

Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes

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Received: 16 January 2010 / Revised: 11 February 2010 / Accepted: 12 February 2010 / Published online: 30 March 2010
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Abstract Phenazines constitute a large group of nitrogen-containing heterocyclic compounds produced by a diverse range of bacteria. Both natural and synthetic phenazine derivatives are studied due their impacts on bacterial interactions and biotechnological processes. Phenazines serve as electron shuttles to alternate terminal acceptors, modify cellular redox states, act as cell signals that regulate patterns of gene expression, contribute to biofilm formation and architecture, and enhance bacterial survival. Phenazines have diverse effects on eukaryotic hosts and host tissues, including the modification of multiple host cellular responses. In plants, phenazines also may influence growth and elicit induced systemic resistance. Here, we discuss emerging evidence that phenazines play multiple roles for the producing organism and contribute to their behavior and ecological fitness.

Keywords Phenazine · Secondary metabolite · Electron shuttling · Antibiotic · Biofilm

Introduction

Phenazines comprise a large group of nitrogen-containing heterocyclic compounds that differ in their chemical and

physical properties based on the type and position of functional groups present. More than 100 different phenazine structural derivatives have been identified in nature, and over 6,000 compounds that contain phenazine as a central moiety have been synthesized (Mavrodi et al. 2006). Bacteria are the only known source of natural phenazines. However, natural and synthetic phenazines are of significant interest because of their potential impact on bacterial interactions and biotechnological processes.

Phenazines have been researched longer than most other bacterial secondary metabolites, with over 5,000 published reports dating from 1954 reported in the National Center for Biotechnology Information (PubMed). These secondary metabolites are produced by a variety of bacteria, especially pseudomonads, and have been studied intensively because of their broad spectrum antibiotic properties and roles in virulence. From a biotechnological perspective, the continuing interest in phenazines is due largely to their physico-chemical properties, including their oxidation–reduction (redox) properties and their bright pigmentation and ability to change color with pH and redox state. Phenazines continue to be used for many diverse applications, including as electron acceptors and donors, as components of fuel cells, as environmental sensors and biosensors, and as central components of antitumor compounds.

In this mini-review, we discuss emerging evidence that phenazines play multiple roles and contribute to the behavior and ecological fitness of the producing bacterium. For example, phenazines modify cellular redox state, act as electron shuttles altering electron flow patterns, contribute to biofilm formation and architecture, act as cell signals that regulate patterns of gene expression, and contribute to the survival of the producer. In eukaryotic hosts and host tissues, phenazines modify numerous host cellular responses. In plants, phenazines also influence growth and elicit induced

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systemic resistance. Of particular interest in defining their functional impact are the observations that bacterial species may produce different and often multiple phenazine derivatives, that the phenazine derivatives are produced in different proportions, and the question of whether the amount or proportion of each derivative produced change during growth or in response to environmental factors. We also will discuss how the properties of phenazines have and continue to be of interest for biological and biotechnological applications.

Phenazines and phenazine producers

Many phenazine-producing bacteria are commonly found associated with host organisms (Table 1). Perhaps the most studied phenazine is pyocyanin (5-N-methyl-1-hydroxyphenazine), which is blue when oxidized. It is produced by *Pseudomonas aeruginosa*, a common soil inhabitant and opportunistic human pathogen. Pyocyanin (PYO) was isolated originally from patient wounds and subsequently demonstrated to be associated with infections caused by *P. aeruginosa*. Its presence is associated with high morbidity and mortality in immuno-compromized patients, such as cystic fibrosis patients (Courtney et al. 2007; Murray et al. 2007). Phenazines produced by fluorescent pseudomonads also are studied extensively for their application in plant disease management. The bias in research toward pseudomonads often leads to the perception that phenazines are produced primarily by this group. However, phenazines are produced by a wide variety of Eubacteria including both Gram-negative and Gram-positive species. Phenazine producers include *Nocardia*, *Sorangium*, *Brevibacterium*, *Burkholderia*, *Erwinia*, *Pantoea agglomerans*, *Vibrio*, *Pelagibacter* (see Mavrodi et al. 2006; Mavrodi et al.

2010; Mentel et al. 2009), and members of the Actinomycetes, especially *Streptomyces* (Turner and Messenger 1986). Additionally, *Methanosarcina*, a member of the Archaea, was shown to contain a phenazine derivative (Abken et al. 1998). This microbe is known to utilize acetate, methylamines, and methanol. New phenazine producers continue to be identified, such as *Brevibacterium* sp. KMD 003 isolated from a marine purple sponge (Choi et al. 2009).

