*, Δphz*HMS*Δ*phz1* and Δphz*HMS*Δ*phz2* deletion strains grown on agar plates. Error bars indicate standard deviation of three independent experiments.

We next evaluated the expression levels of the *phz* operons in biofilms. We quantified fluorescence across the midsection of colonies grown from strains containing our A1YFP and A2YFP reporter constructs. Fluorescence levels for the A2YFP reporter were significantly higher

than background in a colony containing the YFP-encoding gene cloned without a promoter (Pmcs) (Fig.17). The fluorescence levels for the A1YFP colony were indistinguishable from background. The midsection fluorescence quantification gave rise to a “Batman”-shaped plot for all colony types. This is likely due to an increased cell concentration at the colony perimeter, which results from the coffee ring effect [35] when a cell suspension is first spotted onto an agar surface for the colony morphology assay.

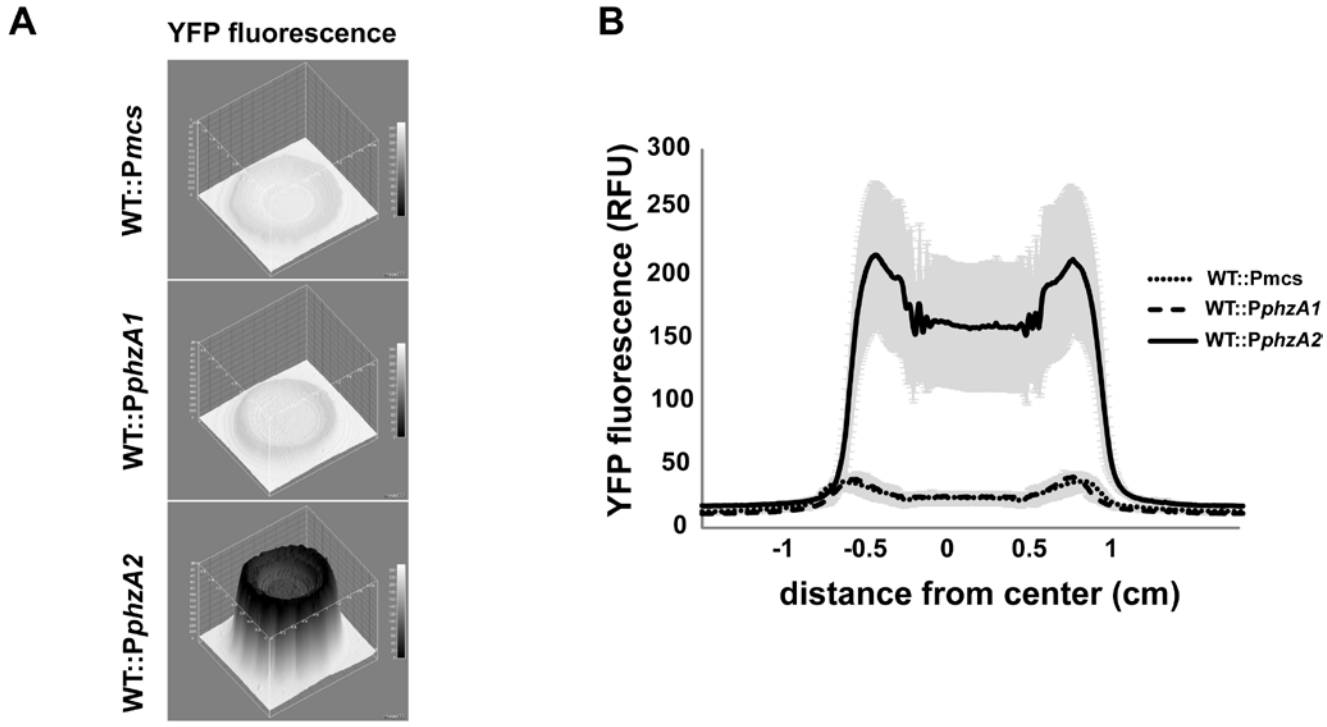

Figure 17. *phz2* is expressed at higher levels than *phz1* in biofilms. (A) 3D surface fluorescence intensity plot and (B) quantification of fluorescence of YFP reporter constructs for *phz1* and *phz2* operons. Fluorescence quantification was performed by using the surface plot analysis across the middle of the colony (Image J). Data represents one experiment performed in biological triplicates. Experiment was repeated three additional times with similar results. Shading indicates standard deviation.

The exclusive expression of *phz2* in biofilms contrasts with the observation made for liquid batch cultures, where *phz1* was expressed at higher levels than *phz2*. In biofilms, the production of PCA could be fully attributed to *phz2*. In planktonic cultures, most, but not all, of the PCA production was *phz2*-dependent. Although the *phz2* operon is consistently the primary contributor to phenazine production, additional regulatory differences between planktonic and biofilm cells likely tune the extent of the *phz1* contribution.

2.2.4. Quinolone-dependent regulation of the *phz2* operon

Although the *P. aeruginosa phz* operons are almost identical, their upstream promoter regions differ significantly. Previous studies have demonstrated that *phz1* expression (1) is upregulated by Las and Rhl [28] and (2) depends on the quinolone PQS and its receptor PqsR (MvfR)[36]. The mechanism whereby PQS controls *phz1* is unknown, as direct binding of PqsR to the *phz1* promoter has not been observed. Even less is known about the regulation of *phz2*, except for its apparent repression by QscR[33]. We sought to identify signals required for the activation of the *phz2* operon. We tested whether quinolones were required for induction of *phz2* in biofilms. PA14 produces three major types of alkyl quinolones: PQS, 2-heptyl-4(1H)-quinolone (HHQ) and 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) [37]. We generated a mutant lacking the genes *pqsABC* (Δ*pqsAC*), which is unable to produce any quinolones. We also created individual mutants with deletions in the genes encoding the monooxygenases PqsL and PqsH, which catalyze the formation of HQNO and PQS respectively[38,39]. The Δ*pqsAC* strain showed the most significant defect in PCA production while the Δ*pqsL* deletion seemed to have no effect (Fig. 18A). The double mutant Δ*pqsHL* showed PCA production similar to that of the Δ*pqsH* strain

suggesting that HHQ and not NQNO is responsible for PCA production. To investigate whether quinolones affect PCA production directly from the *phz2* operon we deleted the *pqsABC* genes in the Δ*HMS*Δ*phz1* and Δ*HMS*Δ*phz2* backgrounds and assayed for phenazine production. The Δ*pqsAC*Δ*HMS*Δ*phz1* mutant showed a drastic reduction in PCA production compared to the Δ*HMS*Δ*phz1* strain (Fig.18B), illustrating quinolone-dependent regulation of *phz2*. Removing quinolones also abolished PCA production from *phz1* (Δ*pqsAC*Δ*HMS2* strain). Finally, to evaluate whether PQS specifically is the quinolone responsible for *phz2* induction (as is the case for *phz1*), we generated the mutants Δ*pqsH*Δ*HMS*Δ*phz1* and Δ*pqsL*Δ*HMS*Δ*phz1*. PCA production in these mutants was identical to that of the Δ*HMS*Δ*phz1* mutant, suggesting that the PQS/HQNO precursor HHQ positively regulates phenazine production from the *phz2* operon (Fig.18C).

To further verify that quinolone signaling affects *phz2* expression, we inserted the P*phzA2*YFP reporter construct into the Δ*pqsAC,* Δ*pqsR* and Δ*pqsHL* mutants and compared fluorescence between the strains. As shown in figure 18D*, phz2* expression levels were significantly reduced in the Δ*pqsAC*::A2YFP and the Δ*pqsR*::A2YFP strains in biofilm environments. However, *phz2* expression levels in the Δ*pqsHL*::A2YFP strain were similar to that of the wild type (Fig.18E). These observations were recapitulated in the planktonic environment (Fig.S4). These results indicate that quinolones, specifically HHQ, can positively regulate transcription of the *phz2* operon.

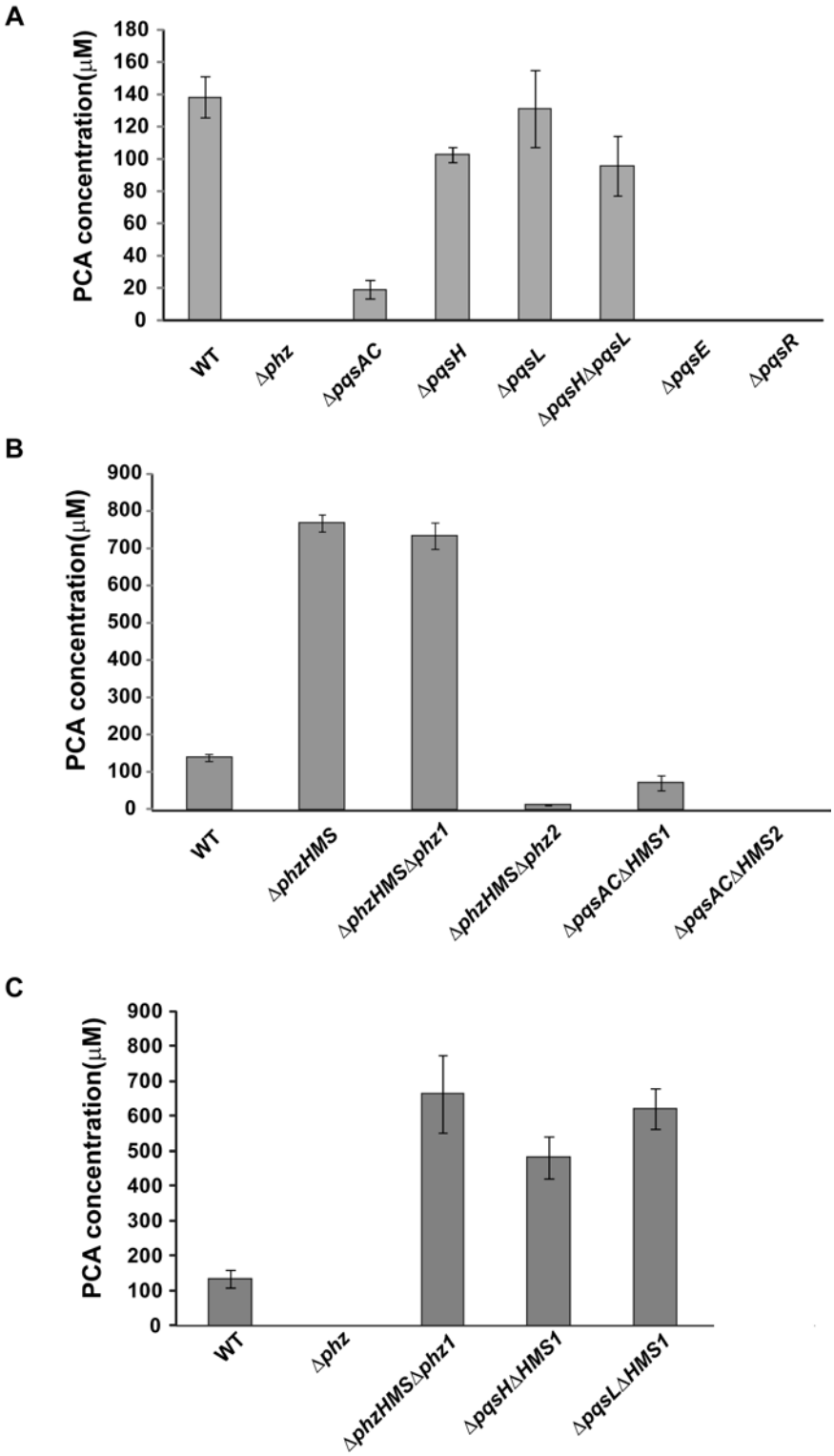

Figure 18A-C. Quinolones regulate the *phz2* operon in biofilms. Quantification of PCA from strains containing deletions in various biosynthetic genes involved in H-alkyl-quinolone (HAQ) production (A) in the Δ*phzHMS*Δ*phz1* and Δ*phzHMS*Δ*phz2* backgrounds (B). PCA production of Δ*phzHMS*Δ*phz1*, Δ*pqsH*Δ*HMS1* and Δ*pqsL*Δ*HMS1* strains (C). Error bars indicate standard deviation of three independent experiments

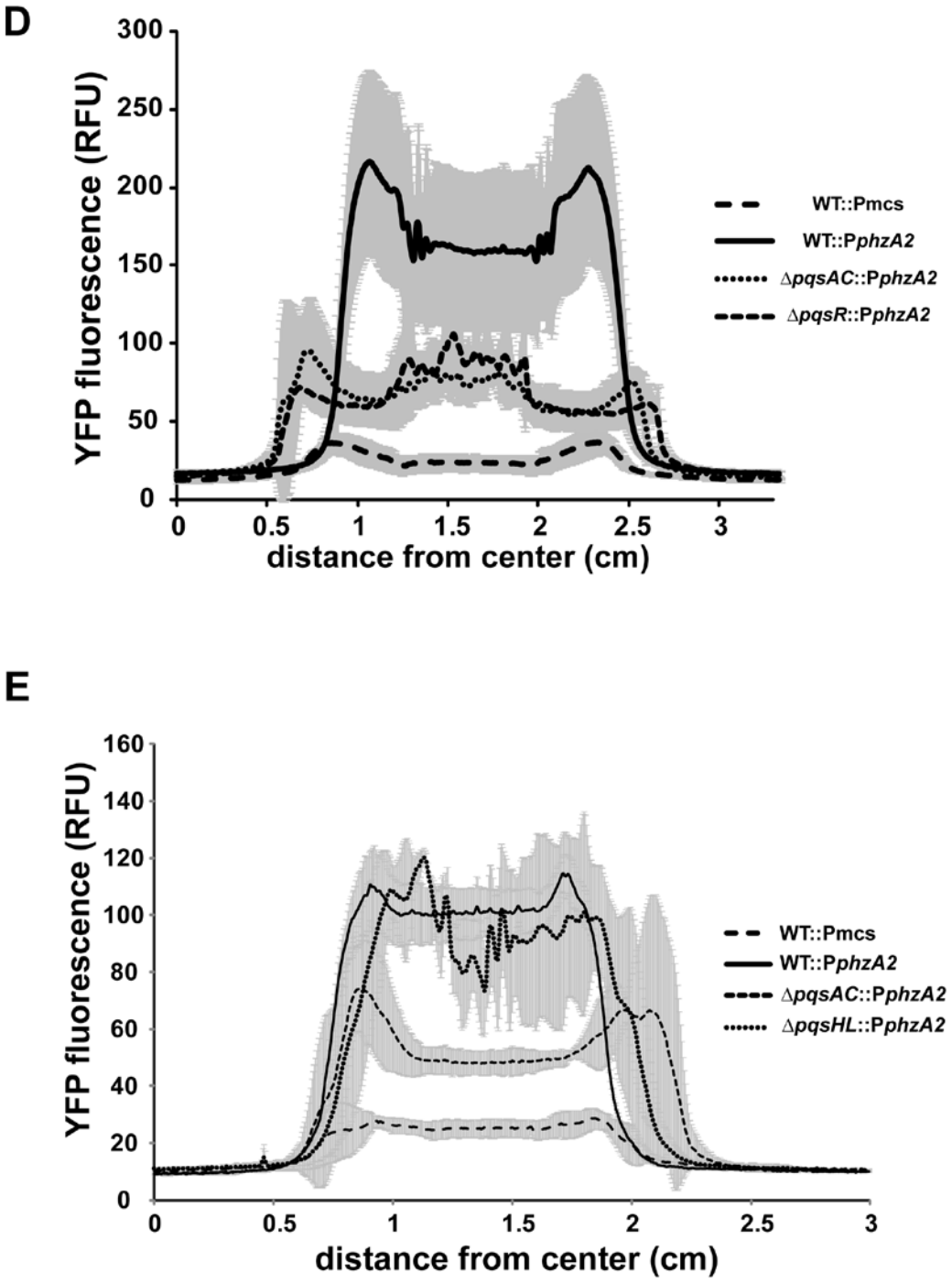

Figure 18D-E. HHQ positively regulates *phz2* expression in biofilms. Expression levels of the *phz2* operon in the Δ*pqsAC*, Δ*pqsR* (D) and in the Δ*pqsHL* strain (E). Representative experiment performed in biological triplicates is shown (for D and E). Experiment was repeated several times with similar results. Shading indicates standard deviation.

2.2.5. The *phz2* operon is required for lung colonization in a murine model of infection

Phenazine production contributes to virulence in diverse infection models (flies, worm, mice and lettuce leaves[14,15,40,41]). Characterizations of the bacterial populations associated with infections have suggested that *P. aeruginosa* assumes a biofilm-like lifestyle during host colonization. We observed that the *phz2* operon was required for phenazine production in biofilms; we therefore set out to test whether the *phz2* operon is required for infection in a mouse lung colonization model[15]. Mice were inoculated with 0.3-1 x 10^5 colony-forming units (cfu) of *P. aeruginosa* PA14 wild-type, Δ*phz1*, or Δ*phz2* and euthanized 18 h after infection. Cfu counts were performed by dilution and plating of whole lung homogenates. In this model, the Δ*phz1* strain had the capacity to colonize the mouse lung to the same extent as the wild type (Fig.19). In comparison, the Δ*phz2* and the Δ*phz* strains showed a significantly decreased rate of infection. The pathogenicity of the panel of mutants was also evaluated in the lettuce leaf assay (data not shown). These results demonstrate that the *phz2* operon is required for *P. aeruginosa* virulence in divergent host systems.

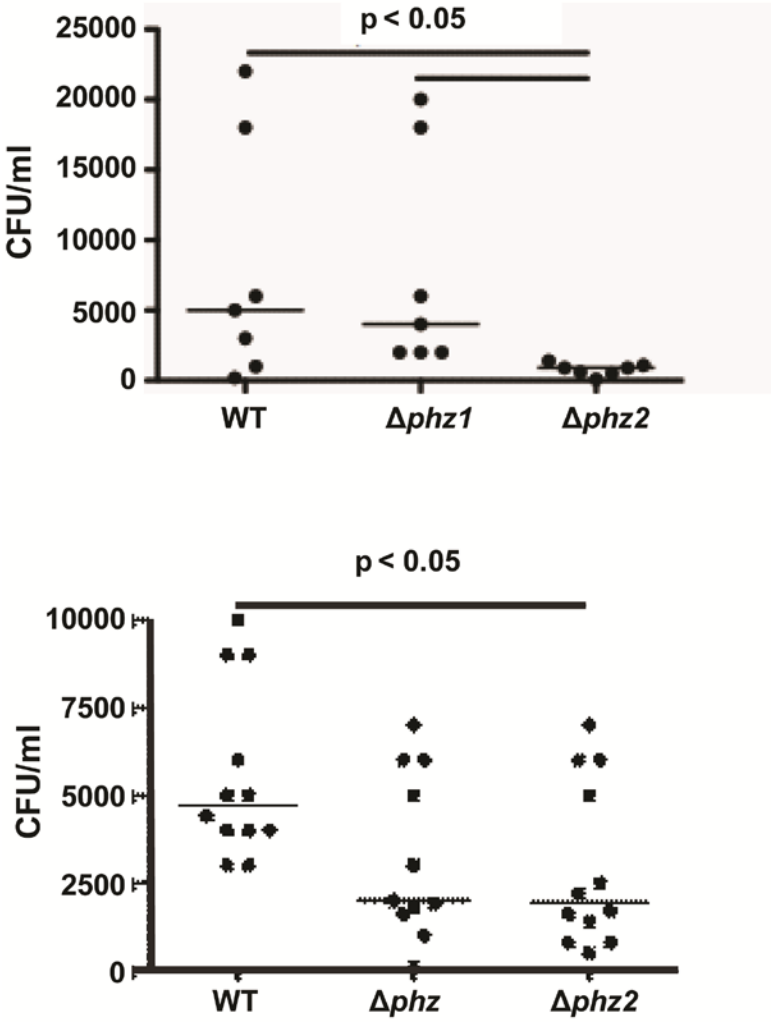

Figure 19. The *phz2* operon is necessary for lung colonization in the murine infection model. CFU counts of the WT, Δ*phz*, Δ*phz1* and Δ*phz2* strains from mouse lungs. Mice were inoculated with 0.3-1 x 10^5 colony-forming units (cfu) of *P. aeruginosa* PA14 wild-type, Δ*phz1*, or Δ*phz2* and euthanized 18 h after infection. Cfu counts were performed by dilution and plating of whole lung homogenates. Straight lines within the data points indicate average of CFU/ml. P-value between data is shown.

2.3. Discussion

Surveys of redundancy across bacterial phylogeny have revealed that gene duplication gives rise to organism-specific phenotypes and adaptive traits [42]. Many of these duplicated genes products have redundant functions. *P. aeruginosa* contains a redundant phenazine biosynthetic operon (*phz2*) whose regulation was previously underappreciated. We hypothesized that the

nonhomologous promoter regions of the redundant *P. aeruginosa phz1* and *phz2* operons allow for condition-dependent regulation of PCA biosynthesis in diverse environments. We generated mutants with deletions in each of these operons, and created fluorescent reporter constructs to monitor operon expression. We also deleted genes required for downstream conversion of PCA to the phenazines PYO and PCN. For planktonic cultures we found that *phz1* was expressed at higher levels than *phz2* (Fig. 13C), and that both contributed significantly to PCA production, with the *phz2* making a greater contribution (Fig. 14B). This result suggests that factors other than transcriptional regulation are controlling the amount of PCA produced in such cultures.

In contrast to planktonic cultures, *phz* expression in colony biofilms correlates well with PCA production. During colony development, *phz1* expression was undetectable, while fluorescence from the *phz2* yfp reporter was detectable throughout the structure (Fig. 17). Biofilm morphogenesis (a process dependent on phenazine production) and PCA synthesis of the *phz1* deletion mutant phenocopied the wild type. With respect to biofilm morphology and PCA production, the *phz2* mutant was indistinguishable from a mutant lacking both *phz* operons, suggesting that *phz2* is sufficient for the wild-type phenotype during multicellular growth and survival.

Our *phz* expression data for planktonically grown *P. aeruginosa* PA14 are consistent with published reports comparing relative expression of *phz1* and *phz2* in *P. aeruginosa* PAO1 and *Pseudomonas* sp. M18[43]. This difference in expression has been attributed to post-transcriptional regulation at the RNA level as part of a feedback loop through PCA production from *phz2* to *phz1*. The orphan repressor QscR has also been shown to negatively affect the transcription of

both phenazine operons through an unknown mechanism[33]. Recently, the small RNA chaperone protein Hfq was shown to positively affect *phz2* transcription by inactivating QscR[44].

It is well established that phenazine production is controlled by the quinolones. *P. aeruginosa* produces at least 50 quinolone derivatives[45]. The best studied quinolones are HHQ, HQNO and PQS. PqsA-D synthesizes HHQ, which can then be converted by the monooxygenases PqsH or PqsL to PQS or HHQ, respectively[46-48]. Previous reports demonstrated that *phz1* induction is dependent on PQS though its transcriptional regulator PqsR[30]. Both PQS and its precursor molecule HHQ activate PqsR although HHQ does this with lower efficiency[49,50]. The connection between PQS and *phz1* is in agreement with our findings that Δ*pqsH* and the Δ*phz1* mutants produce similar amounts of PCA (compare figure 16B to figure 18A). We now show that *phz2* expression is also controlled by quinolones and does not rely on PQS. The production of HHQ is sufficient to allow full expression of *phz2* and that this regulation is PqsR- dependent (Fig. 18). HHQ has been previously shown to act as a signaling molecule responsible for cell-cell communication[50]. It is worth noting that neither the *phz1* nor *phz2* promoters contain an identifiable PqsR binding motif, suggesting that additional regulators may be required for *phz* expression. Interestingly, we also detect some quinolone independent regulation of the *phz2* operon as removing the quinolone biosynthetic genes does not completely abolish PCA production (Fig. 18B).

An important distinction between the quinolone signals HHQ and PQS is that biosynthesis of the latter requires oxygen as it is catalyzed by the oxygen-dependent monooxygenase PqsH[51]. An intriguing idea therefore is that HHQ/phz2 ensures PCA production under anaerobic conditions

because it is oxygen-independent. In contrast PQS/phz1 may be exclusively tuned to aerobic environments. This is further supported by the observation that another oxygen-dependent monooxygenase gene *phzS* is found adjacent to *phz1* and transcription of both is thought to be controlled by the same promoter (unpublished observation). It is somewhat peculiar that we were not able to *phz1* expression in the colony biofilm environment, which is characterized by steep oxygen gradients and therefore should allow the expression of both operons.

From an evolutionary perspective, the maintenance of redundant genes is paradoxical. One might expect redundancy to disappear over time as redundant genes alone do not provide and functional selective advantage[52]. However, subtle functional specialization and differential regulation are features that can render duplicate genes and their products beneficial [53]. The *phz1* and *phz2* operons exhibit environment-specific expression and individual characteristics that account for distinguishing phenotypes. The increased expression and phenazine production from the *phz2* operon in biofilms may allow the bacteria to adapt to its environment as the *phz2* operon is sufficient and necessary for colonization of mouse lungs.

James Thomas and others have put forth several mechanisms for the maintenance of redundant genes[3,54]. Specifically, the divergent function mechanism states that two genes that have overlapping activities are selected for properties that are unique to each. Our findings suggest that the mechanism of maintenance for the redundant phenazine operons of *P. aeruginosa* agrees with this hypothesis. Both the *phz1* and *phz2* operons have the shared function of producing PCA but also have distinct regulatory mechanisms in different environments. It is tempting to speculate that the PQS-dependent regulation of the *phz1* operon is enhanced in the planktonic

environment while the HHQ-dependent regulation of *phz2* operon is dependent on factors that are prominent in the biofilm environment.

2.4. Materials and Methods

2.4.1. Bacterial Strains and Growth Conditions

All strains were grown at 37°C in Luria-Bertani (LB) broth or 1% tryptone as specified. Biofilms were grown on 1% tryptone/1% agar plates. Coomasie Blue (20 µg/mL) and Congo Red (40 µg/mL) were added to plates used for morphology assays.

2.4.2. Construction of mutants

We generated unmarked deletions of the phenazine modifying enzymes *phzH*, *phzM*, *phzS* and of the two redundant phenazine biosynthetic operons *phzA1-G1* and *phzA2-G2* in PA14. Deletions of *phzA1-G1* and *phzA2-G2* genes have been described previously[18]. Here we describe the protocol for generating the unmarked deletion of *phzA2-G2:* The 5′ region (~1 kb in length) of the sequence flanking *phzA2* was amplified using the primer pair #1 and the 3′ region (~1 kb in length) of the sequence flanking *phzG2* with primer pair #2 (Table 3). These flanking DNA fragments were joined using overlap extension PCR. The resulting PCR product, containing a deletion of *phzA2-G2*, was cloned into a unique SpeI site in the mobilizable plasmid pSMV10. pSMV10 is a suicide plasmid for PA14 and contains an oriR6K origin of replication that does not function in PA14 but replicates in *E. coli* strains containing the pir gene; a gentamicin

resistance gene (*aacC1*); an oriT from RP4 that allows for mobilization by *E. coli* strains carrying RP4-derivatives on their chromosome (*E. coli* BW29427); and the counterselectable sacB gene. The resulting deletion plasmid was transformed into E. coli BW29427 and mobilized into PA14 using biparental conjugation[28]. PA14 single recombinants (merodiploid containing the intact *phzA2-G2* operon and the deleted operon) were selected on LB agar containing gentamicin. Potential *phzA2-G2* deletion mutants were generated by selecting for a resolved merodiploid (double recombinant) by identifying strains that grew in the presence of 10% sucrose (these strains lost the *sacB* containing plasmid because *sacB* is toxic in the presence of sucrose). Strains with properties of a double recombination were further analyzed by PCR to determine if *phzA2-G2* has been deleted and one was selected. The deletions of all other strains listed in Table 2 were made in a similar manner using primer pairs shown in Table 3.

2.4.3. Quantification of phenazines from biofilms and liquid cultures

For phenazine quantification from biofilms assay, starter cultures were grown for approximately 16 hours in LB. Ten microliters from these stationary-phase cultures were spotted on 1% tryptone/1% agar plates. These were then grown for 3 and 6 days. On day 3 or 6, five of these colonies were scraped from the plate and the agar was broken into pieces and put into 50ml conical with 3ml of water to extract phenazines. The tubes were then placed on a rotator for approximately 16 hours. Two hundred microliters from the overnight extraction was then centrifuged twice at 13,000 x g for 5min to remove any debris. A final centrifugation of the aliquots in spin columns (0.2 mm filter pore size) at 13 000 x g was done to remove any cells. The cleared extract was then loaded directly onto a Waters Symmetry C18 reverse-phase column

(5 mm particle size; 4.6 x 250 mm) in a Beckman SystemGold set up with a photodiode array detector. Phenazines were separated in a gradient of water-0.01% TFA (solvent A) to acetonitrile-0.01% TFA (solvent B) at a flow rate of 0.6 ml/min in the following method: linear gradient from 0 to 5% solvent B from 0 to 2 min, linear gradient to 83% solvent B from 2 to 22 min, then a linear gradient to 0% solvent B from 22 to 24 min. The total method time was 39 min. Retention times for PYO and PCA averaged 10.933 and 19.918 respectively. System Gold 32 Karat Software was used to calculate the area under each peak in absorbance units in the 366 nm channel. Phenazine standards at known concentrations were used to calculate conversion factors for PYO and PCA and were 8 x 10-6 mM/AU and 9.5 x 10-6 mM/AU respectively.

For liquid cultures grown in 1% Tryptone medium, 200 microliter samples were taken after being grown for approximately 16 hours. These were then prepared for HPLC analysis using the method described above.

2.4.4. Construction of the YFP-reporter plasmids

To generate the yfp reporter construct, we amplified an optimized yfp gene (Venus) from an *E. coli* strain in which the yfp gene is integrated within the chromosome (courtesy of Dr. John Hunt) using primers 1 and 2. The PCR product was digested with KpnI and NcoI and ligated with KpnI and NcoI digested miniTn7 (Gm) PA1/04/03 eyfp-a [55] to give pAKN69-venus, which contains a SpeI site upstream of the engineered SphI site at the Venus start codon. The multiple cloning site from pUCP18-mini Tn7T-Gm-*lacZ* [56] was amplified with primers 3 and 4. The PCR product was digested with SpeI and SphI and ligated with SpeI and SphI digested pAKN69-

venus to give pAKN69-MCS-venus. The multiple cloning site from pAKN69-MCS-venus was then amplified with primers 5 and 6. The PCR product was digested with SalI and MfeI and ligated with XhoI and EcoRI digested pYL122 [57]. This step replaced the *rhlA* promoter in pYL122 with a multiple cloning site to give pSEK101-*yfp*. The *phzA1* promoter was amplified from *P. aeruginosa* PA14 genomic DNA by PCR with primers 7 and 8. The *phzA2* promoter was amplified from *P. aeruginosa* PA14 genomic DNA by PCR with primers 9 and 10. These PCR products were digested with SpeI and SphI and ligated with SpeI and SphI digested pSEK101-*yfp* to give pSEK-P*phzA1-yfp* and pSEK-P*phzA2-yfp*. To integrate the reporter fusions into the PA14 genome, the P*phzA1-yfp* and P*phzA2-yfp* fusions were inserted as single-copies into the chromosomal *attB* site in *P. aeruginosa* PA14 using a modified version of a previously described protocol[57]. Briefly, pSEK-P*phzA1-yfp* and pSEK-P*phzA2-yfp* were transformed into chemically competent *E. coli* BW29427 cells for conjugation with *P. aeruginosa*. Merodiploids were selected with 200 µg/mL tetracycline, and Flp-catalyzed excision of the integrase and TetR cassette was carried out as previously described[58,59].

2.4.5. Yfp fluorescence quantification

Yfp fluorescence was quantified for yfp-reporter strains described above in planktonic and biofilm growth. For planktonic culture, strains were grown in biological triplicates in LB for 16 hours after which, cultures were diluted 1:100 and grown for another 3 hours to assure strains were in logarithmic phase. After 3 hours of growth, strains were diluted to an OD of 0.05 into a 96-well plate (Costar). The OD500 and fluorescence was monitored for 20 hours using Synergy

4 plate reader from BioTek. The excitation wavelength was 488nm and emission was at 520nm. Gen5 program was used to acquire the data.

For yfp-reporter strains grown on solid media, strains were spotted on morphology assay plates (90mL of media) the same as above and followed for 3 days. High resolution images of the plates were acquired using a Typhoon Trio variable mode scanner on Day 3. The excitation wavelength was 488nm and emission was at 520nm. Fluorescent data was quantified using the surface plot analysis in Image J.

2.4.6. Mouse lung colonization assay

P. aeruginosa strains were grown in LB broth or agar at 37°C with appropriate selection when applicable. Lung infections of *P. aeruginosa* were performed using eight-week-old C57BL/6J mice. Mice were anaesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine and inoculated with 0.3-1 x 10^5 colony-forming units (cfu) of organism before euthanasia 18 h after infection. Bacterial cfu were determined by homogenizing the whole lung and plating dilutions of the re-suspended tissue on LB agar. All mouse infections were performed under the guidelines of the Institutional Animal Care and Use Committee of Columbia University.

2.5. References

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2.6. Appendix

Table 2. Strains and plasmids used in Chapter 2

Strain	**Comments/Genotype**	**Source or Reference**
P.aeruginosa		
PA14	Clinical Isolate, UCBPP-14	Dietrich et. al., 2006
Δ*phz*	PA14 with deletions in the *phzA1-G1* and the *phzA2-G2* operons	Dietrich et. al., 2006
Δ*phz1*	PA14 with deletion of the *phzA1-G1* operon	This study
Δ*phz2*	PA14 with deletions of the *phzA2-G2* operon	This study
Δ*HMS*	PA14 with deletions of the *phzM, phzH and phzS genes*	This study
Δ*HMS*Δ*phz1*	PA14 with deletions of *phzM, phzH ,phzS genes* and *phzA1-G1* operon	This study
Δ*HMS*Δ*phz2*	PA14 with deletions of *phzM, phzH ,phzS genes* and *phzA2-G2* operon	This study
Δ*pqsAC*	PA14 with deletions of the *pqsA-C genes*	This study
Δ*pqsR*	PA14 with deletion of the *pqsR gene*	Hogan Lab
Δ*pqsH*	PA14 with deletion of the *pqsH gene*	Hogan Lab
Δ*pqsE*	PA14 with deletion of the *pqsE gene*	This study
Δ*pqsL*	PA14 with deletion of the *pqsL gene*	This study
Δ*pqsAC*Δ*HMS1*	PA14 with deletions of the *pqsA-C genes* in the Δ*HMS1* background	This study

Δ*pqsACΔHMS2*	PA14 with deletions of the *pqsA-C* genes in the Δ*HMS2* background	This study
Δ*pqsHΔHMS1*	PA14 with deletions of the *pqsH gene* in the Δ*HMS1* background	This study
Δ*pqsLΔHMS1*	PA14 with deletions of the *pqsL gene* in the Δ*HMS1* background	This study
Δ*pqsHLΔHMS1*	PA14 with deletions of the *pqsH* and *pqsL* genes in the Δ*HMS1* background	This study
WT::MCS YFP	PA14 with YFP insert with no promoter in the multiple cloning site	This study
WT::P*phzA1* YFP	PA14 with P*phzA1*YFP insert	This study
WT::P*phzA2* YFP	PA14 with P*phzA2*YFP insert	This study
Δ*pqsAC::*P*phzA2* YFP	Δ*pqsAC* with P*phzA2*YFP insert	This study
Δ*pqsR::*P*phzA2* YFP	Δ*pqsR* with P*phzA2*YFP insert	This study
Δ*phz2::*pUCP18	Δ*phz2* with pUCP18 plasmid inserted	This study
Δ*phz2::phz2*	Δ*phz2* with pUCP18 plasmid with *phz2* insert	This study
E.coli		
UQ950	*E. coli* DH5α λ(pir) host for cloning; F-Δ(*argF-lac)169 Φ80 dlacZ58*(ΔM15) *glnV44*(AS) *rfbD1 gyrA96*(Nal^R) *recA1 endA1 spoT1 thi-1 hsdR17 deoR λpir+*	D. Lies, Caltech
BW29427	Donor strain for conjugation: *thrB1*004 *pro thi rpsL hsdS lacZ* ΔM15RP4–1360 Δ(*araBAD*)*567* Δ*dapA*1341::[*erm pir*(wt)]	W. Metcalf, University of Illinois
Plasmids	**Description**	**Source**
pUCP18	Multi-copy plasmid with ColEI ORI;	Schweizer, 1991

	Amp^R; *lacZ* α gene	
pUCP18-*phz2*	pUCP18 plasmid with *phz2* operon inserted	This study

Table 3. Primers used in Chapter 2

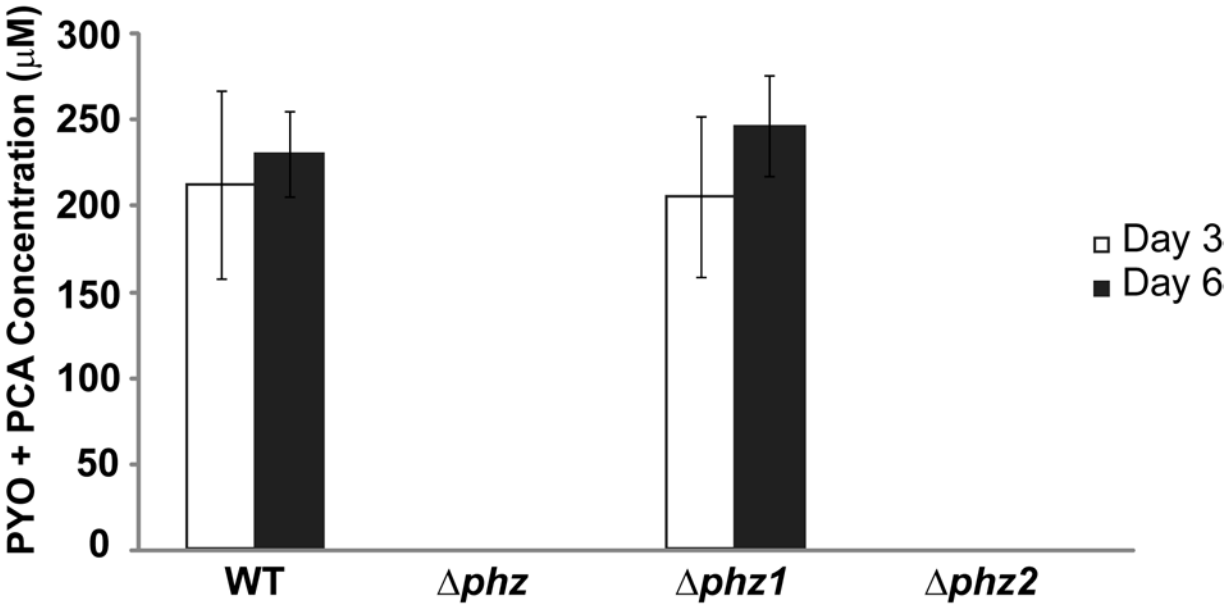
Primer	Sequence	Source
Primers for fluorescent reporter constructs		
1)kpnI-speI-sphI-Venus-FOR	5'-tgaggggtaccactagtagcttgcatgctgagcaagggcgagg-3'	This study
2) ncoI-venus-REV	5'-cgtaccatggttacttgtacagctcgtcca-3'	
3) speI-MCS-FOR	5'-tgcccgaggcatagactgta-3'	This study
4) sphI-MCS-REV	5'-ggatggcatgcctgtttcctgtgtgataaagaaag-3'	
5) salI-MCS-FOR	5'-tgaggtcgactaccgccacctaacaattcg-3'	This study
6) mfeI-MCS-REV	5'-tcgacaattgtaccgggcccaagcttct-3'	
7) speI-P*phzA1*-FOR	5'-cgccactagtttcctgcgtaccgaaagaat-3'	This study
8) xhoI-P*phzA1*-REV	5'-cgagctcgagcgagagggctctccaggtat-3'	
9) speI-P*phzA2*-FOR	5'-cgccactagtgcctgctcaactgaatcgac-3'	This study
10) xhoI-P*phzA2*-REV	5'-cgagctcgagagttcgaatcgactggcatc-3'	
Primers for deletion strains		
phz1-US-1	5'-GGACTAGTAGAACAGCACCATGTC-3'	This study
phz1-US-2	5'-CCCATCCACTAAATTTAAATATGTACC-3'	
phz1-DS-1	5'-TATTTAAATTTAGTGGATGGGCGCTA-3'	This study
phz1-DS-2	5'-GGACTAGTCATGCACACCCAGTTCAC-3'	
phz2-US-1	5'-GCGACTAGTGCTGATCTGGAATGGCG-3'	This study
phz2-US-2	5'-CCCATCCACTAAATTTAAATACAACCGTTGGTACTCTCG-3'	
phz2-DS-1	5'-TATTTAAATTTAGTGGATGGGCACCGCTACCTGCAAC-3'	This study
phz2-DS-2	5'-GCGACTAGTGGGTTTCTTCGATCACTAC-3'	
pqsABC-US-1	5'-ggaattgtgagcggataacaatttcacacaggaaacagctAGAGGC	This study

	TCCGATCACCCTAT-3’	
pqsABC-US-2	5’-CTCAGCACACCAGCACCTCGTCTGGCCCCGATAGTGATA-3’	
pqsABC-DS-3	5’-TATCACTATCGGGGCCAGACGAGGTGCTGGTGTGCTGAG-3’	This study
pqsABC-DS-4	5’-ccaggcaaattctgttttatcagaccgcttctgcgttCTGAACCGTAGGTCAGGACCAG-3’	
pqsL-US-1	5’-ggaattgtgagcggataacaatttcacacaggaaacagctCGCCTGTTCCTCAAGTACG-3’	This study
pqsL-US-2	5’-GCTGATAGGAACGCTCGCCCTGCCCACTACCACCAC-3’	
pqsL-DS-3	5’-GTGGTGGTAGTGGAGCAGGGCGAGCGTTCCTATCAGC-3’	This study
pqsL-DS-4	5’-ccaggcaaattctgttttatcagaccgcttctgcgttCTCGAACAGGTGTTCCTCAATC-3’	
pqsH-US-1	5’-ggaattgtgagcggataacaatttcacacaggaaacagctGATATCCACATCCACGGTGTC-3’	This study
pqsH-US-2	5’-TATTCCTCAGCCAGACGCTCGATGCCTGCCTTGGTGAAT-3’	
pqsH-DS-3	5’-ATTCACCAAGGCAGGCATCGAGCGTCTGGCTGAGGAATA-3’	This study
pqsH-DS-4	5’-ccaggcaaattctgttttatcagaccgcttctgcgttctgatGGAGATGCTCTGCACCTTGT-3’	

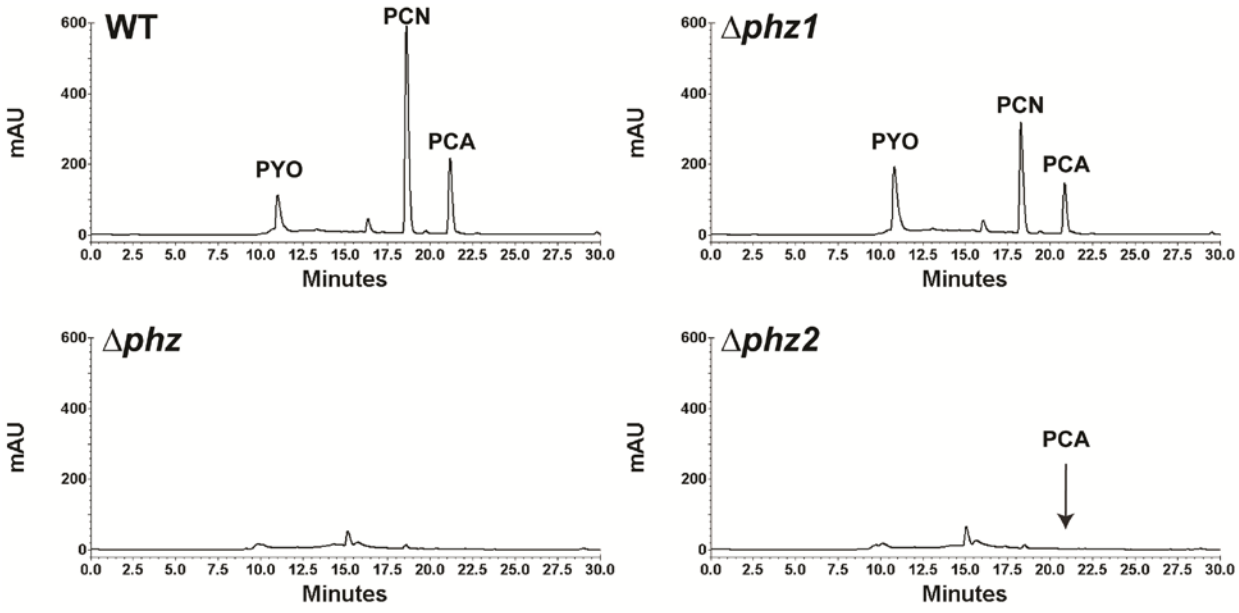
pqsE-US-1	5’-ggaattgtgagcggataacaatttcacacaggaaacagctGCAATCATGACCTGGTAGGG-3’	This study
pqsE-US-2	5’-ATGCTCCCCAGGTGCAGTCCAACAGGCACAGGTCATC-3’	
pqsE-DS-3	5’-GATGACCTGTGCCTGTTGGACTGCACCTGGGGAGCAT-3’	This study
pqsE-DS-4	5’-ccaggcaaattctgttttatcagaccgcttctgcgttCTGACAGGCACAACTGGCGATAG-3’	
phzH-US-1	5’-ggaattgtgagcggataacaatttcacacaggaaacagctGTTTCGACCAAGGAGGTCAG-3’	This study
phzH-US-2	5’-GCTCACCTGGGTGTTGAAGTGTATCGGTCATGGCGAAGAT-3’	
phzH-DS-3	5’-ATCTTCGCCATGACCGATACACTTCAACACCCAGGTGAGC-3’	This study
phzH-DS-4	5’-ccaggcaaattctgttttatcagaccgcttctgcgttCTGATCGCTTCCTCGACTCCATC-3’	
phzM-US-1	5’-ggaattgtgagcggataacaatttcacacaggaaacagctCACTCGACCCAGAAGTGGTT-3’	This study
phzM-US-2	5’-GTTGAGAGTTCCGGTTCAGGTATCAAATTACGCGCAGCAG-3’	
phzM-DS-3	5’-CTGCTGCGCGTAATTTGATACCTGAACCGGAACTCTCAAC-3’	This study
phzM-DS-4	5’-ccaggcaaattctgttttatcagaccgcttctgcgttctgatGCTGGT	