It is increasingly evident that bacteria produce a wide variety of phenazines, and that many bacteria produce multiple phenazine derivatives (Fig. 1). Bioinformatic comparisons of the phenazine biosynthetic genes among several bacteria demonstrate a high degree of conservation of five genes (Mavrodi et al. 2006; Mentel et al. 2009; Gross and Loper 2009). These are considered the ‘core’ biosynthetic genes as each is required for the synthesis of the basic three-ringed phenazine structure. Recent evidence suggests that these ‘core’ biosynthetic genes moved among diverse bacterial genera via horizontal transmission (Mavrodi et al. 2010). In most phenazine-producing bacteria, the core biosynthetic genes are flanked by one or more accessory genes that encode different terminal-modifying enzymes that result in the production of additional phenazine derivatives. For example, *Pseudomonas chlororaphis* 30-84, a root-associated beneficial bacterium produces three phenazines, phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine-1-carboxylic acid (2OHPKA), and 2-hydroxyphenazine (2OHPZ) (Pierson and Thomashow 1992). This *Pseudomonas* species is unique in that it contains *phzO*, a gene that encodes a monooxygenase, located immediately downstream of the core genes (Delaney et al. 2001). The presence of *phzO* converts a small amount (~10%) of the yellow PCA into the bright orange 2OHPKA. Additionally, a third minor derivative, 2OHPZ, is generated spontane-

Table 1 Examples of phenazine-producing microbes commonly associated with hosts

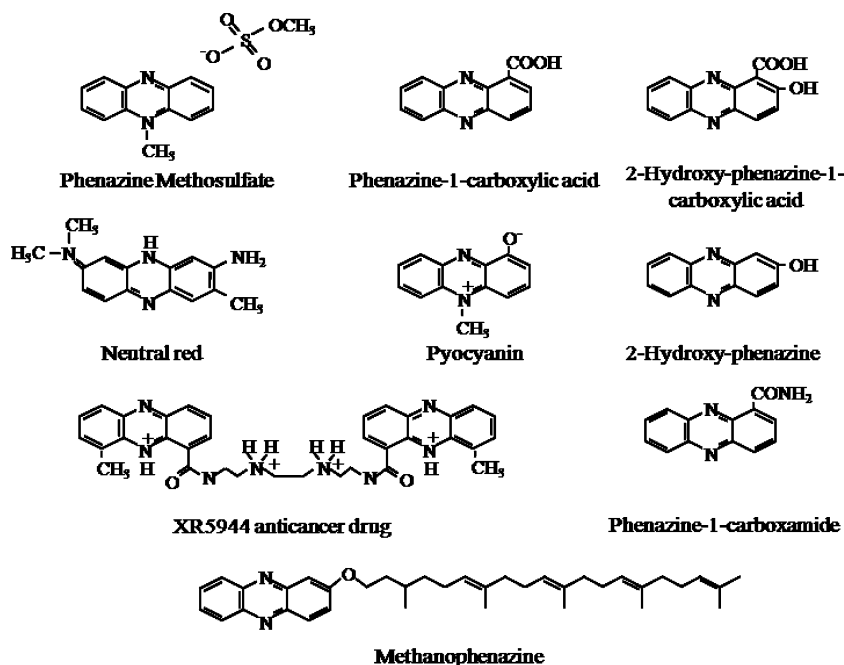
Bacterium	Human	Animal	Plant	Insect
<i>Brevibacterium linens</i>	+ (skin)			
<i>Brevibacterium</i> sp. KMD 003		+ (marine sponge) ^a		
<i>Burkholderia cepacia</i>	+	+	+	
<i>Methanosarcina mazei</i>	+ (intestine)	+ (rumen)		
<i>Mycobacterium abscessus</i>	+ (skin, soft tissues)			
<i>Pantoea agglomerans</i>	+	+	+	
<i>Pectobacterium atrosepticum</i> ^b			+	
<i>Pelagio variabilis</i>		+ (macroalgae)		
<i>Pseudomonas aeruginosa</i>	+	+	+	+
<i>Pseudomonas chlororaphis</i>			+	
<i>Pseudomonas fluorescens</i>			+	
<i>Streptomyces anulatus</i>				+ ^c
<i>Streptomyces cinnamonensis</i>			+	

^a Callysongia (Choi et al. 2009).

^b Formerly *Erwinia carotovora* subsp. *atroseptica*.

^c Arthropod endosymbiont (Gebhardt et al. 2002).

Fig. 1 Representative phenazine structural derivatives. Phenazine methosulfate and neutral red are commonly used phenazine derivatives. Phenazine-1-carboxylic acid, 2-hydroxy-phenazine-1-carboxylic acid and 2-hydroxy-phenazine are produced by *P. chlororaphis* strain 30-84. Pyocyanin is produced by *P. aeruginosa*. MLN944 is a DNA-binding agent inhibitory to marine and human tumors (Sappal et al. 2004). Methanophenazine is produced by the Archaea *Methanosarcina* spp. Descriptions are provided in the text



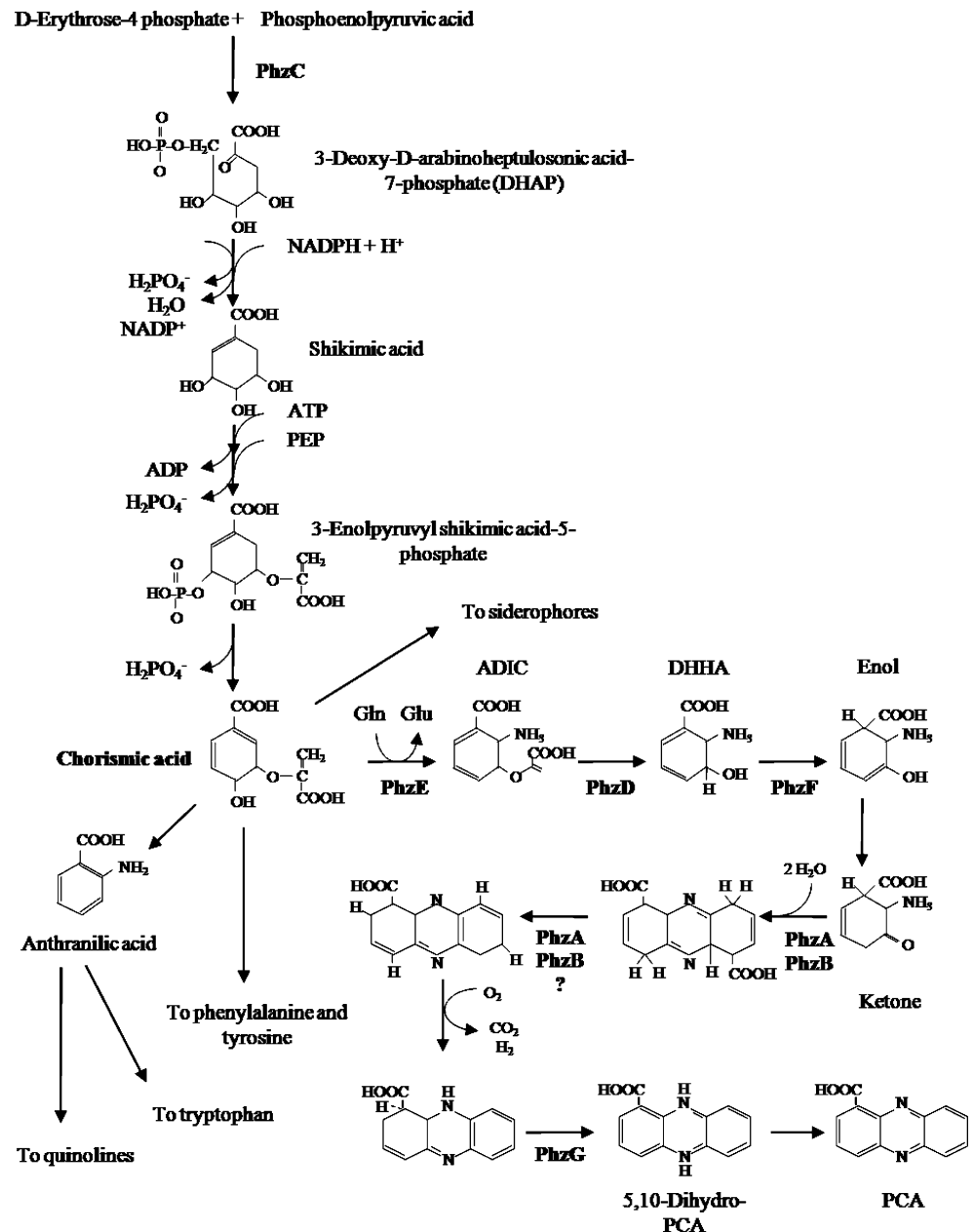
ously from 2OHPCA. *P. chlororaphis* PCL1391 and *P. aeruginosa* PAO1 contain *phzH*, a putative transamidase that converts a portion of PCA into phenazine-1-carboxamide (PCN), enabling these strains to produce both PCA and PCN (Chin-A-Woeng et al. 2001). Two additional genes in *P. aeruginosa* PAO1, *phzM*, a methyltransferase (Parsons et al. 2007), and *phzS*, a flavin-containing monooxygenase, together are responsible for the conversion of PCA to pyocyanin (Mavrodi et al. 2001; Greenhagen et al. 2008). PhzS alone can facilitate conversion of PCA to 1-hydroxy-phenazine (1OHPZ). *P. aeruginosa* PAO1 has two phenazine core biosynthetic gene clusters, separated from each other by ca. 2.6 Mb. One phenazine core region is flanked upstream by *phzM* and downstream by *phzS*. In contrast, the transamidase *phzH* is not closely linked to either biosynthetic locus. It is interesting that one or a few terminal modifying enzymes are responsible for the chemical modifications that result in the majority of natural phenazine derivatives. Since these structural modifications account for many of the biological functions of phenazines, the expression of these individual genes greatly affects the ecological fitness and activities of the producing bacterium.