	ACGCCTGAGCAT-3’	
phzS-US-1	5’-ggaattgtgagcggataacaatttcacacaggaaacagctAAGGTCAACGCGGTACAGAT-3’	This study
phzS-US-2	5’-CCATCGATATCCTCATTGCCGCGACCGAAGACTGAGAAGA-3’	
phzS-DS-3	5’-TCTTCTCAGTCTTCGGTCGCGGCAATGAGGATATCGATGG-3’	This study
phzS-DS-4	5’-ccaggcaaattctgttttatcagaccgcttctgcgttctgatACGCGAACATTTCCGAGTC-3’	

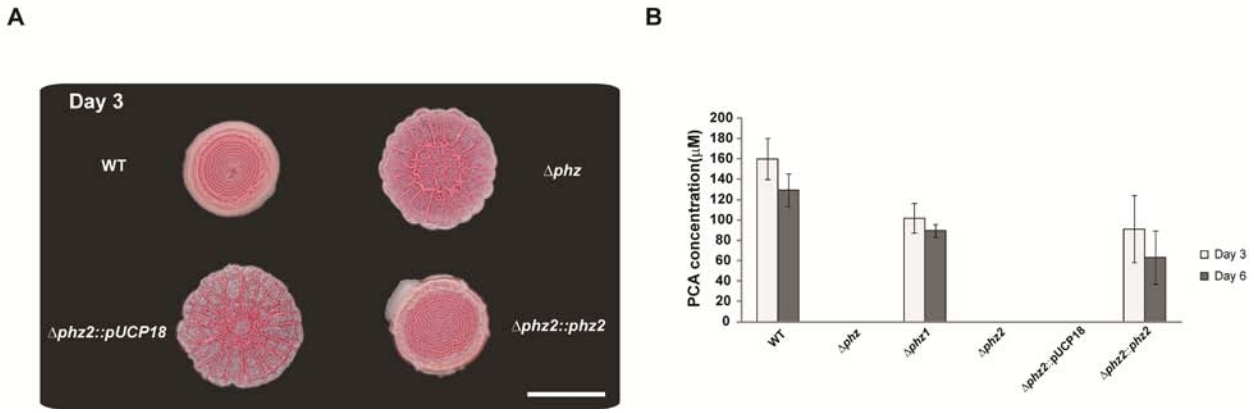
Supplementary Figures



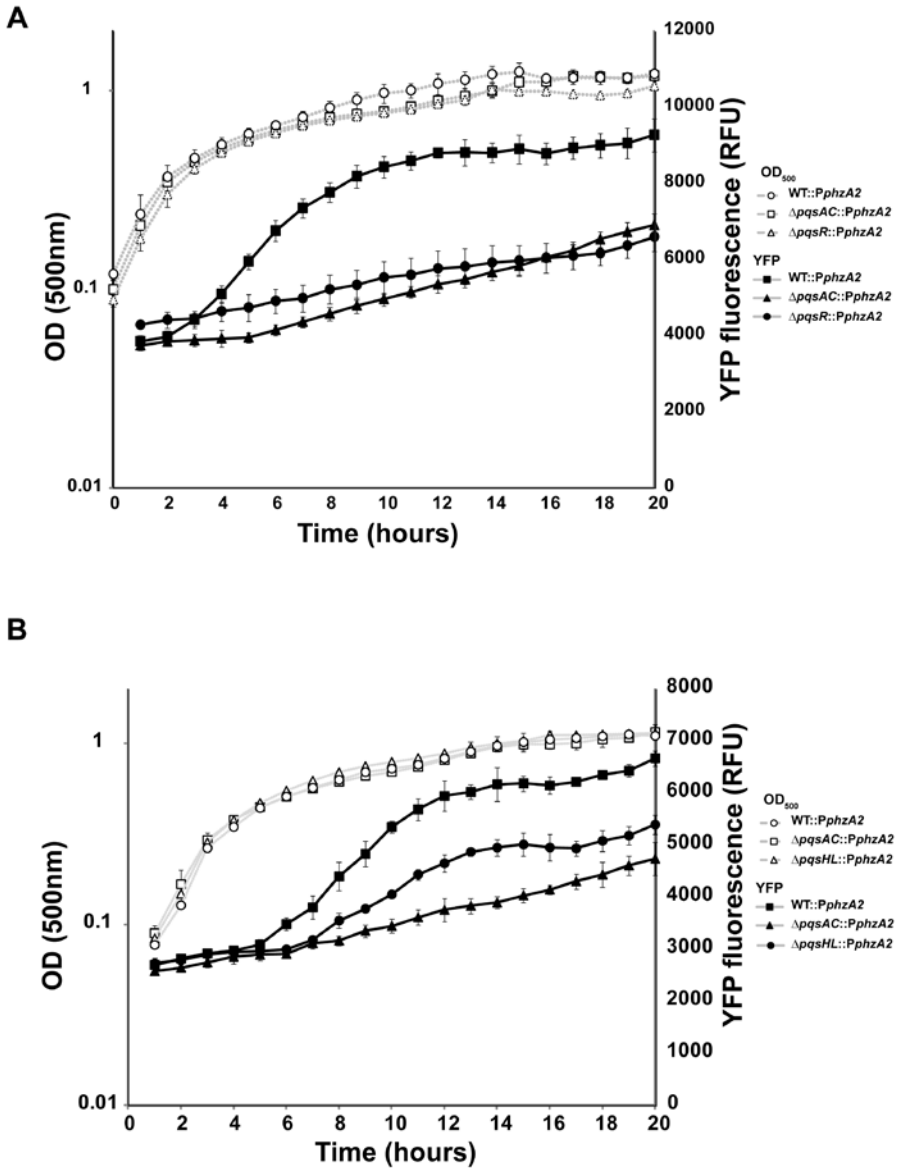
Supplementary Figure 1 (S1). HPLC quantification of PYO and PCA from colonies grown on 1% tryptone and 1% agar plates. Quantification of phenazines extracted from the agar on which biofilms were grown for 3 or 6 days. The PYO+PCA concentrations from the wild type strain are similar to that of the Δ*phz1* strain, indicating that the *phz2* operon is sufficient for production of wild type levels of these phenazines. Error bars indicate standard deviation of three independent experiments



Supplementary Figure 2 (S2). HPLC traces of phenazines extracted from day 6 biofilms. Phenazines were extracted from agar and submitted to HPLC analysis for separation and quantification at a wavelength of 366nm. The phenazines PYO, PCN and PCA were able to be detected in the wild type strain. The Δ*phz* and Δ*phz2* strains did not produce detectable levels of any phenazines. Arrow indicates where PCA peak would be expected in the Δ*phz2* strain. This suggests that *phz2* is necessary for phenazine production in biofilms. HPLC conditions and protocol were adapted from Dietrich *et al*., 2006. Observed retention times for PYO and PCA agree with their results (~10min and ~20min respectively).



Supplementary Figure 3 (S3). Complementation with *phz2* restores PCA production and rescues wrinkled morphology. *phz2* complementation strain was made by inserting multi-copy plasmid containing the entire *phz2* operon into the Δ*phz2* strain. (A) Colony morphology assay shows that the Δ*phz2*::*phz2* strain regains wild type morphology. Control strain containing empty vector (Δ*phz2*::*pUCP18*) shows wrinkled morphology similar to Δ*phz* strain. (B) Quantification of PCA production from deletion and complemented strains shows that complementation with *phz2* restores PCA production. The Δ*phz2* and *Δphz2*::*pUCP18* strains show no detectable phenazines. Error bars indicate standard deviation of three independent experiments



Supplementary Figure 4 (S4). HHQ positively regulates the expression of *phz2* in the planktonic environment. We assayed for expression of the *phz2* operon using a yfp-reporter plasmid containing the 500 bp upstream promoter elements of *phz2*. We inserted this reporter plasmid into the WT, Δ*pqsAC* (no quinolones) and Δ*pqsHL* (HHQ) strains and monitored growth (OD500) and yfp expression in planktonic cultures for 20 hours. (A) Quinolone signaling is necessary for wild type expression of phz2 as Δ*pqsAC*::A2YFP and Δ*pqsR*::A2YFP exhibited a severe reduction in *phz2* expression. (B) Quinolone-dependent expression of *phz2* is achieved specifically though HHQ. The Δ*pqsHL*::A2YFP strain produces exclusively HHQ (unable to produce PQS or HQNO) and is able to induce expression of *phz2* although not to wild type levels. This suggests that HHQ-dependent expression of *phz2* may be more prominent in the biofilm environment. Error bars represent the standard deviation of one experiment performed in biological triplicates. Experiment was repeated three additional times with similar results.

Chapter 3. Individual phenazines perform unique roles in *P. aeruginosa* PA14 biofilm development

This chapter is adapted from a manuscript that is in preparation (Recinos and Okegbe, et al., 2012)

3.1. Introduction

Bacteria typically grow and persist in multicellular communities called biofilms. Biofilm development depends greatly on the availability and production of exogenous and endogenous signals. In addition to their roles as modulators of gene expression, these molecules can also be important substrates for energy metabolism, with one role sometimes taking precedent over the other in a condition- or species-dependent manner[1]. Examples of exogenous signals include the respiratory substrates oxygen and nitrate, while endogenous signals include quorum-sensing compounds such as N-acyl homoserine lactones[2,3].

The opportunistic pathogen *Pseudomonas aeruginosa* produces a class of small redox-active molecules called phenazines. Phenazines are produced in stationary phase during growth in planktonic batch cultures and regulate a specific set of targets that have been implicated in phenazine modification and transport[4]. Phenazines can also act as metabolic substrates by accepting electrons from the intracellular pyridine nucleotide pool[5,6]. This activity enables survival of *P. aeruginosa* batch cultures in the absence of alternate respiratory chain oxidants by mediating electron transfer to an external, inaccessible substrate. Phenazine reduction and redox

cycling may be important in the biofilm context, where consumption of oxygen or nitrate by cells at the surface, combined with poor diffusion through the densely-packed community, leads to steep gradients of substrate availability[7]. Bacteria at depth in biofilms could benefit from the production of redox mediators that shuttle electrons from cells to areas of the biofilm where oxidants are present.

P. aeruginosa biofilm morphogenesis is strongly influenced by the presence of phenazines. *P. aeruginosa* biofilms that are unable to produce phenazines form hyper-wrinkled colonies when grown on solid agar containing a rich, complex growth medium. Such colonies wrinkle earlier during development when compared to those formed by the wild type, which remain relatively smooth[4]. Although recent studies have demonstrated that one phenazine, pyocyanin (PYO), does not restore the wild-type phenotype, the physiological effects of individual pseudomonad phenazines in the biofilm context have not been thoroughly explored. Interestingly, exogenous addition of phenazine-1-carboxylate (PCA), the precursor to PYO, gives rise to a wild-type morphology, indicating that specific phenazines perform distinct roles in colony maturation.

Pseudomonas aeruginosa produces a diversity of phenazines from the precursor molecule chorismic acid. Chorismic acid is converted to PCA, which can then be modified to produce other phenazines[8]. The enzyme PhzM methylates PCA to produce 5-methyl-carboxylic acid (5-MCA). Various modifications to 5-MCA give rise to PYO, aeruginosin A, and aeruginosin B[9]. While the monooxygenase responsible for PYO production, PhzS, is known, enzymes required for aeruginosin production have not been identified [10]. PhzS can act directly on PCA to produce 1-hydroxyphenazine. Finally, PCA can also be transformed by the enzyme PhzH to give rise to phenazine-1-carboxamide (PCN) (Figure 20A).

While *P. aeruginosa* and other phenazine-producing pseudomonads possess mechanisms that allow them to cope with and benefit from the redox activity of phenazines, phenazines are toxic for a diversity of other organisms. In the context of mammalian host infections, PYO and PCA are the primary phenazines that have been evaluated as virulence factors and it has been suggested that they contribute to *P. aeruginosa* pathogenicity in part by inhibiting the host immunological response[11,12] . PYO contributes to the virulence of *P. aeruginosa* during lung infection in mice and humans[13,14]. Other pseudomonads, such as *P. fluorescens* and *P. chlororaphis*, thrive in the plant rhizosphere and produce the phenazines PCA and PCN, respectively, as a defense against fungal phytopathogens. [15-17]. The diverse lifestyles of these pseudomonad species share the common theme of biofilm formation: *P. aeruginosa* forms oxygen-limited cellular aggregates during acute and chronic infections, while pseudomonad plant commensals form biofilms on roots in soil. However, the unique roles of individual phenazines in pseudomonad biofilm physiology have not been evaluated in detail.

Given the varying chemical properties exhibited by individual phenazines, we wondered if different phenazines have distinct physiological effects in the biofilm context. Our laboratory has previously reported increased PCN production in biofilms compared to planktonic cultures. This finding suggests that the environment plays a role in determining the type of phenazines that are made. We hypothesized that specific ratios of phenazines are required for wild-type colony morphogenesis. To address this, we generated mutants in the phenazine biosynthetic pathway that produced altered phenazine profiles. We investigated whether changing the relative amounts of the phenazines produced would have an effect on colony morphology and redox balancing for cells in biofilms. This work revealed primary roles for PCN and 5-MCA in biofilm physiology and development.

3.2. Results

3.2.1. Phenazine-1-carboxamide (PCN) is a major phenazine produced during colony biofilm development

Previous studies have established high pressure liquid chromatography (HPLC) as a method for the accurate separation and quantification of phenazines[8,18]. Using HPLC, we determined the repertoire of phenazines produced by *P. aeruginosa* strain PA14 under different environmental conditions. Planktonic cultures were grown in 1% tryptone liquid medium in glass tubes with constant shaking to stationary phase before sampling. Culture supernatants were filtered through a 0.2 µm pore and analyzed immediately by HPLC. Biofilms were grown for 6 days on 1% tryptone, 1% agar containing the dyes Congo Red Coomassie Blue before the agar was extracted with water and similarly filtered before HPLC analysis. The HPLC solvent system consisted of an aqueous acidic running buffer with a gradient of acetonitrile. This protocol enabled detection of the phenazines PYO, PCA, and PCN (Figure 20B). We are unable to detect 5-MCA and the aeruginosins using this method. For simplicity, we will refer to 5-MCA alone for the remainder of the text with the implicit assumption that Aeruginosins may also be produced whenever this compound is made. PYO and PCA were present in the traces from both planktonic cultures and biofilms with elution peaks at ~10 min and ~20 min, respectively. The biofilm sample showed a large peak at 17 min. Extracts from biofilms of the Δ*phzH* mutant lacked this peak (Figure S5). PCA levels were similar for planktonic cultures and biofilms. In contrast, PYO was the predominant phenazine in planktonic culture, while PCN was the major phenazine produced by biofilms (Figure 20C). This suggests that downstream modification of PCA is regulated in a condition-dependent manner.

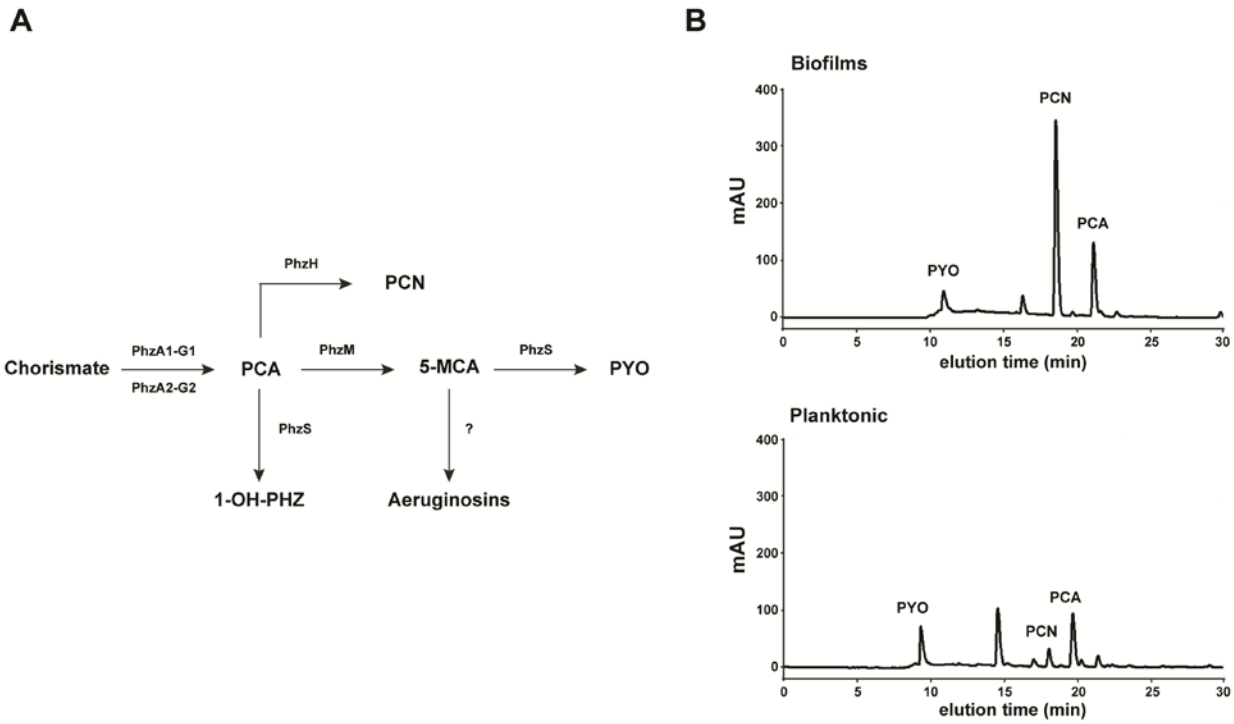


Figure 20 A-B. Influence of growth environment on the phenazine production dynamics of PA14. (A) Diagram showing the enzymes that catalyze the synthesis of phenazine-1-carboxylic acid (PCA) which is the precursor to pyocyanin (PYO), phenazine-1-carboxamide (PCN), 1-hydroxyphenazine (1-OH-PHZ) and Aeruginosin A and B. The intermediate molecule 5-methyl-carboxylic acid (5-MCA) is the precursor to both PYO and the Aeruginosins. (B) HPLC traces showing the separation and detection of phenazines produced by wild type PA14. Phenazines were extracted from planktonic culture supernatants (Bottom) or from agar on which biofilms were grown for 6 days (Top). We can accurately detect PYO, PCN and PCA from these modes of growth. We were not able to detect 1-OH-PHZ or 5-MCA/Aeruginosins under our conditions.

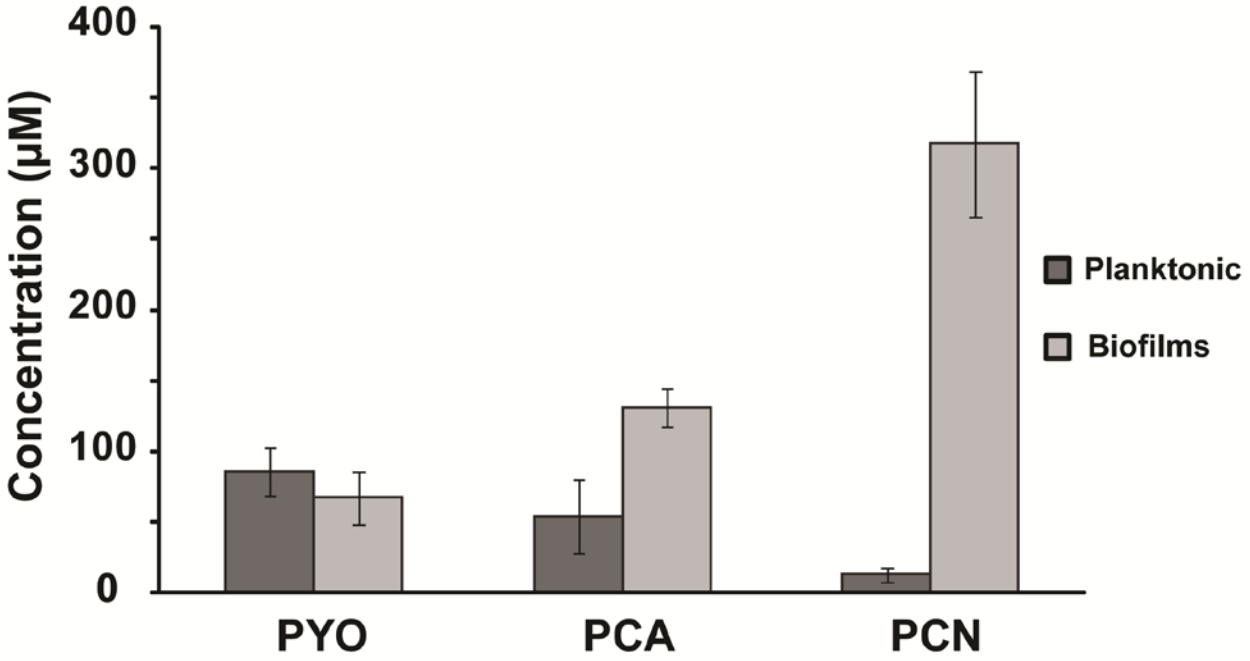


Figure 20C. PA14 shifts from PYO to PCN production in response to growth in biofilm environment instead of planktonically. Quantification of PYO, PCN and PCA using HPLC analysis shows that PCN is produced in large quantities during biofilm growth compared to growth in planktonic cultures. In contrast, PYO is produced in lower quantities in biofilms, suggesting that the PYO:PCN ratio is modulated by the growth environment. Error bars indicate standard deviation of three independent experiments

3.2.2. Characterization of phenazine biosynthetic mutants grown as biofilms

The observation that phenazine profiles vary with growth regime led us to hypothesize that different combinations of phenazines bear functional importance. To test this, we first created a panel of mutants that would only produce distinct combinations of phenazines. We made individual, double and triple deletions of genes encoding the biosynthetic enzymes PhzH, PhzM, and PhzS in order to shift phenazine production towards PCN (Δ*phzMS*), PCA (Δ*phzHM*) or 5-

MCA (Δ*phzHS*)[9,19] production. The combination of phenazines produced by the mutant strains is shown in Table 4.