Recent biochemical work has led to important insights into the mechanisms of phenazine biosynthesis, and several excellent reviews are available (Mavrodi et al. 2006; Mavrodi et al. 2010; Gross and Loper 2009). Some phenazine biosynthetic enzymes have been crystallized, and studies of these structures have led to a greater appreciation of the complexities of phenazine synthesis (Mentel et al. 2009; Mavrodi et al. 2010). A brief overview of phenazine biosynthesis is given below. Phenazines are derived from the shikimic acid pathway, using the interme-

diolate chorismic acid as the branch point for biosynthesis of the basic phenazine aromatic structure (Fig. 2). The shikimic acid biosynthetic pathway is highly conserved and is involved in the production of numerous metabolites necessary for primary growth, including the three aromatic amino acids, and para-aminobenzoic acid. In bacteria, this pathway is under stringent regulation, occurring primarily at the first step involving the condensation of erythrose-4-phosphate and phosphoenolpyruvic acid by a type I-3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase. In many bacteria, isozymes of type I-DAHP synthase exist. Each is subject to feedback inhibition by one of the end products of the pathway. Interestingly, in many phenazine producers, the third gene in the biosynthetic operon (*phzC*) encodes a type II-3-DAHP synthase that is more similar to DAHP synthases of solanaceous plants than that of prokaryotes (Pierson et al. 1995). In plants, type II-3-DHAP synthase enzyme lacks a loop region required for allosteric control (Webby et al. 2005). The activity of this enzyme is not modulated by the three aromatic amino acid products, but its activity is enhanced during specific aspects of plant growth such as during seed germination and shoot formation and by glyphosate exposure (Pinto et al. 1988). Expression of *phzC* may enhance the first condensation step, ensuring sufficient substrate levels for phenazine production. However, unregulated expression of *phzC* may deplete the cell of metabolites required for primary growth and may lead to reduced overall fitness in the rhizosphere (Mavrodi et al. 2006). This need for controlled expression may explain, at least in part, the complex regulation of phenazine production.

Almost all studies on phenazine regulation to date have focused on pseudomonads. These studies show that

Fig. 2 Phenazine biosynthesis [Figure modified from Mentel et al. (2009)]. Phenazines are derived from the shikimic acid pathway that is highly conserved in most organisms. Chorismic acid serves as the phenazine branch point once the phenazine biosynthetic genes (*phzABCDEFG*) are expressed (note *phzB*, *phzD*, *phzE*, *phzF* and *phzG* are considered the five core genes). PhzC, the third enzyme in the phenazine operon, is a type II-3-deoxy-D-arabinoheptulosonate-7-phosphate that probably ensures sufficient substrate flow through the shikimic acid pathway for phenazine biosynthesis. The early steps of phenazine biosynthesis are becoming well elucidated while several of the later steps are not yet completely understood and differences may reflect variation in the final biosynthetic steps among microorganisms. From PCA a number of derivatives are formed by additional terminal modifying genes (not shown). For a thorough discussion of the pathway please see Mentel et al. (2009). Abbreviations: DAHP: 3-deoxy-D-arabinoheptulosonate 7-phosphate, Gln: glutamine, Glu: glutamic acid, ADIC: 2-amino-2-desoxyisochorismic acid, DHHA: *trans*-2,3-dihydro-3-hydroxyanthranilic acid, PCA: phenazine-1-carboxylic acid



different pseudomonads utilize combinations of conserved regulatory systems integrated into sensory networks to control phenazine production in response to environmental, nutritional, population, and metabolic inputs (reviewed in Mavrodi et al. 2006; Mentel et al. 2009; Gross and Loper 2009). For example, phenazine production by the plant-associated bacteria *P. chlororaphis* strain 30-84 and *P. fluorescens* strain 2-79 is regulated at multiple levels. These include transcriptional regulation via quorum sensing (QS) (PhzR/PhzI), two component positive (GacS/GacA) and two component negative (RpeA/RpeB) regulation. Yet additional regulatory genes have been implicated in *P. chlororaphis* phenazine control, including *pip*, and post-transcriptional control by *rsmA* and *rsmZ* (Pierson et al. unpub.).

The opportunistic pathogen *P. aeruginosa* also regulates PYO production by multiple regulatory systems. However, there are distinct differences in the types of regulatory systems and the linkage(s) between regulatory modules. For example, *P. aeruginosa* utilizes the hierarchical LasR/LasI and RhIR/RhII QS systems to activate PYO production, as well as a third signaling system based on production of 2-heptyl-3-hydroxyl-4-quinoline, known as the *Pseudomonas* quinoline system (PQS) (Dubern and Diggle 2008). *P. aeruginosa* contains the gene *mvfR* involved in the regulation of the MexGHI-opmD operon believed to be responsible for secretion of the QS signals required for PYO production. Recently, PA1196 was identified as a regulator of the RhIR/RhII and PQS systems in that mutation of the

open reading frame PA1196 resulted in significant decrease in *rhlI* and *pqsA* expression as compared to the wild type (Liang et al. 2009). Additional regulatory inputs affecting PYO production are likely to be identified. In contrast to *P. aeruginosa*, hierarchical QS systems and analogs of the PQS system have not been identified in *P. chlororaphis* or *P. fluorescens*.

Pseudomonas sp. strain M18, a biological control strain isolated from the melon rhizosphere, has a phenazine biosynthetic locus organization similar to *P. aeruginosa*, including two separate phenazine core gene clusters and the presence of both the methyltransferase *phzM* and the monooxygenase *phzS* genes. However, it produces predominantly PCA, whereas *P. aeruginosa* produces primarily PYO. In strain M18, the expression of *phzM* and *lasI* (and a third gene *ptsP*) is under temperature-dependent regulation such that they are transcribed at 28°C but not at 37°C (Huang et al. 2009). In *P. aeruginosa* strain PAO1, *phzM* expression is not affected by temperature, perhaps reflecting its need for expression when associated with a human host.

Although much is known about the regulation of the core phenazine biosynthetic genes, especially in *Pseudomonas* spp., little is known about the fine tuning of the timing of expression and the relative amounts of the different phenazines produced. For example, fine tuning in *P. aeruginosa* occurs by control of QS signals required for PYO production. *P. aeruginosa* produces two major QS signals, C₄-HSL and 3-oxo-C₁₂-HSL, with short and long acyl chains, respectively (reviewed in Williams and Cámara 2009). *P. aeruginosa* also contains an AHL acylase specific for long acyl chain signals. The presence of this enzyme appears to act as a quorum quencher by turning off the *las* QS system by degradation of the longer acyl chain signal (Sio et al. 2006). The removal of this QS signal results in loss of PYO production. This acylase, also known as *qscI12* or *pvdQ*, is up-regulated during iron deprivation and may repress PYO production during periods of low iron availability. However, it is not produced at high levels at 37°C, the temperature which *P. aeruginosa* would encounter upon infection of a human host. Although more work is required, this may represent a mechanism by which the bacterium modulates PYO production in response to iron and host availability.

The regulatory complexity governing phenazine production likely reflects the complexity of the roles phenazines play for the producing bacterium (Pierson and Pierson 1996). This observation is consistent with evolutionary theory that metabolically costly metabolites are more likely to be maintained if they serve multiple functions (Wink 2003). Wang et al. (2009) hypothesized that phenazine biosynthesis evolved originally during a period of low oxygen availability, and that some of the effects on bacterial

behavior today may not reflect the conditions that drove their early evolution. Evolutionary pressure on bacteria may have selected for different phenazines to serve different roles, and this is reflected by the diversity in the linkage and types of regulatory pathways controlling their expression. Further, since the roles phenazines play for different producers may not be the same, analysis of more than one or two experimental systems may be required to gain a more complete picture of their functions and importance.

Roles in pathogenesis and competition

Most of the described effects of phenazines during pathogenesis and competition are attributed primarily to their ability to generate reactive oxygen species (ROS) in other organisms and tissues. In some cases, this may result in host beneficial effects, such as the inhibition of pathogenic organisms. In other cases, bacterial virulence is enhanced by phenazine production as it interferes with normal host cell functions. Both of these outcomes are due ultimately to the ability of phenazines to accept or donate electrons because of their aromatic structure. Whether they accept or donate electrons is dependent on their redox potential relative to that of other electron transfer molecules in the cell or in the environment. Early studies on cellular respiratory chains demonstrated the ability of phenazines to uncouple oxidative phosphorylation by shunting electrons from the endogenous pathway in mammalian cells (Stewart-Tull and Armstrong 1971) and in *B. subtilis* (Bisschop et al. 1979).