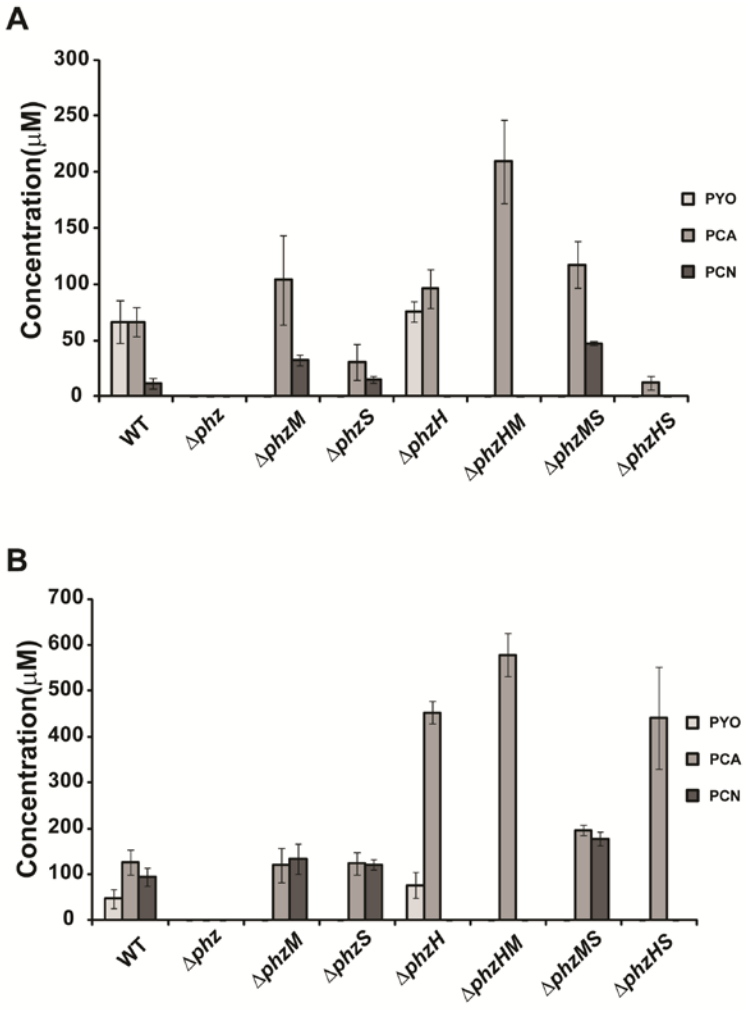


Figure 21. Phenazine quantification of *phz* deletion mutants grown in *P. aeruginosa* PA14 planktonic cultures or biofilms. (A) Quantification of extracted phenazines after 16 h of growth. PYO is produced at higher levels than PCN in this environment. (B) Quantities of PYO, PCN and PCA released into the agar by colony biofilms grown for 2 days. Error bars indicate standard deviation of three independent experiments

We assessed phenazine production of these mutants grown planktonically (Figure 21A) and as biofilms (Figure 21B). Phenazine production dynamics differed in these two modes of growth.

PCA levels were elevated in biofilms of strains lacking the glutamine amidotransferase PhzH compared to those of wild-type biofilms. Furthermore, Δ*phzH* mutant biofilms showed an increase in PYO production, and the Δ*phzS* and the Δ*phzHS* strains also show an increase in production of the 5-MCA and aeruginosins compared to wild type (data not shown). Together with the results that PCN is produced in large quantities in biofilms, these results suggest that the pool of PCA is divided into two pathways. One pathway represents the large portion of the PCA pool that is used by PhzH to make PCN, while the other pathway consists of the portion of the PCA pool that is used by PhzM to synthesize 5-MCA. The *P. aeruginosa* biofilm phenazine profile shifts toward 5-MCA and/or PYO when PCN production is not possible. These results demonstrate that a large portion of the PCA pool is utilized for PCN production during growth in biofilms.

3.2.3. PCN and 5-MCA play major roles in colony morphogenesis

We next tested the effects of different *phz* gene mutations on colony morphology. Previous studies have shown that mutant strains that are unable to produce any phenazines exhibit a wrinkled morphology[4]. Using our mutant panel, we asked which phenazines were needed to maintain smooth colony morphology. We found that both PCN and 5-MCA were required for wild-type (smooth) development of colonies (Figure 22), as only the wild type and Δ*phzS* biofilms maintained smooth morphologies for 2 days of incubation. Further support for the synergy between PCN and 5-MCA was exhibited by the Δ*phzS* and Δ*phzHS* strains. Abolishing PCN synthesis in the Δ*phzS* (smooth) background led to a wrinkled morphology. Strains that contain deletions of the *phzH* and/or the *phzM* genes, leading to loss of PCN and/or 5-MCA

production, respectively, began wrinkling earlier and more severely than strains that produce both PCN and 5-MCA. On the other hand, comparison of the wild type and the Δ*phzS* strains showed that PYO was not necessary for smooth colony development. This is in agreement with the previously published finding that complementation with PYO could not rescue a wrinkled morphology[20]. Additionally, PCA production alone (Δ*phzHMS* strain) was not sufficient to support wild-type development, suggesting that the development of colony morphology is governed by the synergistic activity of PCN and 5-MCA.

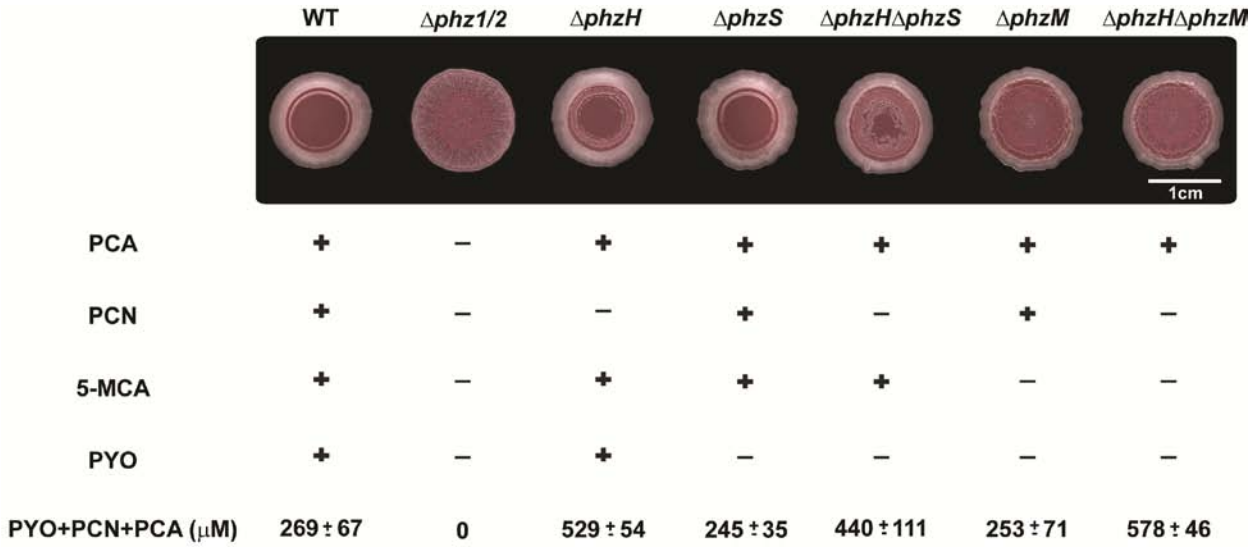


Figure 22. PCN and 5-MCA function synergistically to maintain a wild type (smooth) colony morphology. Phenazine deletion mutants were grown on 1% tryptone, 1% agar plates supplemented with the dyes Congo red and Coomassie blue for 2 days. The graph shows the phenazines that are produced by each of the phenazine mutant strains. Only the strains that produce both 5-MCA and PCN (WT and Δ*phsS*) are able to maintain a wild type colony morphology. Strains with *phzH* and *phzM* deletions exhibit earlier and more severe wrinkling. Quantification of additive levels of PYO, PCA and PCN are shown. As the total levels of PYO, PCA and PCN produced do not correlate with wrinkling, this suggests that it is the type of phenazines produced and not the total amount that govern colony morphology. It should be noted that we have no suitable method to quantify 5-MCA. Standard deviation of three independent experiments are shown for quantification of phenazines.

3.2.4. PCN and 5-MCA affect the intracellular redox state of cells in biofilms

Previous work has shown that redox metabolism is a key driver of colony morphogenesis, and that phenazine production alters the intracellular redox state of cells in biofilms. Different phenazines have different redox potentials and other chemical properties, and thus vary in their ability to act as electron acceptors. We wondered whether the colony morphotypes arising from mutations in specific *phz* genes might correlate with production of specific phenazines and/or the average intracellular redox state of bacteria in the community. We extracted NADH and NAD+ from our phenazine mutants grown planktonically and as biofilms, and measured their levels using an enzymatic cycling assay.

In planktonic cultures, most mutants that produced at least one phenazine maintained an $NADH/NAD^+$ ratio similar to that of the wild type (Figure 23A). Only the Δ*phzH* mutant (lacking PCN) had a significantly lower ratio compared to the wild type, indicating that the phenazines produced by this strain (likely the PYO, 5-MCA, and aeruginosins in particular) are significantly stronger oxidizers of NADH than PCN. This result also suggests that the small amount of PCN produced by wild-type planktonic cultures lessens the production levels of these stronger oxidizers. In biofilms, however, the effects of mutations in various *phz* genes on the intracellular redox state were different from those observed in planktonic cultures. The NADH/NAD+ ratio of the Δ*phzH* mutant was similar to that of the Δ*phz* strain, indicating that the phenazines produced by the Δ*phzH* strain are not sufficient to maintain the NADH/NAD+ balance. This is in contrast to the findings in planktonic culture, which indicated that the combination of PYO, PCA and 5-MCA produced by the Δ*phzH* strain was sufficient to oxidize a

large portion of the NADH pool. These data suggest that PCN plays a major role in redox balancing in biofilms, but not in the planktonic environment.

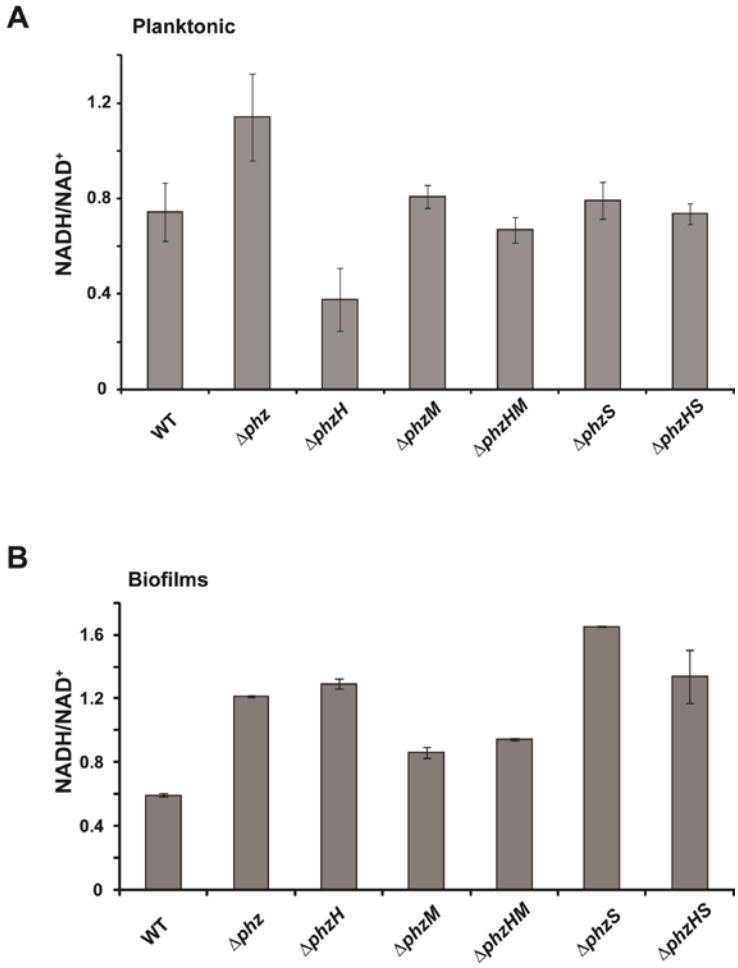


Figure 23. PCN balances the intracellular redox state in biofilms but not in the planktonic environment. Phenazines are redox-active molecules that can maintain redox homeostasis. Ratio of extracted of NAD^+ and NADH from phenazine mutant strains grown in planktonic cultures (A) and in biofilms (B). Mutants that produce a combination of PCA, PCN and 5-MCA produce NADH/NAD+ ratios similar to that of the wild type strain in planktonic culture. In biofilms, loss of PCN (Δ*phzH*, Δ*phzHS* strains) leads to a higher NADH/NAD+ ratios, suggesting that PCN is important for maintaining redox homeostasis in biofilms. Error bars represent the standard deviation of one experiment performed in biological triplicates. Experiment was repeated three additional times with similar results.

Previous studies evaluating the Δ*phz* mutant and the effects of exogenously added oxidants on morphology have suggested that colony wrinkling is an adaptation that allows bacteria in biofilms to cope with electron acceptor limitation. The NADH/NAD+ ratio of cells in Δ*phz* mutant biofilms reaches a maximum immediately before or coinciding with the induction of wrinkling, leading to the hypothesis that a reduced intracellular redox state can trigger the smooth-wrinkled transition. After wrinkling, the NADH/NAD^+ ratio in Δ*phz* mutant biofilms is similar to that of the wild type. It therefore appears that the either the production of phenazines or the increased availability of oxygen (that arises from the increased surface area) associated with the wrinkled morphology can serve to oxidize the intracellular redox state of cells in biofilms.

Interestingly, although the NADH/NAD^+ ratios of cells from our Δ*phzH* and Δ*phz* mutants were similar, the colony morphotypes were different, with the Δ*phzH* mutant exhibiting an intermediate morphology that bore more resemblance to the wild type. This suggests that PCN is necessary for the maintenance of a wild-type intracellular redox state, but that the increased NADH/NAD^+ ratio in this biofilm is not sufficient to induce wrinkling to the extent observed in the Δ*phz* mutant. A further exaggerated version of this result was observed for the Δ*phzS* mutant, as this mutant phenocopied with wild type with respect to colony morphology, but showed an even more pronounced increase in the NADH/NAD^+ ratio than the Δ*phzH* mutant. This suggests that the small amount of PYO produced in biofilms is also necessary for oxidation of the intracellular redox state. That the Δ*phzHS* mutant exhibited a similar NADH/NAD^+ ratio as the Δ*phz* mutant combined with its increased wrinkling suggests that lack of PYO and PCN is sufficient to induce the morphotypic transition toward the Δ*phz* morphology.

Because the Δ*phzM* and Δ*phzHM* mutants exhibited almost as much wrinkling as the Δ*phz* mutant, it is difficult to decouple the effects of the phenazines they produce from the effects of increased colony surface area on the intracellular redox state. However, their increased $NADH/NAD^+$ ratios relative to the wild type combined with their wrinkled morphologies suggests that 5-MCA is an important oxidant and regulator of morphogenesis in the *P. aeruginosa* biofilm context.

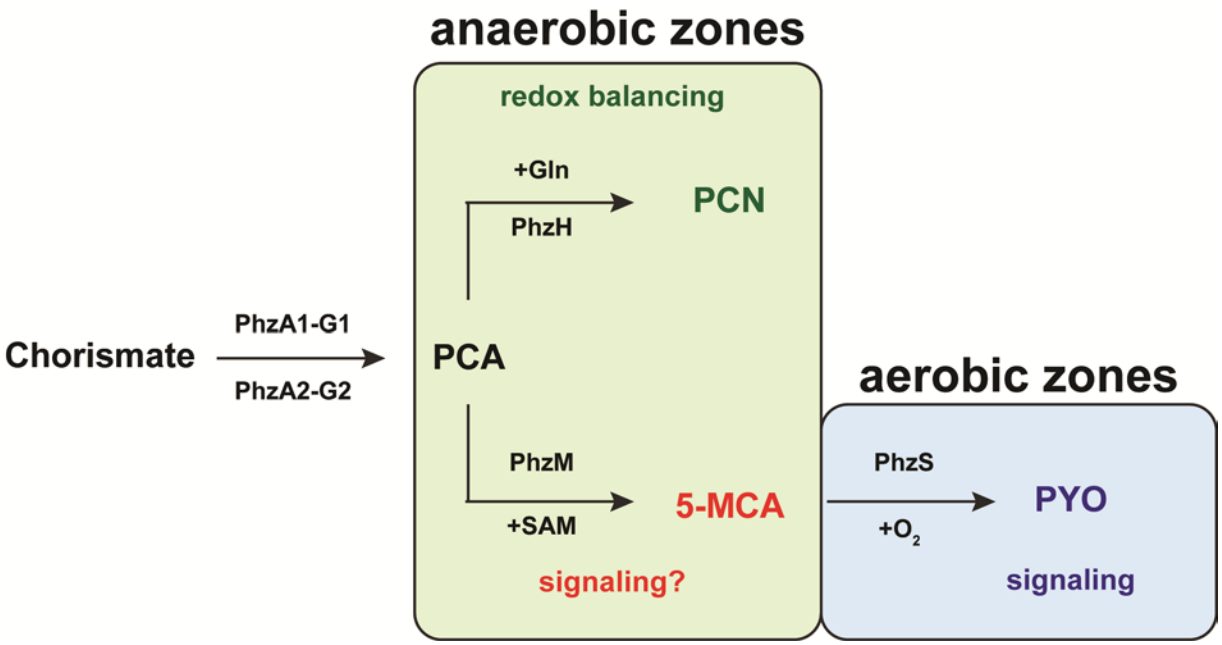


Figure 24. Synergistic effect of phenazines within biofilms. Proposed model of how the synergistic effects of individual phenazines affect colony development. Growth in the biofilm environment leads to the creation of different microenvironments, including oxygen and nutritional gradients. Based on our results, we propose that the functions of PYO, PCN and 5-MCA are governed by the different environmental niches present within biofilms. For example, the signaling and redox balancing properties of PYO may be important in aerobic zones while PCN and 5-MCA may assert their functions in anaerobic zones. PCN affects colony morphology through redox balancing while 5-MCA may affect colony development through non-redox balancing properties such as signaling.

3.3. Discussion

The phenazine PCA can be converted to PCN or 5-MCA and subsequently PYO. We found that in planktonic cultures the majority of PCA modification leads to production of PYO. In contrast, in biofilms a substantial amount was converted to PCN, suggesting an adaptation of phenazine combinations to different environments.

Phenazine production is important for *P. aeruginosa* wild-type colony biofilm development[4,20]. We evaluated the contributions of individual phenazines to biofilm morphogenesis through a detailed physiological characterization of mutants lacking the specific phenazine biosynthetic enzymes PhzH, PhzS and PhzM. We found that wild-type colony morphogenesis required production of PCN and 5-MCA, two phenazines that are often overlooked in the context of *P. aeruginosa* pathogenicity. Strains with deletions of the enzymes *phzH* and *phzM*, which are responsible for PCN and 5-MCA synthesis, respectively, exhibited wrinkling significantly earlier in development than strains able to produce PCN and 5-MCA (Figure 22). This finding suggests that there may be a temporal aspect to phenazine-dependent biofilm formation. In support of this hypothesis, Maddula *et al.* found that altering the ratio of phenazines had significant effects on initial attachment, architecture and dispersal of biofilms in *Pseudomonas chlororaphis* 30-84[21].

Closer examination of the morphologies of the mutants shows that while both the Δ*phzH* and Δ*phzM* mutants wrinkled, the wrinkling of the *ΔphzM* mutants was more severe. The Δ*phzM* wrinkling phenotype also seems to be dominant over the Δ*phzH* wrinkling phenotype, as seen in the Δ*phzHM* mutant strain (Figure 22). Although the Δ*phzHM* mutant showed more rugosity than either of the individual mutants, it still exhibited a phenotype that differed from the Δ*phz*

mutant in that it lacked the ability to spread to the same extent as this strain. This observation suggests a role for PCA in inhibition of colony spreading.

Interestingly, deletion of *phzS* alone suggested that PYO production does not play a role in maintaining a smooth colony architecture (Figure 22). This agrees with previous work that found PYO cannot rescue the wrinkled morphology [20]. However, deleting *phzS* in the Δ*phzH* background lead to a more wrinkled morphology than was observed for either mutant alone, implying that the effects of PYO can substitute for those of PCN.

The observation that mutants unable to produce 5-MCA and PCN form more structurally complex communities (similar to that of the Δ*phz* strain) with increased surface area lead us to propose that this morphogenetic switch is a response to redox imbalance. Our results support this hypothesis for PCN, as a mutant unable to produce PCN could not balance the $NADH/NAD^+$ ratio in biofilms. It is very interesting that these results were not recapitulated in the planktonic environment, as all phenazines should have the same properties in both environments. However, the ratio of the phenazines produced in each environment may be important. A possible explanation for the difference in ratios between the two environments is that PYO production from 5-MCA requires molecular oxygen. Bacterial cultures grown planktonically with shaking are sufficiently aerated, which may lead to a higher PYO::PCN ratio. In the biofilm environment, where steep oxygen gradients exist, PYO is not produced in large quantities and does not have an effect on colony morphology (Figure 23). It is possible that the microenvironments within the colony dictate the ratio of phenazines produced. The idea that different types and concentrations of phenazines affect the morphological features of a community is reminiscent of morphogens affecting development in eukaryotic organisms. Further studies are needed to elucidate the

connection between individual phenazines and colony morphology and establish these already multi-faceted molecules as morphogens within the bacterial community.

Based on our findings we propose a model that highlights the synergistic functions of phenazines in *P. aeruginosa* biofilms (Figure 24). Conversion of PCA to PCN, 5-MCA, and PYO requires the addition of functional groups derived from glutamine, S-adenosylmethionine, and molecular oxygen, respectively. The oxygen limitation experienced by cells in biofilms may be responsible for the increased production and/or importance of PCN and 5-MCA in this context. Due to the heterogeneity of the biofilm environment, there may be further variation in the ratios of phenazines present in specific biofilm microenvironments. Cell in biofilms may depend on the redox balancing properties of PCN in regions lacking sufficient oxygen. Intercellular signaling may be the more relevant physiological role of PYO in the biofilm context as its production would be limited in regions where redox balancing is most needed. The redox potentials and reactivity of individual phenazines are somewhat consistent with their apparent roles in redox balancing. These properties have not been assessed for 5-MCA. Whether a role in signaling or redox balancing is more significant for the functionality of 5-MCA remains an open question.

3.4. Future Directions

Our model shows the two essential appropriations of the PCA pool in response to the heterogeneous microenvironment of biofilms (Figure 24). However, additional investigations need to be performed in order to confirm our model. First, we need to confirm that 5-MCA is produced under anaerobic conditions. This would prove that both PCN and 5-MCA are needed

under anaerobic conditions to maintain the development of the colony. Investigations into the enzymatic properties of the methyltransferase PhzM show that this enzyme is not oxygen dependent. However, it has also been suggested that PhzM necessitates a transient interaction with the oxygen dependent PhzS for its activity[22,23]. To show that 5-MCA is present anaerobically we could: (1) probe for the presence of 5-MCA in anaerobically grown planktonic cultures and (2) create reporter constructs for PhzM and PhzH in order to visualize their localization using colony thin sections. The latter method would be performed using fluorescent probes that can function under anaerobic conditions as GFP requires oxygen to function[24].

Secondly, we will confirm the functions of PCN and 5-MCA in the biofilm environment. Our results show that PCN is needed to balance the intracellular NADH/NAD^+ ratio of cells within biofilms. We can confirm that the PCN effects on redox homeostasis are wide ranging by measuring the extracellular redox potential within biofilms. This will establish a more direct connection between the phenazine-reduced extracellular environment and morphological changes of the colony. By inserting a redox sensitive microelectrode probe within the biofilm we can measure the environmental redox potential inside biofilms of phenazine mutants that isolate PCN and 5-MCA production. One caveat to this assay is that the microelectrode is sensitive to any redox changes in the environment and cannot discriminate as to their cause (pH, other redox active molecules, etc.).

Our results suggest that the role of 5-MCA in colony development may not depend on its redox properties. 5-MCA is transformed by an unknown enzyme to produce the aeruginosins. The aeruginosins are hydrophilic and difficult to isolate from *P. aeruginosa* cultures[9,19]. Novel isolation techniques will be employed so that we may accurately purify and quantitate 5-MCA.