Several recent reviews discuss the formation of ROS and oxidative stress by phenazines (Laursen and Nielsen 2004; Mavrodi et al. 2006). For example, PYO production by *P. aeruginosa* plays important roles in pathogenesis during lung infection (Lau et al. 2004a, b; Winstanley and Fothergill 2009). When growing on airway epithelial cells, PYO (and PCA) produced by *P. aeruginosa* can be reduced via the oxidation of glutathione and NADH resulting in increased oxidation levels. The reduced PYO reacts with free oxygen in the lungs, generating ROS. Airway epithelial cells produce lactoperoxidase and related dual oxidases that produce mild oxidants as a defense effective against a number of bacterial intruders, including *Staphylococcus aureus*, *Burkholderia cepacia* and *P. aeruginosa*. However, PYO production by *P. aeruginosa* negates this host defense mechanism by competing with epithelial cell Duox activity for NADPH (Rada and Leto 2009). The generation of these ROS contributes to the virulence of the infection. ROS also may contribute to successful host invasion and disease due to their negative effects on a range of host cell functions, including respiration, ciliary beating, epidermal cell growth, calcium homeostasis, prostaglandin release, neutrophil

apoptosis, interleukin-2 release, Immunoglobulin G secretion, and a protease–antiprotease activity (Hassan and Fridovich 1980; Ran et al. 2003; Laursen and Nielsen 2004).

PYO production may inhibit the growth of competing microorganisms during mixed lung infections (Machan et al. 2001). In fact, the effects of phenazines can be simultaneously positive and negative within the same host. For example, while PYO production by *P. aeruginosa* during lung infection of cystic fibrosis patients is correlated with a poor prognosis, it is also highly correlated with a lower incidence of yeast infections (Lau et al. 2004a, b; Cogen et al. 2008). However, a more fundamental role for PYO in the producing bacterium is suggested by the observation that PYO production is important for persistence even in the absence of competitors (Hassett et al. 2009; Kobayashi et al. 2009; Price-Whelan et al. 2006). PYO-producing cells protect themselves by the production of high levels of superoxide dismutase activity (SOD). Mutants of *P. aeruginosa* in *sodA* or *sodB* are defective in SOD production and produced half or no PYO, respectively, as compared to the wild type (Hassett et al. 1995). Recent work with *P. aeruginosa* and *Bacillus subtilis* further supports the role of ROS in growth inhibition and bacterial competition. *B. subtilis* cells containing intact *nos* genes that produce nitric oxide (NO) that stimulates SOD expression were more resistant to PYO in competition studies compared to isogenic *nos* mutants (Gusarov et al. 2009). Although the multiple roles of PYO in virulence are being recognized, it should be noted that in addition to PYO, *P. aeruginosa* produces several other phenazine derivatives including 1-hydroxyphenazine, PCA, PCN, aeruginosin A, and aeruginosin B (see Mavrodi et al. 2006) for which the impacts on virulence are less well understood.

In addition to their involvement in pathogenesis and virulence, interest in phenazines as beneficial compounds emerged in the 1980s due to their effectiveness as antimicrobial compounds in plant disease control. Phenazine production by a number of soil-borne bacteria was shown to control a wide range of plant pathogenic fungi (Chin-A-Woeng et al. 2003; Mavrodi et al. 2006). For example, *P. chlororaphis* strain 30-84 produces three PZ derivatives PCA, 2OHPCA, and 2OHPZ (Pierson and Thomashow 1992). *P. fluorescens* strain 2-79 produces only PCA (Thomashow and Weller 1988), whereas *P. chlororaphis* strain PCL1391 produces phenazine-1-carboxamide (PCN) and some PCA (Chin-A-Woeng et al. 1998). PCN was shown to control *Fusarium oxysporum* f. sp. *radicis lycopersici*, the causative agent of tomato foot and root rot. Both PCA and PCN are involved in control of *Pythium myriotylum*, the causative agent of root rot of cocoyam (Tambong and Hofte 2001) whereas PYO produced by *P. aeruginosa* inhibited *Septoria tritici* of wheat (Flaishman et

al. 1990). PCA and PCN produced by *Pseudomonas* strain PCL1391 induced several ABC transporters in *Botrytis cinerea* (Schoonbeek et al. 2002). Additionally, the ability to produce phenazine was strongly correlated with bacterial persistence in natural soil in the presence of the indigenous microbial community (Mazzola et al. 1992).