Once 5-MCA is isolated, we can investigate its properties including its potential role as a signaling factor. This can be investigated using DNA microarray analysis of cells that have been treated with exogenous 5-MCA, or by using mutants that are only able to produce 5-MCA.

3.5. Materials and Methods

3.5.1. Bacterial Strains and Growth Conditions

All strains were grown at 37°C in Luria-Bertani (LB) broth or 1% tryptone (Teknova) and shaken at 250rpm. Biofilms were grown on 1% tryptone/1% agar plates. Coomasie Blue (20 µg/mL) and Congo Red (40 µg/mL) were added to plates used for morphology assays. Both were purchased from EMD.

3.5.2. Construction of mutants

We generated unmarked deletions of the phenazine modifying enzymes *phzH*, *phzM*, *phzS* in PA14. Deletion of phenazine biosynthetic genes has been described previously[18]. Here we describe the protocol for generating the unmarked deletion of *phzH:* The 5′ region (~1 kb in length) of the sequence flanking *phzH* was amplified using the primer pair #1 and the 3′ region (~1 kb in length) of the sequence flanking *phzH* with primer pair #2 (Table 6). These flanking DNA fragments were joined using overlap extension PCR. The resulting PCR product, containing a deletion of *phzH*, was cloned into BamHI and EcoRI site in the mobilizable plasmid pMQ30. pMQ30 is a suicide plasmid for PA14 and contains an ColEI origin of replication that

does not function in PA14 but replicates in *E. coli* strains containing the pir gene; a gentamicin resistance gene (*aacC1*); an oriT from RP4 that allows for mobilization by *E. coli* strains carrying RP4-derivatives on their chromosome (*E. coli* BW29427); and the counterselectable sacB gene. The resulting deletion plasmid was transformed into E. coli BW29427 and mobilized into PA14 using biparental conjugation[25]. PA14 single recombinants (merodiploid containing the intact *phzH* gene and the deleted gene) were selected on LB agar containing gentamicin. *PhzH* deletion mutants were generated by selecting for a resolved merodiploid (double recombinant) by identifying strains that grew in the presence of 10% sucrose (these strains lost the *sacB* containing plasmid because *sacB* is toxic in the presence of sucrose). Strains with properties of a double recombination were further analyzed by PCR to determine if *phzH* has been deleted and one was selected. The deletion of *phzM, phzS* and the rest of the strains in Table 5 were made in a similar manner using primer pairs shown in Table 6.

3.5.3. Quantification of phenazines from biofilms and liquid cultures

For phenazine quantification from biofilms assay, starter cultures were grown for approximately 16 hours in LB. Ten microliters from these stationary-phase cultures were spotted on 1% tryptone/1% agar plates. These were then grown for three days. Five of these colonies were then scraped from the plate and the agar was broken into pieces and put into a 50ml conical with 3ml of water to extract phenazines. The tubes were then placed on a rotator for approximately 16 hours. Two hundred microlitres from the overnight extraction was then centrifuged twice at 13,000 x g for 5min to remove any debris. A final centrifugation of the aliquots in spin columns (0.2 µm filter pore size) at 13 000 x g was done to remove any cells. The cleared extract was

then loaded directly onto a Waters Symmetry C18 reverse-phase column (5 mm particle size; 4.6 x 250 mm) in a Beckman SystemGold set up with a photodiode array detector. Phenazines were separated in a gradient of water-0.01% TFA (solvent A) to acetonitrile-0.01% TFA (solvent B) at a flow rate of 0.6 ml/min in the following method: linear gradient from 0 to 5% solvent B from 0 to 2 min, linear gradient to 83% solvent B from 2 to 22 min, then a linear gradient to 0% solvent B from 22 to 24 min. The total method time was 39 min. Retention times for PYO, PCA and PCN averaged 10.9, 20.9 and 17.5 respectively. System Gold 32 Karat Software was used to calculate the area under each peak in absorbance units in the 366 nm channel. Phenazine standards at known concentrations were used to calculate conversion factors for PYO and PCA and were 8 x 10-6 mM/AU and 9.5 x 10-6 mM/AU respectively as has been previously found[18].

For liquid cultures grown in 1% Tryptone medium, 200 microliter samples were taken after being grown for approximately 16 hours. These were then prepared for HPLC analysis using the same method as above.

3.5.4. 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[26]. For planktonic cultures grown in 1% tryptone, cells were first grown for 16 hours after which they were diluted to an OD500 of 0.05. Cells were then grown for 5 hours to an OD500 of 0.9. Two 1-ml samples of culture were placed in two separate microcentrifuge tubes and centrifuged at 16,000 g for 1 min. For colonies grown as biofilms, samples were taken over the course of development. At each time point, three colonies growing on 1% tryptone and 1% agar plates

amended with 40µg/ml Congo red and 20µg/ml coomassie blue dyes were scraped off the agar plate using sterile razor blades and resuspended in 1ml of 1% tryptone. The more developed colonies were disrupted using a pellet disrupter. For each resuspended colony, two 450-µl samples were placed into two separate microcentrifuge tubes and centrifuged at 16,000 rcf for 1 min. Supernatant was removed and pellets were resuspended in 300 microliter of 0.2 M NaOH (for NADH extraction) or 0.2 M HCl (for NAD^+ extraction). These lysates were incubated for 10 min at 50°C, then for 10 min on ice. While vortexing, 300 microliter 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 min at 16,000 rcf. Supernatants were removed to fresh tubes and stored at -80°C until quantification.

Relative or absolute NADH and NAD^+ were quantified using a modification of the enzyme cycling assay developed by Bernofsky and Swan[27]. Briefly, a master reagent mix was prepared with 1x Bicine buffer (1.0 M, pH 8.0), 3x water, 1x 40 mM EDTA, 1x100% ethanol, 1x 4.2 mM thiazolyl blue (MTT), and 2x 16.6 mM phenazine ethosulfate (PES); 90 microliter aliquots were dispensed into individual wells of a 96-well microtiter plate. Five microliter of standard or sample was added to each well. The plate was warmed to 30°C, then the cycling reaction was started by the addition of 5 microliter 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, and values were normalized to optical density of the original cell culture sample where appropriate.

3.6. References

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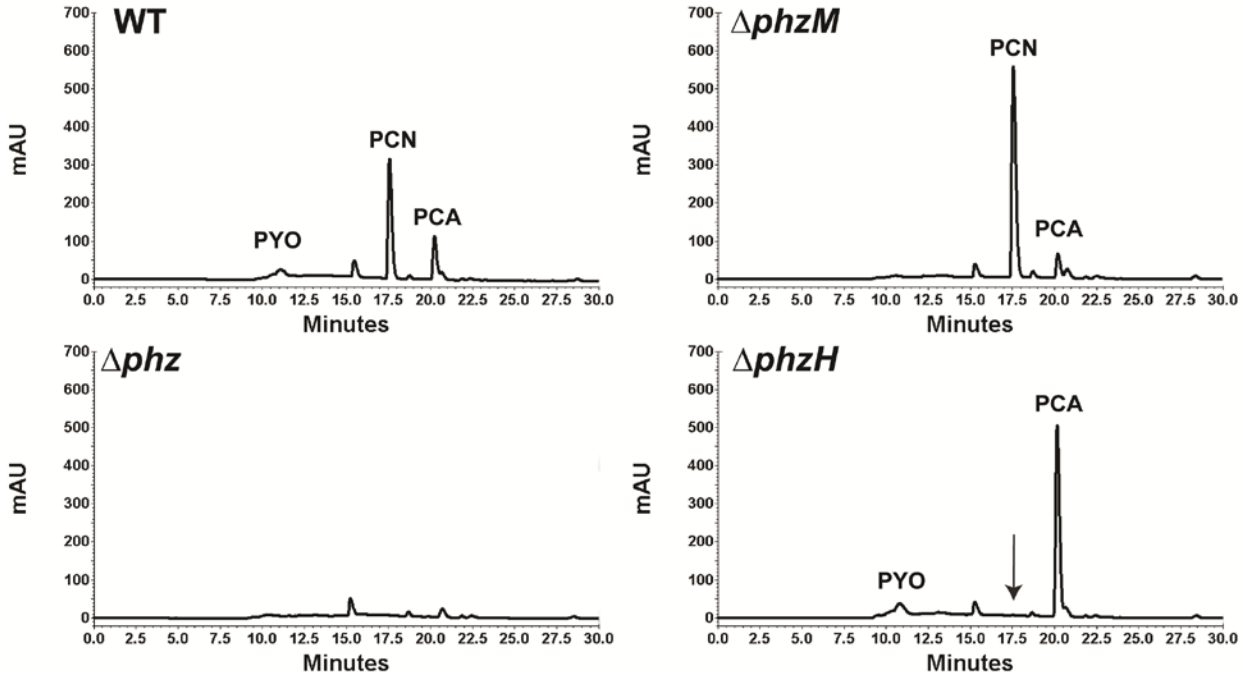
3.7. Appendix

Supplementary Figure



Supplementary Figure 5 (S5). HPLC traces show that the large 17min peak is that of PCN. HPLC analysis of phenazines produced by WT, Δ*phz*, Δ*phzM* and Δ*phzH* strains extracted from day 6 biofilms. A strain with a deletion of the glutamine amidotransferase PhzH known to catalyze the formation of PCN (Δ*phzH* strain) does not produce PCN. Arrow indicates where PCN peak would appear.

Tables

Table 4. Phenazines produced by mutant strains

Strain	Phenazines Produced
WT	PCA, PCN, PYO, 5-MCA
Δ*phz*	No phenazines
Δ*phzH*	PCA, PYO, 5-MCA
Δ*phzM*	PCA, PCN
Δ*phzS*	PCA, PCN, 5-MCA
Δ*phzHM*	PCA
Δ*phzMS*	PCA, PCN
Δ*phzHS*	PCA, 5-MCA

Table 5. Strains used in Chapter 3

Strain	Comments/Genotype	Source or Reference
P.aeruginosa		
PA14	Clinical Isolate, UCBPP-14	Dietrich et. al., 2006
Δ*phz*	PA14 with deletions in the *phzA1-G1* and the *phzA2-G2* operons	Dietrich et. al., 2006
Δ*phzH*	PA14 with deletion of the *phzH* gene	This study
Δ*phzM*	PA14 with deletions of the *phzM* gene	This study
Δ*phzS*	PA14 with deletions of the *phzS* gene	This study
Δ*phzMS*	PA14 with deletions of *phzM and phzS* genes	This study
Δ*phzHM*	PA14 with deletions of *phzM, phzH* genes	This study
Δ*phzHS*	PA14 with deletions of *phzH and phzS* genes	This study

E.coli		
UQ950	*E. coli* DH5α λ(pir) host for cloning; F-Δ(*argF-lac)169 Φ80 dlacZ58*(ΔM15) *glnV44*(AS) *rfbD1 gyrA96*(Nal^R) *recA1 endA1 spoT1 thi-1 hsdR17 deoR λpir+*	D. Lies, Caltech
BW29427	Donor strain for conjugation: *thrB1*004 *pro thi rpsL hsdS lacZ* ΔM15RP4–1360 Δ(*araBAD*)*567* Δ*dapA*1341::[*erm pir*(wt)]	W. Metcalf, University of Illinois

Table 6. Primers used in Chapter 3

Primer	Sequence	Source
phzH-US-1	5’-ggaattgtgagcggataacaatttcacacaggaaacagctGTTTCGACCAAGGAGGTCAG-3’	This study
phzH-US-2	5’-GCTCACCTGGGTGTTGAAGTGTATCGGTCATGGCGAAGAT-3’	
phzH-DS-3	5’-ATCTTCGCCATGACCGATACACTTCAACACCCAGGTGAGC-3’	This study
phzH-DS-4	5’-ccaggcaaattctgttttatcagaccgcttctgcgttCTGATCGCTTCCTCGACTCCATC-3’	
phzM-US-1	5’-ggaattgtgagcggataacaatttcacacaggaaacagctCACTCGACCCAGAAGTGGTT-3’	This study
phzM-US-2	5’-GTTGAGAGTTCCGGTTCAGGTATCAAATTACGCGCAGCAG-3’	
phzM-DS-3	5’-CTGCTGCGCGTAATTTGATACCTGAACCGGAACTCTCAAC-3’	This study
phzM-DS-4	5’-ccaggcaaattctgttttatcagaccgcttctgcgttctgatGCTGGTACGCCTGAGCAT-3’	
phzS-US-1	5’-ggaattgtgagcggataacaatttcacacaggaaacagctAAGGTCAACGCGGTACAGAT-3’	This study
phzS-US-2	5’-CCATCGATATCCTCATTGCCGCGACCGAAGACTGAGAAGA-3’	
phzS-DS-3	5’-TCTTCTCAGTCTTCGGTCGCGGCAATGAGGATATCGATGG-3’	This study
phzS-DS-4	5’-ccaggcaaattctgttttatcagaccgcttctgcgttctgatACGCGAACATTTCCGAGTC-3’	

Chapter 4. Species-specific residues calibrate SoxR sensitivity to redox-active molecules

This chapter is adapted from a manuscript that has been submitted for publication (Sheplock, M.S., Recinos D.A. et al., 2012). Most of the work was done in Dr. Monica Chander's laboratory. I contributed the experiments addressing the sensitivity of P. aeruginosa SoxR to redox active drugs and the ability of E. coli and S. coelicolor SoxR to modulate P. aeruginosa colony morphology (Figure 25C and Figure S6).

4.1. SUMMARY

In enterics, the transcription factor SoxR triggers a global stress response by sensing a broad spectrum of redox-cycling compounds. In the non-enteric bacteria *Pseudomonas aeruginosa* and *Streptomyces coelicolor,* SoxR is activated by endogenous redox-active small molecules and only regulates a small set of genes. We investigated if the more general response in enterics is reflected in the ability of SoxR to sense a wider range of redox-cycling compounds. Indeed, while *E. coli* SoxR is tuned to compounds that span a redox range of -450 to +80 mV, *P. aeruginosa* and *S. coelicolor* SoxR are less sensitive to molecules with redox potentials below -300 mV. Using a mutagenic approach, we pinpointed three amino acids that contribute to the reduced sensitivity of *P. aeruginosa* and *S. coelicolor* SoxR. Notably these residues are not conserved in enteric homologs. We further identified a motif within the sensor domain that tunes the redox-reactivity of SoxR from enterics – inhibiting constitutive activity while allowing

sensitivity to drugs with low redox potentials. Our findings highlight how small alterations in structure can lead to the evolution of proteins with distinct redox-sensing properties.

4.2. INTRODUCTION

Iron-sulfur clusters (Fe-S) are remarkably diverse in structure and chemistry. Different cluster types span a wide range of redox potentials and the redox potential of a single cluster type can be further tuned by changing its molecular environment (Beinhert, 2000). These features enabled the evolution of Fe-S proteins that perform crucial and versatile functions as metabolic enzymes, components of electron transport chains, and redox-sensing regulators of gene expression. The latter act as molecular switches that are either activated or inactivated by specific redox signals (such as oxygen, hydrogen peroxide, superoxide, nitric oxide, or redox-active small molecules) to regulate important aspects of bacterial development and physiology. For example, FNR, a [4Fe-4S] protein, controls the switch between aerobic and anaerobic metabolism in *Escherichia coli* in response to molecular oxygen (Khoroshilova *et al.*, 1997); IscR, also a [4Fe-4S] protein, increases the production of Fe-S cluster biogenesis machinery under conditions of oxidative stress (Zheng *et al.*, 2001; Yeo *et al.*, 2006); and SoxR, a [2Fe-2S] protein, mediates an oxidative stress response to redox-cycling drugs in the enteric bacteria *E. coli* and *Salmonella enterica* (Hidalgo and Demple, 1996). Within this group of redox-sensing transcription factors SoxR is unique in that, unlike the other proteins that are regulated by assembly/disassembly of their Fe-S clusters, the activity of SoxR is modulated by reversible one-electron oxidation-reduction of its [2Fe-2S] clusters (Ding *et al.*, 1996; Gaudu and Weiss, 1996; Ding and Demple, 1997; Gaudu *et al.*, 1997).

In *E. coli* (and related enteric bacteria), SoxR senses redox stress imposed by a broad collection of redox-active compounds including viologens, phenazines and quinones (Table 8; Gu and Imlay, 2011). SoxR transduces these redox signals into a global defense program via a second transcription factor, SoxS. SoxR is a constitutively expressed regulator bound to the *soxS* promoter poised to detect stress. In the absence of oxidants, SoxR exists in a quiescent state with reduced [2Fe-2S] clusters and *soxS* is not expressed. Exposure to redox-cycling drugs causes oxidation of SoxR's [2Fe-2S] centers, and the oxidized protein activates *soxS* expression by mediating structural changes in the promoter DNA that allow RNA polymerase to initiate transcription (Hidalgo *et al.*, 1995). SoxS in turn recruits RNA polymerase to transcribe >100 genes, some of which encode proteins that reestablish redox balance and repair oxidant-induced damage (Pomposiello *et al.*, 2001). The SoxRS system in enterics allows for rapid amplification of the stress signal into a stress response geared towards oxidants.

The SoxRS regulon is unique to enterics. Although *soxR* is widely distributed (and highly similar at the amino acid level) across the Gram-negative Proteobacteria and the Gram-positive Actinobacteria, *soxS* is present exclusively in enterobacteria. An extensive bioinformatic analysis of *soxS*-deficient genomes predicted that in non-enterics SoxR directly regulates a relatively small set of genes that encode putative oxygenases, oxidoreductases, or transporters (Dietrich *et al.*, 2008*).* This has been verified for the γ-Proteobacterium *Pseudomonas aeruginosa* and the Actinomycete *Streptomyces coelicolor*, both soil-dwelling organisms notable for producing redox-active secondary metabolites. The SoxR regulon in *P. aeruginosa* consists of a Resistance-Nodulation-Division (RND) efflux pump MexGHI-OmpD (*PA4205-4208)*, a major facilitator superfamily (MFS) transporter (*PA3718)*, and a monooxygenase (*PA2274*) (Palma *et al.*, 2005).

In *S. coelicolor*, SoxR regulates a monooxygenase (*SCO1909*) with homology to *PA2274*, two oxidoreductases (*SOC2478, SCO4266),* an epimerase/dehydratase (*SCO1178*), and an ABC transporter (*SCO7008*) (Dela Cruz *et al.,* 2010; Shin *et al.*, 2011). In these bacteria SoxR-regulated genes are induced in stationary phase during the production and secretion of redox-active metabolites – phenazines in the case of *P. aeruginosa* and the benzochromanequinone polyketide actinorhodin in the case of *S. coelicolor* (Dietrich *et al.,* 2006; Dela Cruz *et al.,* 2010; Shin *et al.*, 2011). This is not a mere correlation as expression of each SoxR regulon is dependent on production of the redox-active compounds by the microbe (Dietrich *et al.,* 2006; Dela Cruz *et al.,* 2010; Shin *et al.,* 2011). These observations support the view that SoxR evolved to regulate the machinery that processes/transports endogenous redox-active metabolites in producer organisms. The enterobacteria (which do not produce redox-active secondary metabolites) are unique in that SoxR regulates only one gene, *soxS*. They may have acquired *soxR* via lateral gene transfer, taking advantage of its redox-sensing abilities to regulate a generalized stress response (SoxS regulon) against toxic redox-cycling compounds.

Given that SoxR performs distinct functions in different bacteria, we asked if the differences in SoxR functionality are manifested only by its regulons, or if SoxR from different species also sense different inputs. We hypothesized that SoxR from *P. aeruginosa* and *S. coelicolor* sense redox molecules that resemble their endogenous activators (phenazines and anthraquinones, respectively), while *E. coli* SoxR, given its involvement in a general stress response, senses a broader spectrum of redox-active compounds. Here we report that the non-enteric SoxR proteins are indeed more restricted in the range of molecules they sense compared to their *E. coli* counterpart, and we have identified key features that contribute to the differential sensitivities.

This study provides insight into the evolutionary fine-tuning of this redox-sensing transcription factor that adapted it to serve the needs of organisms with different physiologies.

4.3. RESULTS

4.3.1. SoxR protects *E. coli*, but not *P. aeruginosa* or *S. coelicolor*, against redox-cycling drugs

In *E. coli* and related enterobacteria, SoxR mediates a general stress response against redox-cycling compounds by activating the SoxS regulon. By contrast, non-enterics lack a SoxS regulon. Instead, SoxR directly regulates a small set of genes, making a general stress response unlikely. To test this, we exposed *soxR* deletion mutants of *E. coli* (which contains *soxS*), the Gram-negative *P. aeruginosa* PA14, and the Gram-positive *S. coelicolor* M145 (both of which lack *soxS*) to a diverse array of redox-cycling compounds using a filter disk assay. In agreement with previous reports, an *E. coli ΔsoxR* mutant was more sensitive to most tested redox-cycling agents such as pyocyanin (Pyo), plumbagin (PB), and 4-nitroquinoline-N-oxide (4NQO), compared to wild type (Greenberg *et al.* 1990; Tsaneva and Weiss, 1990; Fig. 1A). The *E. coli* Δ*soxR* mutant was no more sensitive to the viologen diquat (DQ) than wild type, and a previous study had shown this to also be true for paraquat (PQ), another viologen (Greenberg *et al.*, 1990). This appears to be a strain-specific phenomenon, since a different *E. coli* Δ*soxR* mutant strain was more sensitive to PQ than wild type (Tsaneva and Weiss, 1990). In contrast to *E. coli*, the *S. coelicolor ΔsoxR* mutant and wild type were equally sensitive to all tested compounds (Fig. 25B). *P. aeruginosa* was generally more resistant to all drugs tested in this study. The wild type

and *ΔsoxR* mutant were resistant to Pyo, PB, and 4NQO, and were equally sensitive to DQ (Fig. 25C). It is worth noting that the *P. aeruginosa* Δ*soxR* mutant has a colony morphology phenotype (Dietrich *et al.*, 2008), which can be reverted by complementation with *E. coli* or *P. aeruginosa* SoxR (Fig. S6).

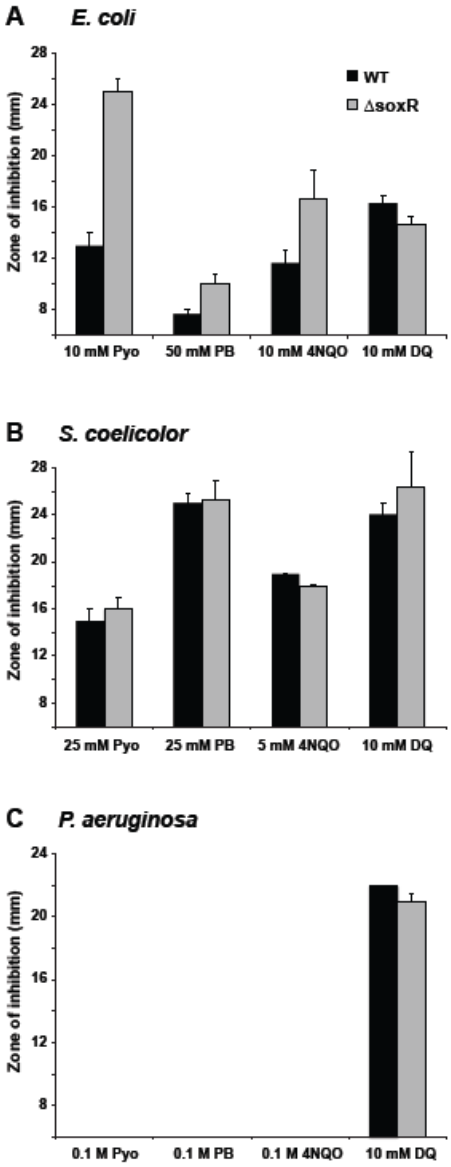


Figure 25. *S. coelicolor* and *P. aeruginosa* Δ*soxR* mutants are not hypersensitive to superoxide-generating agents. Paper disks soaked with solutions of the indicated compounds were placed on bacterial lawns of wild type (black columns) or Δ*soxR* mutant (grey columns) growing on nutrient agar plates. Zones of growth inhibition around the disks were recorded after 24 h at 37°C for *E. coli* (A) or 48 h at 30°C for *S. coelicolor* (B) and *P. aeruginosa* (C). The data represent the means of 3 to 5 replicates ± standard deviations (bars; some not visible).