These results are consistent with the commonly held belief that the primary role of phenazines produced by these soil-borne bacteria is as antibiotic compounds that aid in their competitive survival in natural systems. Yet, despite the observations that phenazines produced by biological control agents do inhibit a broad spectrum of target fungal pathogens, in many cases, the same phenazine derivatives are ineffective at inhibiting the growth of co-occurring bacteria, probably their most immediate competitors (Fernando and Pierson, unpub.; Beifuss and Tietze 2005). Within rhizosphere communities, the effects of phenazine production on the indigenous rhizosphere community are negligible, with little effect on the microorganisms that compete with the producer (Mavrodi et al. 2006; Dwivedi et al. 2009). More recent studies on phenazine function suggest that, although phenazines may assist the producing bacterium in competitive survival, this is probably not their primary function. As described below, phenazines also induce plant defense pathways, play roles in electron shuttling, iron chelation, biofilm formation, and even serve as signals that modulate gene expression (Fig. 3).

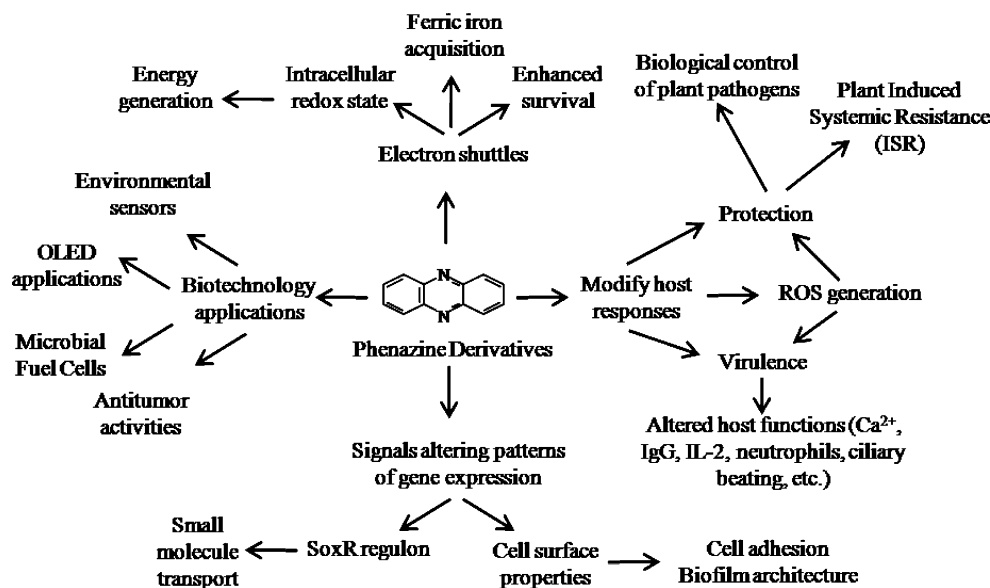
Induction of plant defense pathways

Phenazines have been shown to activate induced systemic resistance (ISR) in plants. ISR is widespread and can be induced by a number of bacterial components, including outer membrane components, lipopolysaccharide, flagella, siderophores, and volatile compounds (Van Wees et al. 2008). ISR is dependent on expression of the plant ethylene and jasmonic acid pathways, leading to a rapid systemic expression of broad spectrum resistance against numerous pathogens (Verhagen et al. 2004). Inoculation of *P. aeruginosa* onto rice plants elicited ISR, as evidenced by resistance to the fungal rice blast pathogen *Magnaporthe grisea*. PYO was shown to be critical for this induction as loss of PYO production resulted in the loss of ISR against *M. grisea* (De Vleeschauwer et al. 2006). However, the production of PYO resulted in increased susceptibility of rice to another fungal pathogen, *Rhizoctonia solani*.

Electron shuttling and iron chelation

Microorganisms often experience environments in which terminal electron acceptors are limited or may become

Fig. 3 Representative effects of phenazines on cellular physiology, gene expression, host functions and biotechnological applications. In the center is the basic phenazine structure lacking any additional modifications. Details of the effects of phenazine derivatives on specific functions are described in the text



limited under specific conditions. The lack of available terminal electron acceptors severely limits bacterial growth and survival due to low energy generation. In most bacteria, this also results in a highly reduced intracellular redox state as indicated by a high NADH/NAD⁺ ratio (de Graef et al. 1999). *P. aeruginosa* is capable of maintaining a NADH/NAD⁺ ratio slightly greater than one due to the production of PYO, which serves as an alternate electron acceptor that reoxidizes NADH to NAD⁺. This enables the cell to balance intracellular redox in the absence of other electron acceptors (Price-Whelan et al. 2007). Recently, PYO production was shown to be important for bacterial survival, but not growth, under anaerobic conditions (Wang et al. 2009). In this work, wild type *P. aeruginosa* PA14 and a phenazine null derivative were grown as planktonic cultures to stationary phase and electron flow measured in anaerobic bioreactors. The pyocyanin-producing wild type strain remained viable for 7 days, although its population did not increase from its initial level. In contrast, the non-pyocyanin-producing mutant population decreased logarithmically after 3 days. This effect was specific to pyocyanin, as several other redox compounds with similar structures did not support survival. These results demonstrate that phenazines are essential for long term survival under anaerobic conditions, for example, as occurs below the outer surface layer of biofilms (Drago 2009).