4.3.2. *P. aeruginosa* and *S. coelicolor* SoxRs sense a narrower spectrum of redox drugs than *E. coli* SoxR

Our finding that SoxR did not contribute to resistance against redox-cycling agents in *P. aeruginosa* and *S. coelicolor* is consistent with the notion that the enteric-specific SoxS regulon governs a general stress response. To explore the hypothesis that the SoxR regulons in *P. aeruginosa* and *S. coelicolor* may be specific to phenazines and actinorhodin, respectively, we posited that SoxR itself might be optimized to sense specific redox inputs. Given the role of SoxR in *E. coli* as a general stress-response regulator, we predicted this protein would respond to a broad spectrum of redox-cycling molecules, while *P. aeruginosa* and *S. coelicolor* SoxRs would only respond to molecules that resemble their endogenous activators, i.e. phenazines and anthraquinones, respectively.

To quantify the SoxR response to a wide spectrum of redox-cycling compounds, we employed a ß-galactosidase assay in *E. coli*. The three *soxR* genes (each with an N-terminal histidine-tag) were transformed into an *E. coli* Δ*soxRS* mutant lysogenized with a λ[*soxS* promoter-*lacZ* reporter] (Table 9). Transformed cells were grown to exponential phase and then treated with representative drugs that span a wide range of redox potentials and belong to different structural classes (Table 8). The phenazines Pyo and phenazine-1-carboxylic acid (PCA) are produced by *P. aeruginosa*, while phenazine methosulfate (PMS) is synthetic.

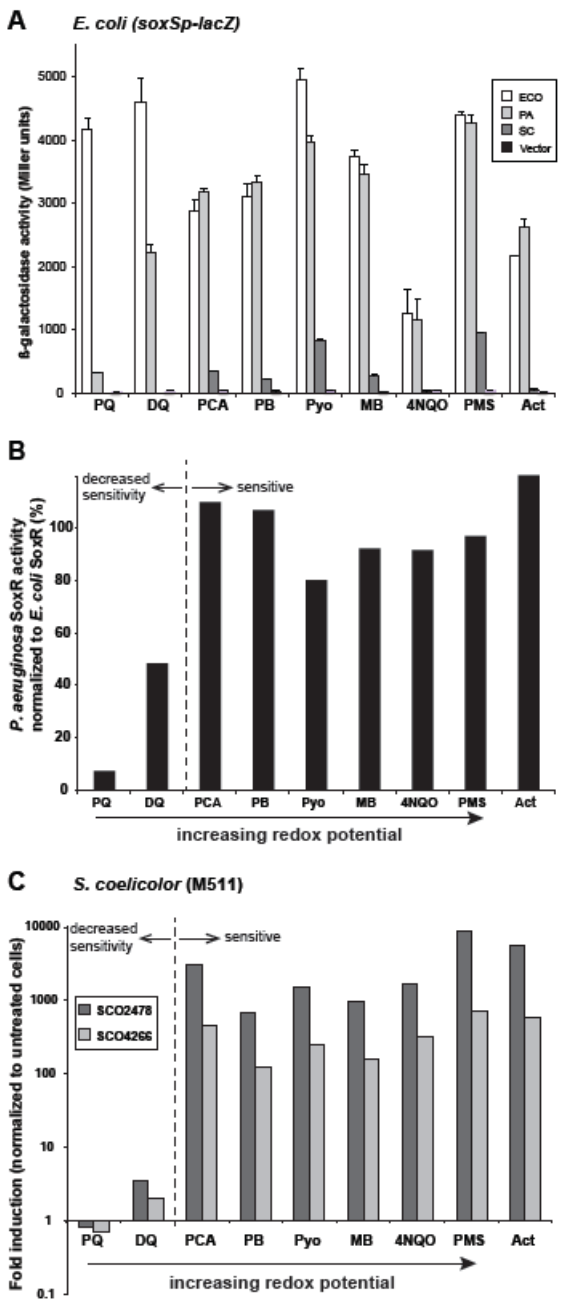


Figure 26. *P. aeruginosa* and *S. coelicolor* SoxRs sense a narrower spectrum of redox-active compounds than *E. coli* SoxR.
(A). E. *coli* strain EH46 expressing histidine-tagged *E. coli* SoxR (white columns), *P. aeruginosa* SoxR (light grey columns), *S. coelicolor* SoxR (dark grey columns) or empty vector (black columns) were treated with 200 µM PQ, 200 µM DQ, 500 µM PCA, 25 µM PB, 20 µM Pyo, 25 µM MB, 50 µM 4NQO, 20 µM PMS or 25 µM Act for 1 h before the assay for ß-galactosidase activity.
(B). *P. aeruginosa* SoxR activity normalized to that of *E. coli* SoxR indicates that the former displays reduced sensitivity to drugs with redox potentials below -300 mV.
(C). The Act-deficient *S. coelicolor* strain M511 was grown for 20 h in R5^{-} medium, then exposed for 30 min to 1 mM PQ, 1 mM DQ, 500 µM PCA, 100 µM PB, 10 µM Pyo, 25 µM MB, 1 mM 4NQO, 10 µM PMS or 10 µM Act. qRT-PCR was performed on RNA extracted from these cells to detect induction of SoxR-target genes *SCO2478* (dark grey columns) and *SCO4266* (light grey columns). Signals were standardized to the level of the housekeeping sigma factor, *hrdB*, and fold-induction was normalized to untreated M511 cells.

E. coli SoxR was strongly activated (albeit to different extents) by all nine drugs tested (Fig. 26A). *P. aeruginosa* SoxR was activated at levels comparable to *E. coli* SoxR by PCA, PB, Pyo, methylene blue (MB), 4NQO, PMS, and actinorhodin (Act), but at significantly lower levels by

the viologens PQ and DQ (Fig. 26A). PQ elicited 15-fold lower ß-galactosidase activity in cells expressing *P. aeruginosa* SoxR compared with *E. coli* SoxR. Ethyl viologen, which has a similar midpoint redox potential as PQ (-480 mV), was also a weak inducer of *P. aeruginosa* SoxR activity (data not shown). The response to DQ, which has a higher redox potential than PQ (-360 mV) was more robust (2200 Miller units), but still only about half that of *E. coli* SoxR (4600 Miller units). Thus *P. aeruginosa* SoxR has low sensitivity to drugs with redox potentials more negative than ~ -300 mV (Fig. 26B).

In stark contrast to the high levels of ß-galactosidase activity produced by *E. coli* and *P. aeruginosa* SoxR, *S. coelicolor* SoxR produced very low signals (Fig. 26A). As such we were unable to draw any meaningful conclusions about *S. coelicolor* SoxR activation using the heterologous *E. coli* system. We therefore investigated this transcription factor's activity in its native background. Because this protein is activated by the endogenous metabolite Act, it was necessary to monitor its response to exogenous drugs in *S. coelicolor* M511, a strain that does not synthesize Act (Table 9). Cells were grown for 20 h in liquid culture before a 30 min exposure to the redox-cycling drugs listed in Table 8. SoxR activity was assessed by monitoring the expression levels of two of its target genes, *SCO2478* and *SCO4266*, by quantitative real-time PCR (qRT-PCR). In addition to Act, PCA, PB, Pyo, MB, 4NQO and PMS induced SoxR-target gene expression to high levels over background (Fig. 26C). Drug-induced expression of *SCO2478* and *SCO4266* was SoxR-dependent since these mRNAs were not detectable in M511Δ*soxR* cells that were similarly treated (data not shown). Similar to *P. aeruginosa* SoxR, only the viologens PQ and DQ failed to activate SoxR to any appreciable level (Fig. 26C). Thus,

S. coelicolor and *P. aeruginosa* SoxR sense redox-active molecules in the same range of redox potential.

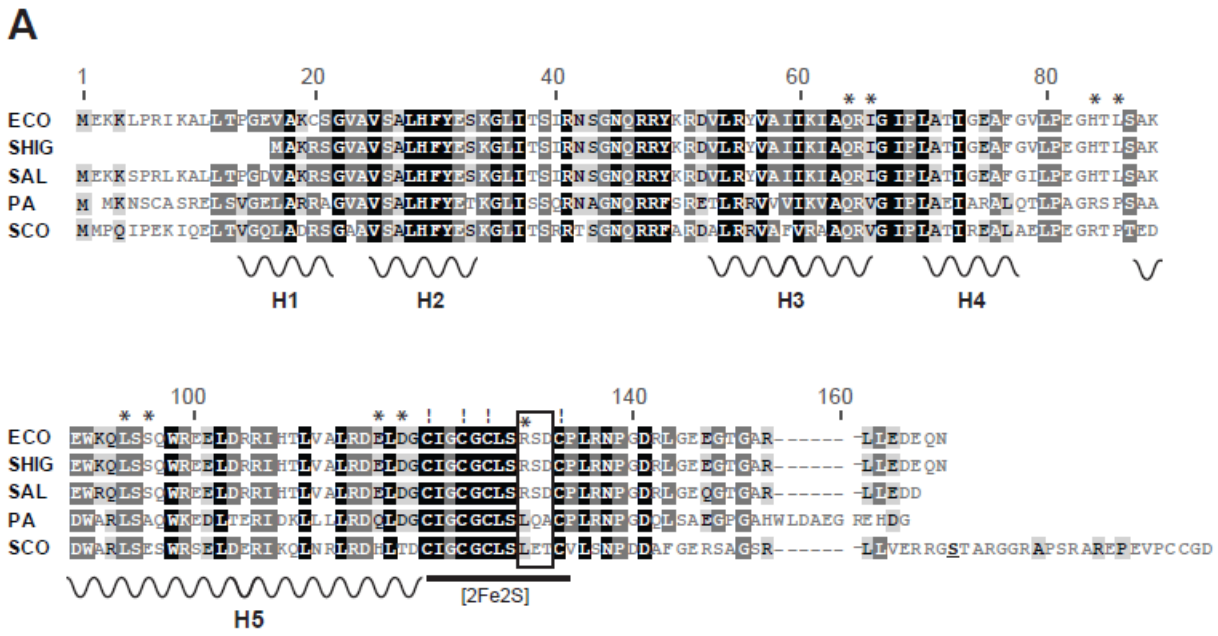


Figure 27 A. Sequence comparison of SoxR homologs. A BLAST analysis was performed for *E. coli* SoxR against all available bacterial genomes. The 250 closest homologs were aligned using ClustalW, and shown is an alignment of five of these SoxR proteins from the enterics *E. coli* (ECO), *Shigella flexneri* (SHIG), *Salmonella enterica* (SAL), and the non-enterics *P. aeruginosa* (PA), and *S. coelicolor* (SCO). Black, dark grey or light grey boxes surrounding residues indicate 100%, 80-100% or 60-80% similarity between all 250 SoxRs (Blosum62 score matrix with threshold of 1). H1-H4 indicate the two helix-turn-helix motifs that form the DNA binding domain; H5 indicates the dimerization helix. The four conserved cysteine residues that anchor the [2Fe-2S] cluster are indicated by exclamation marks (!). A three-residue, hypervariable motif in the [2Fe-2S] region is indicated by a box. In most enterics this motif is RSD. Other residues that influence the redox-sensing properties of *E. coli* SoxR are marked by asterisks (Chander and Demple, 2004). The serine residue that was changed to a stop codon to construct the C-terminal truncated *S. coelicolor* SoxR mutant is underlined.

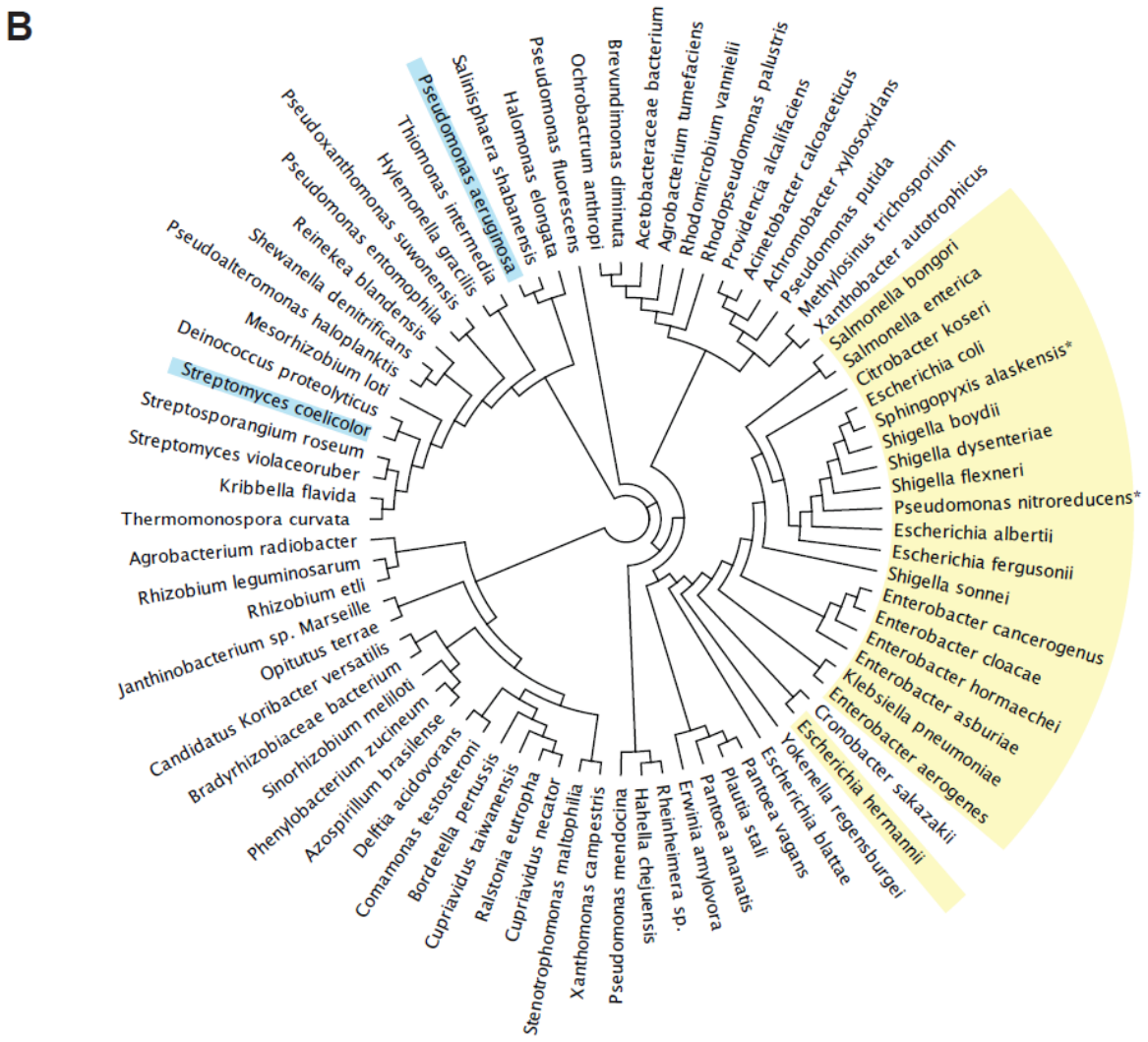


Figure 27 B. Alignment tree of SoxR homologues. Based on the alignment of 250 SoxRs (A) a tree was generated using the Geneious neighbor joining method. For clarity only one member of each represented species is shown. Species that contain the RSD motif in SoxR are highlighted in yellow; these are all enterics excepting *Pseudomonas nitroreducens* and *Sphingopyxis alaskensis*. *P. aeruginosa* and *S. coelicolor* SoxR are highlighted in blue.

The inability of *S. coelicolor* SoxR to complement an *E. coli* Δ*soxR* mutant could result from inefficient protein expression or failure to effect *soxS* transcription. We confirmed that this protein is stably expressed in *E. coli* by immunoblot analysis (Fig. S7A). Furthermore, *S.*

coelicolor SoxR binds efficiently to the *E. coli soxS* promoter *in vitro* (Fig. S7B) and *in vivo* (Fig. S7C), and stimulates transcription of the *soxS* gene *in vitro* (Fig. S7D). Interestingly, the C-terminus of *S. coelicolor* SoxR has an additional 22-residues not present in homologs from enterics or pseudomonads (Fig. 27A). In fact, an extended C-terminal region is found in SoxR proteins from several other *Streptomyces* species (data not shown) and is peculiar to this genus. Given that this is the most obvious structural difference between *S. coelicolor* SoxR and its *E. coli* and *P. aeruginosa* counterparts, we asked if this region could be involved in the regulation of *S. coelicolor* SoxR. To test this, we constructed a mutant that lacks the extreme C-terminal 22-residues by engineering a stop codon at position 154 (see Fig. 27A). We confirmed that the mutant is expressed in *E. coli* and interacts with the *soxS* promoter (Fig. S7B). The transcriptional activity of this protein in response to PMS was measured in the *E. coli* Δ*soxRS* mutant lysogenized with λ[*soxS* promoter-*lacZ* reporter] (Table 9). Figure 28A shows that PMS induced similar ß-galactosidase levels in cells expressing wild type or truncated *S. coelicolor* SoxR proteins. Deletion of the C-terminus also did not affect the activity of this protein when expressed in a *S. coelicolor ΔsoxR* strain. Wild type SoxR and the truncated mutant were equally efficient at activating the SoxR-target genes *SCO4266* and *SCO1178* as assessed by qPCR (Fig. 28B). Thus, the extreme C-terminal region is dispensible for *S. coelicolor* SoxR function, and at this point we have no ready explanation for why *S. coelicolor* SoxR failed to complement the *E. coli* Δ*soxR* mutant.

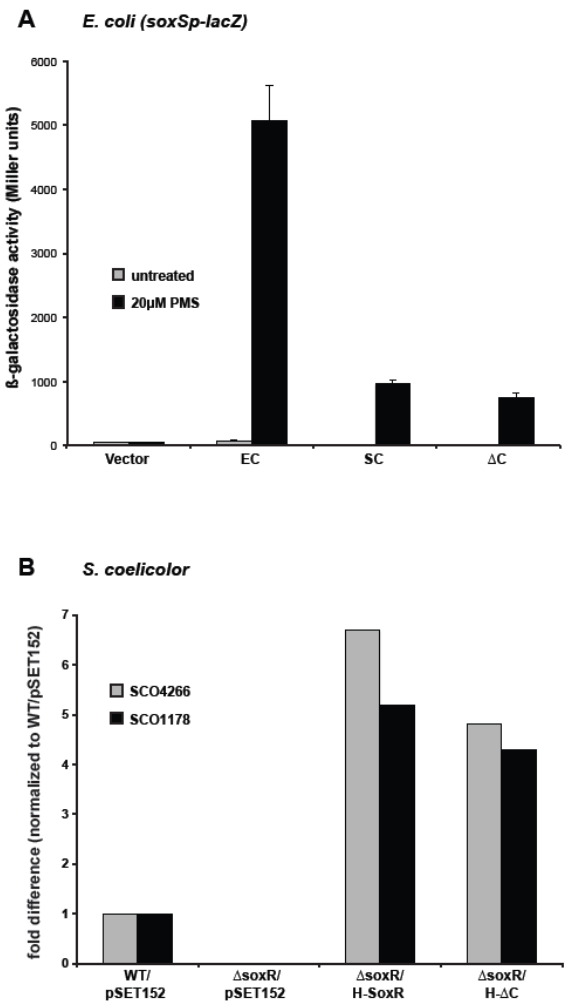


Figure 28. The extended C-terminal region of *S. coelicolor* SoxR is not important for function.
(A) *E. coli* cells (strain EH46) expressing histidine-tagged wild type *E. coli* or *S. coelicolor soxR* alleles, or the *S. coelicolor* C-terminal truncated mutant from pSE380-based plasmids were either untreated (grey columns) or treated with 20 µM PMS (black columns) for 1 h before the assay for ß-galactosidase activity.
(B) qRT-PCR was performed on RNA isolated from the following *S. coelicolor* strains: WT/pSET152, Δ*soxR*/pSET152, and a Δ*soxR* strain complemented with wild type *soxR* (H-SoxR) or the C-terminal truncated mutant (H-ΔC), grown in R5^{-} liquid medium for 3 days. The expression levels of SoxR target genes, *SCO4266* (grey columns) and *SCO1178* (black columns) were standardized to the level of *hrdB* and normalized to expression in WT/pSET152.

4.3.3. Mutations in specific residues alter the redox-sensitivity of *P. aeruginosa* SoxR

The transcriptional assays described in figure 26 demonstrated that *P. aeruginosa* and *S. coelicolor* SoxR were more selective than *E. coli* SoxR, with reduced sensitivity to compounds with low redox potentials (viologens). The activation profiles for *P. aeruginosa* and *S. coelicolor* SoxR (responsive to PMS but not PQ) were reminiscent of *E. coli* SoxR mutant proteins that were reported several years ago (Chander *et al.* 2003; Chander and Demple, 2004). In those

studies, nine residues were identified that, when individually changed, rendered *E. coli* SoxR insensitive to PQ, but fully responsive to PMS (residues marked by asterisks in Fig. 27A). It was suggested that changes in these residues alter the redox-reactivity of SoxR, rendering the protein hyposensitive to certain redox signals. Thus, while these mutant proteins are still activated by the strongly oxidizing drug PMS (redox potential of +80 mV), they are unresponsive to the less oxidizing drug PQ (redox potential of -440 mV). Only two of these residues are conserved in *P. aeruginosa* and *S. coelicolor* SoxR (Fig. 27A). We hypothesized that changing the other seven residues in *P. aeruginosa* or *S. coelicolor* SoxR to those found in *E. coli* SoxR might decrease their drug-selectivity, i.e. that the mutant proteins would respond to PQ. To exclude any SoxR-independent differences between the species, such as drug uptake, we expressed the mutant proteins in *E. coli.* Because *S. coelicolor* SoxR is not active in *E. coli*, we focused our comparison on *P. aeruginosa* and *E. coli* SoxR.

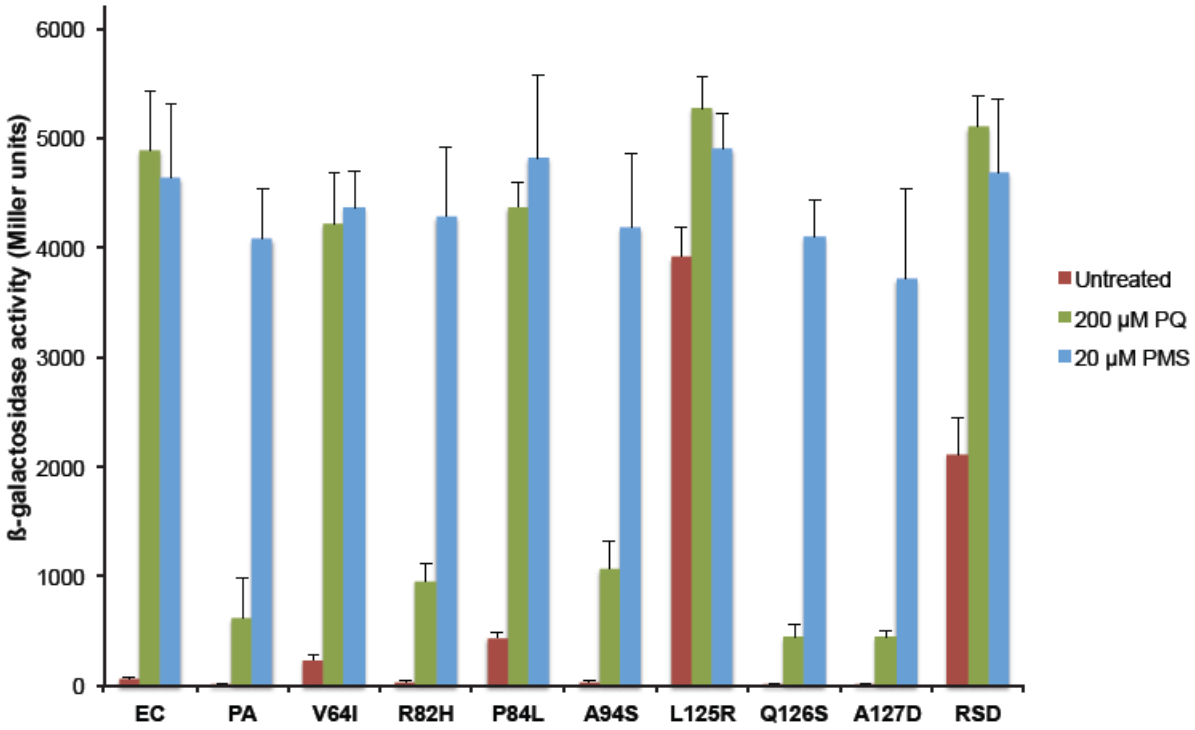


Figure 29. Mutations that alter drug-selectivity of *P. aeruginosa* SoxR. EH46 cells expressing wild type *E. coli* or *P. aeruginosa soxR* alleles, or *P. aeruginosa soxR* mutant alleles from pSE380-based plasmids were untreated (red columns), or treated with 200 µM PQ (green columns) or 20 µM PMS (blue columns) for 1 h before the assay for ß-galactosidase activity. The results represent the means and standard errors of three independent experiments.

We individually mutated five of the aforementioned residues in *P. aeruginosa* SoxR (V64I, R82H, P84L, A94S, L125R) and analyzed the resulting variants using the ß-galactosidase assay described previously. As shown before, while *E. coli* SoxR was activated with similar efficiency by both PQ and PMS, wild type *P. aeruginosa* SoxR was strongly activated by PMS but very weakly by PQ (Fig. 29). Mutant proteins R82H and A94S resembled wild type *P. aeruginosa* SoxR (Fig. 29). Two mutations, V64I and P84L, conferred PQ-sensitivity to *P. aeruginosa* SoxR, essentially converting this protein into its *E. coli* counterpart (Fig. 29). The L125R mutation, which alters a residue in the [2Fe-2S] cluster region, rendered *P. aeruginosa* SoxR

constitutively active; in untreated cells, this variant displayed ~80% of the activity obtained in the presence of PQ or PMS (Fig. 29).

The constitutive activity displayed by the L125R *P. aeruginosa* SoxR variant was unexpected, given that *E. coli* SoxR (which has an Arg in this position) is not constitutive. It is interesting that only SoxR homologs from enteric species contain an Arg in this position, which is replaced by a hydrophobic residue (typically Leu) in SoxR from every non-enteric species analyzed (Fig. 27A and data not shown). We hypothesized that the presence of Arg within the [2Fe-2S] domain makes SoxR constitutively active, as was observed with the L125R *P. aeruginosa* SoxR variant. The fact that *E. coli* SoxR is not constitutive might be attributed to other amino acids in this vicinity that modulate *E. coli* SoxR activity, tuning it so that it is only active in the presence of redox-active drugs. A closer examination of the SoxR sequences from enteric and non-enteric bacteria revealed that while the [2Fe-2S] cluster domain is highly conserved, SoxR homologs from enteric species all contain the hydrophilic "RSD" motif within this region that is absent in SoxRs from other bacteria including *P. aeruginosa*, which instead contains the sequence LQA (Figs. 27A and 27B). In fact, only two non-enteric species, *Pseudomonas nitroreducens* and *Sphingopyxis alaskensis* contain RSD within the sensor domain (Fig. 27B). If the Ser and Asp residues within this motif are indeed responsible for preventing constitutive activity, then one would predict that introduction of these residues into the L125R *P. aeruginosa* variant would dampen the constitutive phenotype, while still allowing sensitivity to PQ. To test this hypothesis, we introduced a triple mutation in *P. aeruginosa* SoxR simultaneously replacing L125, Q126, A127 with RSD, such that the [2Fe-2S] cluster domain was now an exact replica of *E. coli* SoxR, and assayed the ability of this protein to activate *soxS* transcription in the absence of redox-

cycling drugs, or in the presence of PQ or PMS. Fig. 29 shows that the RSD triple mutant more closely resembles *E. coli* SoxR than *P. aeruginosa* SoxR, in that it is responsive to PQ. While this variant still displays partial constitutive activity, this is considerably diminished when compared to the L125R single mutant. This can be attributed to introduction of the additional two mutations Q126S and A127D. The Q126S or A127D mutations alone did not alter the activity of *P. aeruginosa* SoxR (Fig. 29).

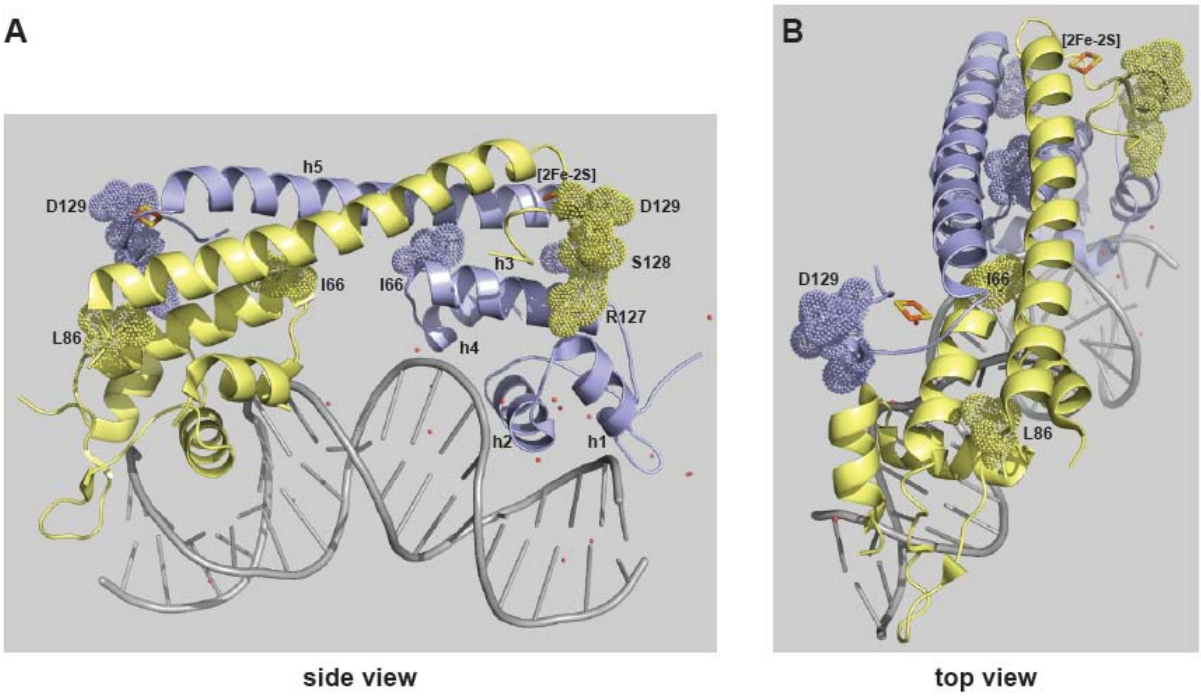


Figure 30. Location of key residues in *E. coli* SoxR. The structure of *E. coli* SoxR protein complexed with the *soxS* promoter is depicted side-on (A) or from the top (B) (Watanabe *et al.*, 2008). Helices labeled h1-h4 comprise the DNA binding domain; h5 is the dimerization domain; the [2Fe-2S] cluster in one monomer is labeled. Residues (I66, L86, R127, S128, D129) identified as playing an important role in tuning the redox-reactivity of SoxR are shown. The images were created using PyMOL.

4.4. DISCUSSION

SoxR regulates a global stress response against redox-cycling drugs in *E. coli*. As such this protein is engineered to sense and respond to a wide spectrum of redox-active molecules that vary in structure and redox potentials. This is not the case for *P. aeruginosa* and *S. coelicolor* in which SoxR performs a more specific role as suggested by the small number of genes it regulates in response to endogenous redox-active signals. Given the functional differences of SoxR homologs across species, we asked if the sensitivities of *P. aeruginosa* and *S. coelicolor* SoxR were tuned towards compounds that resemble phenazines and Act, respectively. We found this to be partially true. While *E. coli* SoxR was activated by structurally distinct drugs that span a range in redox potentials from ~-450 to ~+80 mV, *P. aeruginosa* and *S. coelicolor* SoxR were less sensitive to compounds with redox potentials below ~-300 mV. Thus SoxR appears tuned to sense drugs based on their redox potentials rather than a particular structure.

What is the mechanism underlying SoxR's differential selectivity for drugs? An earlier study on *E. coli* SoxR had identified residues that, when mutated, reduce the protein's reactivity to drugs with low redox potentials (such as PQ). Five of these residues (Ile66, His84, Leu86, Ser96, Arg127) are conserved in SoxR homologs from enterics (which display broad drug selectivity), but not in those from non-enterics (which show narrower drug selectivity). Mutation of the corresponding residues in *P. aeruginosa* SoxR to those found in *E. coli* SoxR revealed that three of the five residues individually affected drug-sensitivity. Mutations V64→Ile and Pro84→Leu both increased the sensitivity of *P. aeruginosa* SoxR to PQ, and the variants were indistinguishable from *E. coli* SoxR. Mutation Leu125→Arg resulted in constitutive activity.

These amino acids are conserved in *S. coelicolor* SoxR (V65, P85, L126; Fig. 3A), which like *P. aeruginosa* SoxR showed reduced sensitivity to viologens (Fig. 26C). These findings emphasize the importance of these amino acids in SoxR redox-reactivity, and suggest structural changes that *E. coli* SoxR may have evolved if its *soxR* gene was acquired by horizontal gene transfer.

SoxR forms a homodimer. Each subunit contains three distinct domains: a DNA binding domain composed of four helices (h1-h4), a coiled-coil dimerization helix (h5) and the C-terminal sensor domain that contains the [2Fe-2S] clusters (Fig. 30). The crystal structure for the oxidized SoxR dimer bound to DNA shows that helices 3 and 4 within the DNA binding domain make hydrophobic contacts with helix 5 within the same subunit (Watanabe *et al.*, 2008). Furthermore, the metal binding domain of one subunit is stabilized by interactions with helices 3, 4 and 5 of the other monomer. The structure of reduced SoxR is unknown, but using Raman spectroscopy, Kobayashi and colleagues (2011) showed that the relative orientations of helices 3 and 4 (in the DNA binding domain) and helix 5 (dimerization domain) depend on the redox state of SoxR. It is tempting to speculate that transmission of oxidative signals from the [2Fe-2S] clusters to the DNA involves an orchestrated rearrangement of the metal binding, dimerization and DNA binding domains, thereby explaining how the redox signal may be propagated from the [2Fe-2S] clusters to the DNA. Conversely, potential structural changes that result from DNA binding of SoxR have dramatic effects on the redox potential of its [2Fe-2S] cluster, highlighting the fine-tuned feedback between the DNA binding and sensory domains (Gorodetsky *et al*., 2008). It is therefore not surprising that even small changes in the protein structure impact sensing and activation. We propose that Ile66, His84, Leu86, Ser96, Arg127 in *E. coli* SoxR, and their counterparts in other species, are some of the key residues that mediate the functional interaction

between DNA binding and sensing. Ile66, His84 and Leu86 are located at the interface between helix 3 in the DNA binding domain and the dimerization helix 5 (Fig. 30). Leu86, for example, which is located just upstream of helix 5, forms hydrophobic interactions with Tyr56 and Ile59 in helix 3 (Watanabe *et al.*, 2008). Interestingly, helix 3 also interacts with the sensor domain of the second dimer subunit (Fig. 30). Considering that the redox potentials of Fe-S clusters are modulated by their immediate environment, any changes affecting these interactions may ultimately tune SoxR sensitivity to redox-active compounds. Our findings demonstrate that even point mutations can change the sensitivity towards specific compounds. A particularly intriguing example is the [2Fe-2S] binding site itself. Although it is remarkably conserved among SoxR homologs, it contains a hypervariable stretch of three residues (Fig. 27A). Strikingly, within almost all enterics we found it to be conserved as the charged RSD motif (Fig. 27B). In contrast, in *P. aeruginosa* it is replaced by LQA (Fig. 27A). We suggest that these hydrophobic amino acids shield the [2Fe-2S] clusters from the solvent, making them less accessible to weakly oxidizing drugs like PQ.

When *P. aeruginosa* SoxR was mutated to replace the LQA motif with RQA, the resulting variant displayed strong constitutive activity. Thus having an Arg residue within the [2Fe-2S] domain makes SoxR constitutively active. However, when the original LQA motif was mutated to RSD (so that the [2Fe-2S] cluster was now identical to that in *E. coli* SoxR), the level of constitutive activity significantly decreased, but the protein still retained the ability to respond to PQ. Thus, the RSD motif in enteric SoxRs is essential for fine-tuning the protein's redox activity – preventing constitutive activity while retaining low selectivity for drugs.

Our findings give insight into the diversity of SoxR proteins with respect to their ability to sense redox-active compounds. They demonstrate how minor changes in the primary sequence can lead to the evolution of SoxR proteins with narrow- or broad-range sensing capacities.

4.5. EXPERIMENTAL METHODS

4.5.1. Bacterial strains and plasmids

Bacterial strains and plasmids that were utilized or constructed in this study are listed in Table 9.

4.5.2. Redox-cycling drugs

The redox-cycling drugs used in this study are listed in Table 1 along with their chemical structures and midpoint redox potentials. All chemicals were purchased from Sigma, with the exception of PCA which was purchased from Princeton Biomolecular Research, and γ-actinorhodin which was extracted from *S. coelicolor* cells as described by Bystrykh *et al* (1996). PQ, DQ, MB, and PMS were dissolved in water; PB, Pyo, PCA and Act in dimethylsulfoxide; 4NQO in acetone.

4.5.3. Drug susceptibility tests

The effects of various redox active drugs on the growth of wild type and Δ*soxR E. coli*, *P. aeruginosa*, and *S. coelicolor* cells were determined using a disk diffusion assay (strains are listed in Table 9). *E. coli* and *P. aeruginosa* cells were grown for 16 h at 37°C in LB medium, 100 µL added to 4 mL of melted soft nutrient agar (Difco), then plated on nutrient agar plates (Difco). *S. coelicolor* spores (~10^8) were similarly plated. Six-millimeter Whatman paper disks impregnated with 15 µL of drug were placed onto the agar. *E. coli* plates were incubated at 37°C for 24 h, and *P. aeruginosa* and *S. coelicolor* plates at 30°C for 48 h, after which the zone of growth inhibition around each disk was recorded.

4.5.4. Cloning of his-tagged *soxR* genes for complementation analysis in *E. coli* and *P. aeruginosa*

For complementation analysis in *E. coli*, the *soxR* alleles from *E. coli*, *P. aeruginosa*, and *S. coelicolor* were expressed as N-terminally histidine-tagged proteins from the plasmid pSE380 under the control of the *trc* promoter (Table 9). The coding region of the *soxR* alleles (including the histidine-tag) was PCR-amplified from pET16b-based vectors (Chander and Demple, 2004, Gorodetsky *et al.*, 2008, Dela Cruz *et al.*, 2010) using primers pET-F and pET-R (Table 7) and Pfu Polymerase (Stratagene). The PCR fragments were digested with BamHI and SalI and ligated into pSE380. The resulting plasmids containing *soxR* alleles with a 10-histidine tag attached to the N-terminus were sequenced on both strands and transformed into *E. coli* strain EH46 or EH86 for ß-galactosidase assays (Table 9).

For complementation analysis in *P. aeruginosa*, the histidine-tagged *soxR* proteins were subcloned from pSE380 into the BamHI/SalI site of the vector, pUCp18 (Table 9), and expressed under the control of the *lac* promoter. The resulting clones were sequenced on both strands and transformed into *P. aeruginosa* strain PA14*ΔsoxR* (Table 9).

4.5.5. Construction of *soxR* mutant alleles

Mutations in the *P. aeruginosa soxR* gene were generated using the GENEART site-Directed mutagenesis kit from Invitrogen according to the manufacturer's instructions. Mutations in the *S. coelicolor soxR* gene were generated using the QuikChange site-directed mutagenesis kit from Stratagene following manufacturer's recommendations. Plasmid pSE380, containing the histidine-tagged *soxR* genes from *P. aeruginosa* or *S. coelicolor* were used as templates for mutagenesis along with the mutagenic primers listed in Table 7. All mutations were verified by DNA sequence analysis.

For expression of the histidine-tagged C-terminal truncated *soxR* gene in *S. coelicolor*, the coding region (along with the 10-histidine tag) was PCR-amplified from pSE380 using Pfu polymerase and primers 380F-Bam and 380R-Bam (Table 7), and subcloned into the BamHI site of the integrating vector pSET152, to yield H-ΔC. The histidine-tagged WT *S. coelicolor soxR* gene was similarly constructed to yield H-SoxR. The pSET152-based plasmids were introduced into the *S. coelicolor ΔsoxR* strain M145-1A by intergenic conjugation from *E. coli* ET12567/pUZ8002.

4.5.6. ß-galactosidase assay to measure complementation in an *E. coli ΔsoxR* mutant

The ability of the various SoxR homologs (and mutant derivatives) to complement an *E. coli* Δ*soxR* strain was assessed by measuring ß-galactosidase activity in EH46 cells (Table 9) expressing the various histidine-tagged SoxR proteins from pSE380-based plasmids as previously described (Chander *et al.*, 2003). Cells were treated with various redox-active drugs for 1 h with shaking at 220 rpm.

ß-galactosidase assays were also used to analyze the stable production and *soxS* promoter binding ability of the various SoxR proteins *in vivo.* Strain EH86 (Table 9) was transformed with the aforementioned plasmids and grown for 2.5 h in the absence of oxidative stress before the lysates were assayed for ß-galactosidase activity.

4.5.7. qRT-PCR assay in *S. coelicolor*

Liquid R5$^-$ medium (Huang *et al.* 2001) was inoculated with 10^7 *S. coelicolor* spores mL^{-1} and grown at 30°C with shaking at 220 rpm for the indicated times. Cells were harvested by incubating with RNAprotect bacterial reagent (Qiagen) for 5 min at room temperature, centrifuging for 10 min at 5,000 x g, and frozen at -80°C. Total RNA was extracted and qRT-PCR assays conducted as previously described (Dela Cruz *et al.*, 2010). The primers used for qRT-PCR are listed in Table 7.

4.5.8. Construction of *P. aeruginosa mexG-gfp* reporter strains and Gfp fluorescence quantification

The *mexG* promoter region was PCR-amplified from PA14 genomic DNA using primers pmexG-F and pmexG-R (Table 7), and cloned into the HindIII/EcoRI site of the vector pYL122 (Table 9). The p*mexG-gfp* reporter fusion was integrated into the *attB* site of *P. aeruginosa* PA14 or PA14Δ*soxR* using a previously described protocol (Lequette and Greenberg, 2005).

To quantify Gfp fluorescence, the p*mexG-gfp* reporter strains expressing histidine-tagged *E. coli* or *P. aeruginosa soxR* from pUCp18, were grown in LB medium supplemented with carbenicillin (300 µg mL^{-1}) for 16 h at 37°C. Cultures were then diluted 100-fold and grown for an additional 3 h (to logarithmic phase), before finally diluting to an optical density of 0.05 at 500 nm into a 96-well plate (Costar). The optical density and fluorescence was monitored for 19 h using a Synergy 4-plate reader (BioTek). The excitation wavelength was 488 nm; emission wavelength was 520 nm. Data was acquired using the Gen5 program.

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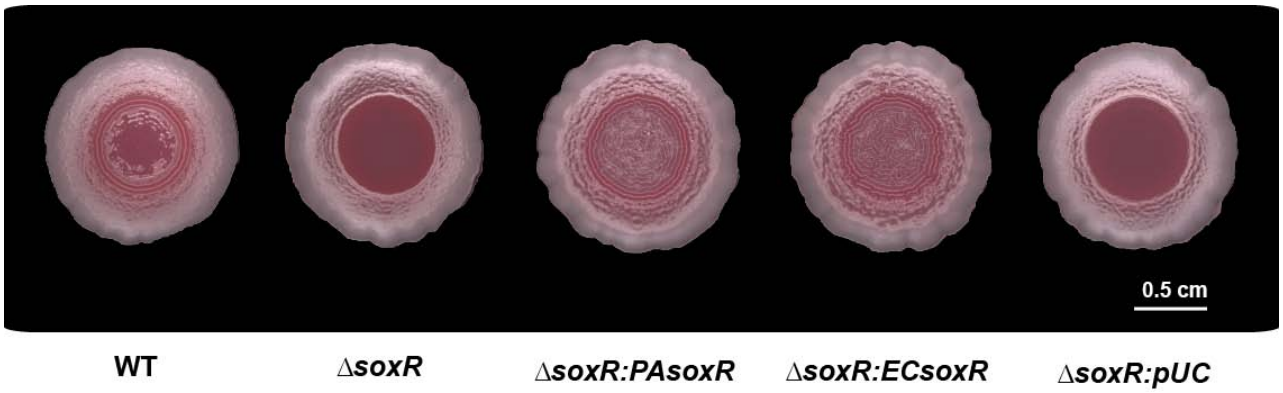
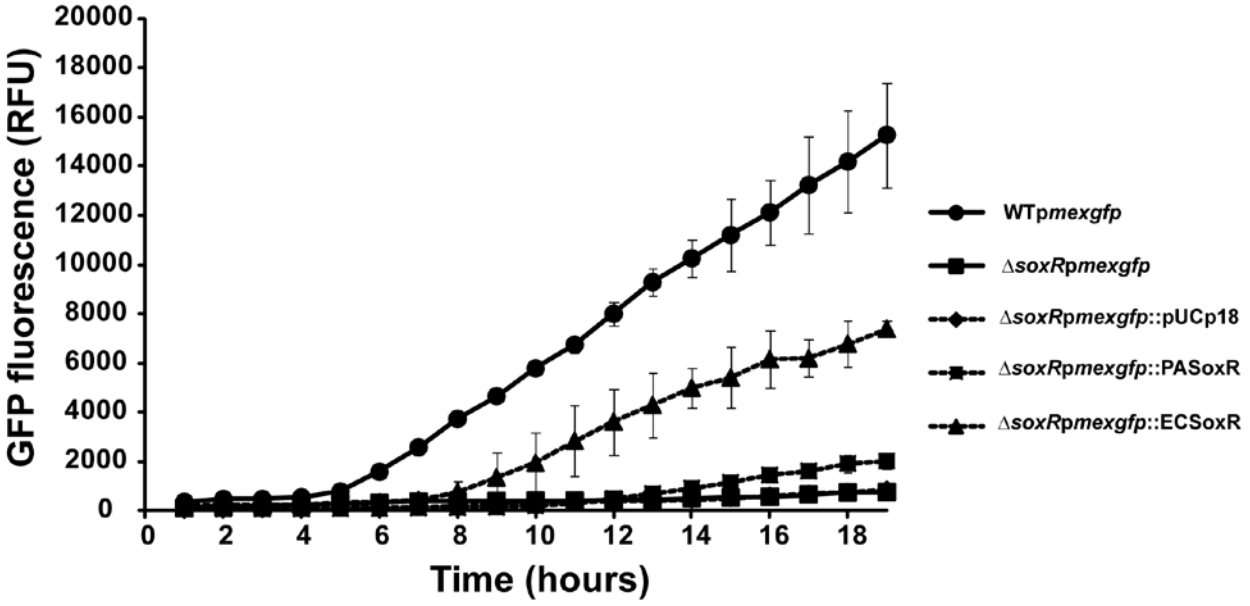
4.7. Appendix

Supplementary Figures

A.

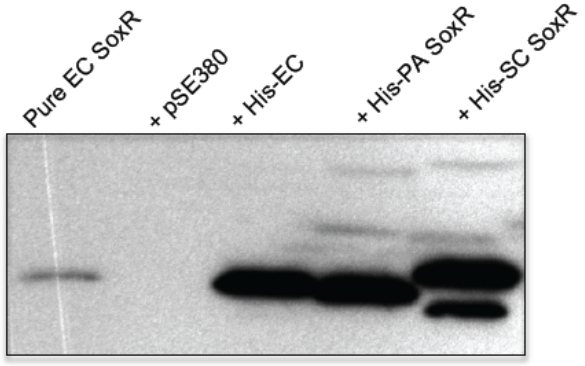


B.

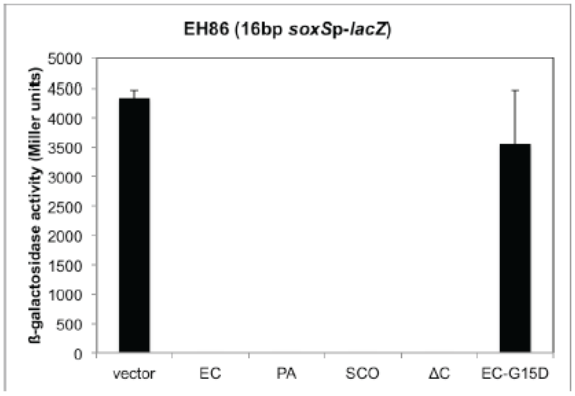


Supplementary Figure 6 (S6). Complementation of *P. aeruginosa* Δ*soxR* mutant by *E. coli* or *P. aeruginosa* SoxR.
(A) Growth of WT, Δ*SoxR*, Δ*SoxR::PAsoxR*, Δ*SoxR::ECsoxR*, Δ*SoxR::pUC* strains on agar plates. Colony development was followed for 6 days. Images for day 2 are shown (B) GFP fluorescence quantification of Δ*soxR*p*mexgfp* strains complemented with plasmids containing PA and EC SoxR in planktonic cultures. The *mexGHI-opmD* operon encodes a transporter that regulates the export of phenazines (Dietrich, *et al*., 2008). The phenazine pyocyanin regulates the expression of this operon through SoxR. Quantifying *mex*gfp expression is a proxy for signaling through SoxR. Strains were diluted to a 0.05 OD. The OD500 and gfp fluorescence were then monitored for 19 hours.

A. His-SoxR expression in *E. coli*

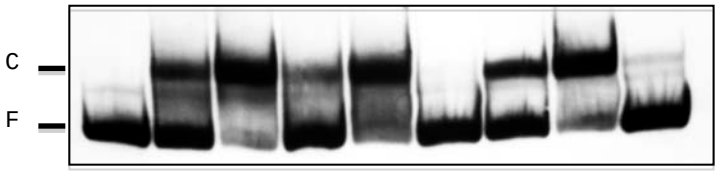


B. *soxS* promoter binding *in vivo*



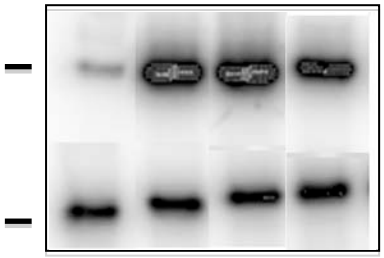
C. soxS promoter binding *in vitro*

SoxR	-	EC	EC	SC	SC	SC	PA	PA	PA
nM	0	1	10	1	10	10	1	10	10
Comp.DNA	-	-	-	-	-	+	-	-	+



D. In vitro transcription of soxS gene

SoxR	-	EC	PA	SC
nM	0	250	250	250



% of EC	12	100	103	80

Supplementary Figure 7 (S7). SoxR protein expression, *soxS* promoter binding, and in vitro transcription of the *soxS* gene.
(A) SoxR protein levels in E. coli. EH46 cells expressing empty vector (pSE380) or histidine-tagged SoxR proteins from E. coli (His-EC SoxR), P. aeruginosa (His-PA SoxR), or S. coelicolor (His-SC SoxR) were grown at 37°C for 2.5 h. Total cell extract (50 µg per lane) was resolved on a 15% SDS-polyacrylamide gel and subjected to immunoblot analysis using anti-histidine antibody (GE Healthcare). Purified histidine-tagged E. coli SoxR (10 ng) was loaded as a control. His-SC SoxR migrates as a higher molecular weight species than His-EC and His-PA SoxR.

(B) SoxR binding to soxS promoter in vivo. E. coli strain EH86 (ΔsoxRS lysogenized with λ[16bp soxS promoter-lacZ reporter) was transformed with vector control, histidine-tagged E. coli, P. aeruginosa or S. coelicolor soxR genes, C-terminal truncated S. coelicolor soxR, or the E. coli DNA binding variant G15D. The wild type soxS promoter has a 19-bp spacer separating the -10 and -35 elements. The shortened (16-bp) soxS promoter renders soxS transcription constitutive and promoter occupation by SoxR prevents access to RNA polymerase (Hidalgo and Demple, 1997). Low ß-galactosidase activity in this background thus indicates specific promoter binding by SoxR, while high ß-galactosidase activity indicates defective promoter binding as demonstrated by cells expressing the E. coli SoxR DNA-binding mutant G15D (Fig. 2D, Chander et al. 2003). The values shown represent the means and standard errors of three independent experiments.

(C) SoxR protein binding to soxS promoter in vitro. A DIG-end-labeled fragment (180 bp) containing the soxS promoter was incubated with 1 nM or 10 nM purified histidine-tagged SoxR proteins from E. coli (EC), S. coelicolor (SC), or P. aeruginosa (PA). Protein-bound complexes [C] were separated from free DNA [F] on a 5% native polyacrylamide gel. SoxR binding specificity was demonstrated by the addition of a 500-fold molar excess of unlabeled probe (Comp. DNA).
(D) Transcription of the soxS gene in vitro. Purified histidine-tagged SoxR proteins (250 nM) from E. coli (EC), P. aeruginosa (PA), or S. coelicolor (SC) were incubated with a plasmid containing the soxS and bla genes, E. coli σ^{70}-RNA polymerase, and four ribonucleotide triphosphates for 15 min at 37°C. The soxS and bla transcripts were quantified by primer extension analysis as described (Chander and Demple, 2004). Reactions were electrophoresced on 8% polyacrylamide, 6 M urea gels and quantified on a Storm phosphorimager. The bla gene is a SoxR-independent transcript and serves as a loading control. The amount of soxS mRNA is reported as a percent of the amount obtained with E. coli SoxR.

TABLES

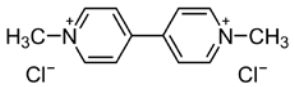
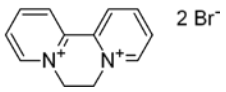
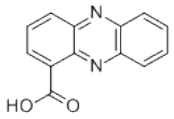
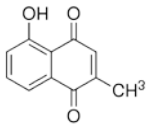
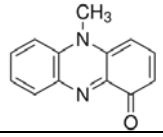
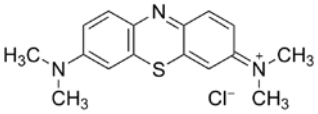
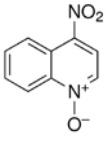
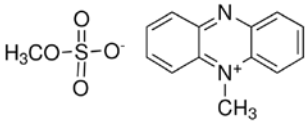
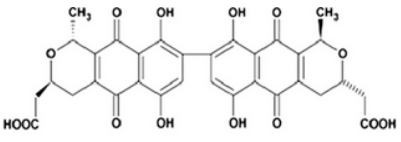
Table 7. Primers used in Chapter 4

Cloning	Sequence (5’ - 3’)		
pET-F	CGC GTC GAC TCA CTA TAG GGG AAT TGT G		
pET-R	GCT TTG TTA GCA GCC G		
380F-Bam	CCG CCG GAT CCG ACA TCA TAA CGG TTC TGG C		
380R-Bam	GCA GAT CTG TCA TGA TG		
pmexG-F	TAC CAA GCT TCT CGT GGC CAA CCA GAA TAG		
pmexG-R	TTG CGA ATT CGT CGT TCCT TGT GCT GGT C		
PA Mutagenic*	Sequence (5’ - 3’)		
PA-V64I	AAG GTC GCC CAG CGG ATC GGC ATT CCC CTC G		
PA-R82H	CCC TGC CGG CCG GGC ACA GCC CTA GCG CGG C		
PA-P84L	CGG CCG GGC GCA GCC TTA GCG CGG CGG ACT G		
PA-A94S	TGG GCG CGC CTG TCG TCG CAG TGG AAG GAG G		
PA-L125R	GCG GCT GCC TGT CGC GCC AGG CCT GCC CGT TG		
PA-Q126S	GGC TGC CTG TCG CTC TCG GCC TGC CCG TTG CG		
PA-A127D	GCC TGT CGC TCC AGG ACT GCC CGT TGC GCA AC		
PA-RSD	GCG GCT GCC TGT CGC GCT CGG ACT GCC CGT TGC GCA AC		
SCO Mutagenic*	Sequence (5’ - 3’)		
SCO-S154stop	GGA GCG CCG CGG CTG AAC CGC CAG GGG C		
qRT-PCR	Sequence (5’ - 3’)	Amplicon	Size (bp)
hrdB-F	CAT GCG CTT CGG ACT CA	hrdB	95
hrdB-R	ACT CGA TCT GGC GGA TG		
1178-F	TCA AGG TCC GGC AGG TCT A	SCO1178	82
1178-R	CCG TCC TCC TGC TTG GT		
2478-F	GAG ATC ACC CCG AAA CTG G	SCO2478	104
2478-R	AAG TGC CAG TCG ATG ACG TT		
4266-F	GAT GGG CAT CCT CCA GTT C	SCO4266	104
4266-R	CGT TCT TCG CGT ACT GCA C		

* Sequence of forward primers used to mutagenize P. aeruginosa and S. coelicolor soxR genes cloned in plasmid pSE380 using either Invitrogen’s GENEART site-directed mutagenesis kit (for P. aeruginosa) or

Stratagene QuikChange site-directed mutagenesis kit (for S. coelicolor). Underlined sequence indicates change from original nucleotide. Reverse primers are complementary to forward primers.

Table 8. Redox drugs used in Chapter 4*

Class	Drug	Structure	Redox potential (mV)
Viologen	Paraquat (PQ)		-440 (Skeckhan and Kuwana, 1974)
Viologen	Diquat (DQ)		-361 (Steckhan and Kuwana, 1974)
Phenazine	Phenazine-1-carboxylic acid (PCA)		-177 (Price-Whelan *et al.*, 2006)
Napthoquinone	Plumbagin (PB)		-135 (Hakura *et al.*, 1994)
Phenazine	Pyocyanin (Pyo)		-34 (Friedheim and Michaelis, 1931)
Phenothiazine	Methylene blue (MB)		+11 (Kamat *et al.* 1987)
Quinoline	4-Nitroquinoline-N-oxide (4NQO)		+74 (Biaglow *et al.* 1978)
Phenazine	Phenazine methosulfate (PMS)		+80 (Moffet *et al.*, 2003)
Anthraquinone	γ-Actinorhodin (Act)		Unknown

* Drugs are arranged in order of increasing midpoint redox potential. The redox potentials are reported versus the normal hydrogen electrode (NHE).

Table 9. Bacterial strains and plasmids used in Chapter 4

Strain/plasmid	Genotype/description	Source/reference
E. coli		
GC4468	K12 *rpsL thi soxR*$^+$ *soxS*$^+$	Greenberg *et al.* (1990)
DJ901	Δ(*soxRS*) derivative of GC4468	Greenberg *et al.* (1990)
EH46	DJ901 lysogenized with λ(*soxS* promoter-*lacZ*)	Hidalgo and Demple (1997)
EH86	DJ901 lysogenized with mutant *soxS* promoter mutant-*lacZ*)	Hidalgo and Demple (1997)
P. aeruginosa		
PA14	Clinical isolate UCBPP-PA14	Rahme *et al*. (1995)
PA14Δ*soxR*	PA14 with a deletion in *soxR*	Dietrich *et al.* (2006)
WTp*mexgfp*	PA14 with insert of *mexG* promoter fused to *gfp* reporter	This study
Δ*soxR*p*mexgfp*	PA14Δ*soxR* with insert of *mexG* promoter fused to *gfp* reporer	This study
S. coelicolor		
M145	SCP1$^-$, SCP2$^-$	Kieser *et al.* (2000)
M145-1A	M145 with a deletion in *soxR*	Dela Cruz *et al.* (2010)
M511	Δ*act*II-ORF4 derivative of M145	Floriano and Bibb (1996)
M511Δ*soxR*	Δ*soxR* derivative of M511	Dela Cruz *et al.* (2010)
Plasmids		
pSE380	*trc* promoter-containing plasmid with *lacI*^{q} gene (Ampicillin^r)	Invitrogen
pSE380:H-ECO	N-terminally histidine-tagged *E. coli soxR* gene in pSE380	This study
pSE380:H-PA	N-terminally histidine-tagged *P. aeruginosa soxR* gene in pSE380	This study
pSE380:H-SCO	N-terminally histidine-tagged *S. coelicolor soxR* gene in pSE380	This study
pSET152	Apramycin^r *lacZα* MCS *rep*^{pUC}	Bierman (1992)
pSET152:H-SCO	N-terminally histidine-tagged *S. coelicolor soxR* gene in pSET152	This study
pSET152:H-ΔC	N-terminally histidine-tagged truncated *S. coelicolor soxR* gene	This study
pUCp18	Carbenicillin^r, Broas host vector	Schweizer (1991)
pUC:*ECsoxR*	N-terminally histidine-tagged *E. coli soxR* gene in pUCp18	This study
pUC:*PAsoxR*	N-terminally histidine-tagged *P. aeruginosa soxR* gene	This study
pYL122	Ampicillin^r, *rhlA-gfp* transcription fusion in mini-CTX-*lacZ*	Lequette (2005)

Chapter 5. Conclusions and Future Directions

Pseudomonas aeruginosa is an opportunistic pathogen that has the capacity to inhabit a variety of environments. One of the defining characteristics of *P. aeruginosa* is its ability to produce a class of redox-active molecules known as phenazines. Phenazines are multi-faceted molecules with a variety of biochemical properties. Recent research has found that phenazines are not only virulence factors but can also act as signaling molecules to aid in *P. aeruginosa's* adaptation to its environment[1,2,3]. For example, phenazines modulate the development of multicellular communities known as biofilms[4]. Like many bacteria, *P. aeruginosa* forms biofilms in diverse environments. The formation of biofilms involves a concerted effort by billions of bacteria and the process involves many signaling molecules including phenazines. However, exactly how phenazines modulate biofilm formation has yet to be elucidated.

In this work, we have investigated how phenazines affect biofilm development. It is well established that growth in a sessile biofilm is different from a planktonic environment and leads to global transcriptomic and proteomic changes[5-7]. I began by investigating how growth in the biofilm affected phenazine production when compared to growth in the planktonic environment. *P. aeruginosa* contains two redundant 7-gene operons that are responsible for phenazine production[8]. The role and regulation of phenazine production through one of the operons (*phz1*) has been studied in the planktonic environment[9]. The role and regulation of the second phenazine operon (*phz2*), however, had yet to be elucidated. We investigated the regulation of the *phz2* operon and its contribution to colony biofilm development in *P. aeruginosa* strain PA14. We found that phenazine production in biofilms is mediated exclusively through the *phz2* operon, *phz2* expression is required for biofilm development and host colonization and *phz2* is regulated

by quinolones, which are prominent signaling molecules in *P. aeruginosa*'s QS system. We then investigated the roles of individual phenazines in colony development and the specificity of SoxR activation by redox-active molecules. We found that the effects of individual phenazines are not redundant and may be used in combination to modulate colony development (Chapter 3). SoxR is a transcription factor that is activated by redox-active molecules including phenazines[3,10]. Our investigations into SoxR specificity showed that SoxR activation in *P. aeruginosa* is tuned to specific redox potentials, which are similar to those of phenazines (Chapter 4). Together, these findings have expanded our knowledge about the role of phenazine production in biofilms. When biofilms form in the host, they enhance *P. aeruginosa's* already heightened ability to resist antibiotics. This is thought to be due to the steric hindrance caused by the polysaccharides that compose the biofilm matrix as they prevent antibiotics from reaching the bacterial cells[11]. Elucidating the mechanism of how phenazines are regulated and how they modulate colony development may lead to new strategies for designing therapeutics that prevent biofilm maturation.

One of the major questions we addressed in this work is how phenazine production is affected by growth in the biofilm environment. More specifically, we addressed the regulation of *phz2* and its role in phenazine production. Previous work had established that the Pseudomonas quinolone signal (PQS) regulates the *phz1* operon in the planktonic environment[12,13]. Due to the fact that the upstream regulatory elements between the *phz1* and *phz2* are different, we hypothesized that phenazine production and regulation may differ in an environment-dependent manner. We found that *phz2* is responsible for all of the phenazine production in the colony and host environments. We also found that both PQS and its precursor 2-heptyl-4-quinolone (HHQ) regulate the *phz2* operon. Since production of PQS, but not HHQ, is oxygen-dependent, we hypothesize that the

HHQ/*phz2* regulation may have evolved as an adaptation to allow for phenazine production under the micro- or anaerobic regions of the biofilm environment. These results suggest that the environment-dependent expression of the *phz2* operon aids *P. aeruginosa* in adapting to its environment. It is tempting to speculate that the phenazine operons have been maintained in the *P. aeruginosa* genome based on their environment-specific activities. Further experiments are required to validate this hypothesis. First we need to address the expression of the *phz2* operon in anaerobic environments. Using reporter constructs that function without oxygen, we will quantify the expression of *phz2* in a spatial and temporal manner within biofilms. This will tell us if there is a difference in expression between aerobic and anaerobic zones. Another test of our hypothesis are fitness assays. For example, competition assays can be performed on agar plates or in lung infection models using our phenazine operon mutants.in co-culture with other bacteria. This will test if the second phenazine operon gives *P. aeruginosa* an advantage and allows it to thrive in the biofilm or host environments.

Another major question we addressed is which phenazines are important for colony development. In other words, why does *P. aeruginosa* produce different phenazines? Previous work had established that phenazines are important for and may have distinct effects on colony development[4]. We found that phenazine-1-carboxamide (PCN) and 5-methylphenazium (5-MCA) have synergistic effects and are both necessary for normal colony development. Based on our results, we propose that the functions of phenazines are governed by the different environmental niches present within biofilms. For example, the signaling and redox balancing properties of pyocyanin (PYO), whose production is oxygen-dependent, may be important in aerobic zones while the oxygen-independent phenazines PCN and 5-MCA may assert their functions in anaerobic zones. Based on our results, we hypothesize that PCN affects colony

morphology through redox balancing while 5-MCA may affect colony development through non-redox balancing properties such as signaling. However, more work needs to be done to validate our model. First, we need to confirm that 5-MCA is produced under anaerobic conditions. We could probe for the presence of 5-MCA in anaerobically grown planktonic cultures or create reporter constructs for PhzM and PhzH in order to visualize their localization using colony thin sections. The latter method would be performed using fluorescent probes that can function under anaerobic conditions.

Our results suggest that the role of 5-MCA in colony development may not depend on its redox properties. 5-MCA is transformed by an unknown enzyme to produce the aeruginosins[14,15]. The aeruginosins are hydrophilic and difficult to isolate from *P. aeruginosa* cultures. Novel isolation techniques will be employed to accurately purify and quantitate 5-MCA. Once 5-MCA is isolated, we can investigate its properties including its potential role as a signaling factor. This can be investigated using DNA microarray analysis of cells that have been treated with exogenous 5-MCA, or by using mutants that are only able to produce 5-MCA.

Lastly, we investigated how phenazines elicit a response that affects colony development. Phenazines are signaling molecules that affect the expression of genes involved in many cellular processes through several transcription factors[3]. The best-studied phenazine-dependent transcription factor is SoxR. In enterics, SoxR triggers a global stress response by sensing a broad spectrum of redox-cycling compounds[16,17]. In the non-enteric bacteria *Pseudomonas aeruginosa* and *Streptomyces coelicolor,* SoxR is activated by endogenous redox-active small molecules and only regulates a small set of genes[3,18]. We investigated the specificity of SoxR and its ability to respond to molecules with specific redox potentials. We found that while *E. coli*

SoxR is tuned to compounds that span a redox range of -450 to +80 mV, *P. aeruginosa* and *S. coelicolor* SoxR are less sensitive to molecules with redox potentials below -300 mV. Our findings give insight into the diversity of SoxR proteins with respect to their ability to sense redox-active compounds. They demonstrate how minor changes in the primary sequence can lead to the evolution of SoxR proteins with narrow- or broad-range sensing capacities. Future studies can be directed at elucidating the specific residues within the iron-sulfur core of SoxR that transduce the redox signals. Our results pointed to five residues that may be important for the functional interaction between DNA binding and response. Further mutagenesis studies of these residues and their counterparts in other species may help pinpoint the key residues needed for the conformational change in SoxR.

In summary, *Pseudomonas aeruginosa* is a versatile bacterium that can inhabit diverse environments such as water, air, soil and host organisms. Phenazine production and formation of multi-cellular communities are two important aspects of its physiology that help this bacterium adapt to different environments. The work presented in this thesis represents novel and important findings towards an elucidation of the mechanism of phenazine regulation and function in the biofilm and host environments. The knowledge gained by these studies can be used as the basis for further investigation into *P. aeruginosa* community formation as well as the development of new therapeutics against *P. aeruginosa* infections.

5.1. References

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