In soils, water saturation and microbial and root respiration limit available O₂ as a terminal electron acceptor. The use of alternative electron acceptors such as ferric (Fe³⁺) iron allows continued energy generation and enhanced microbial survival. The root-associated bacterium *P. chlororaphis* PCL1391 utilizes phenazine-1-carboxamide (PCN) to efficiently convert ferric Fe³⁺ hydroxides to ferrous Fe²⁺ under acidic conditions. The dissimilatory iron-reducing bacterium *Shewanella oneidensis* MR1 also

is capable of utilizing exogenously added PCN to mineralize poorly crystalline Fe³⁺ hydroxides, enabling it to grow under conditions with limited electron acceptors (Hernandez et al. 2004). Different phenazine structural derivatives have different reactivities to ferrihydrite and hematite (pH 5–8), suggesting that particular phenazines may react preferentially with specific forms of iron (Wang and Newman 2008). The recent finding that addition of strong iron chelators inhibited *P. aeruginosa* biofilms, especially under anaerobic conditions (O'May et al. 2009), reinforces the probable role of phenazines as electron shuttles to alternative electron acceptors.

One benefit of phenazine-mediated iron reduction is the increased bioavailability of this limited element to the bacterium. For example, PYO produced by *P. aeruginosa* can acquire iron from the human iron chelator transferrin, and the highest rate of iron acquisition occurs under low O₂ conditions (Cox 1986). In soils, PYO production may increase iron availability to other organisms. PYO itself was shown recently to act as an iron chelator (Newman unpub.).

The use of phenazines as electron shuttles is not limited to Eubacteria. *Methanosarcina* spp. generate methane using anaerobic respiration. These Archaea contain a unique energy-saving electron transport system that utilizes methanophenazine as a membrane-localized component. This chain is proposed to consist of two systems: reduced coenzyme F₄₂₀ (F₄₂₀H₂: heterodisulfide oxidoreductase) and H₂: heterodisulfide oxidoreductase. The net result of either system is the sequential transfer of electrons from F₄₂₀H₂ or H₂ to the membrane-localized methanophenazine (MP) with the concurrent release of two protons outside the cell membrane. The reduced methanophenazine (MPH₂) subsequently donates electrons via the enzyme heterodisulfide reductase (Hdr) to the heterodisulfide of coenzyme M and

coenzyme B (CoM–S–S–CoB), which serves as the terminal electron acceptor and is involved subsequently in methane production. The net result of each system is the translocation of four protons outside the cell for each pair of electrons. The proton motive force generated is used for ATP synthesis (Abken et al. 1998; Beifuss and Tietze 2005; Kulkarni et al. 2009).

Cell adhesion, biofilm development and dispersal

Biofilm formation is important for the persistence and survival of bacteria (Davies 2002; Balasubramanian and Mathee 2009; McBain 2009). Many Gram-negative bacteria require functional QS systems in order to form biofilms (Mavrodi et al. 2006; De Sordi and Mühlshlegel 2009; Dobretsov et al. 2009). Recent work with *P. chlororaphis* 30-84 reinforced the importance of QS in biofilm formation (Maddula et al. 2006). This work also demonstrated that it was specifically the production of phenazines controlled by the PhzR/I QS that was critical. Mutants defective in the PhzR/I QS system failed to establish biofilms even after 6 days, similar to a phenazine structural mutant. Introduction of functional copies of *phzR/phzI* in trans failed to rescue the phenazine structural mutant for adhesion and biofilm development, whereas constitutive phenazine expression resulted in earlier biofilm formation after 1–3 days.

P. chlororaphis strain 30-84 produces primarily phenazine-1-carboxylic acid (PCA) and only about 10% is converted into 2-hydroxy-phenazine-1-carboxylic acid (2OHPCA). Therefore, the effect of altering the ratio of PCA to 2OHPCA produced by strain 30-84 on biofilm development was examined (Maddula et al. 2008). A derivative in which *phzO* was inactivated was constructed along with a derivative in which additional copies of *phzO* were introduced in trans, resulting in strains that produced only PCA or increased levels of 2OHPCA. Compared to wild type strain 30-84 or the PCA-only producer, the 2OHPCA overproducer adhered more quickly and more uniformly to glass surfaces (44% total coverage as compared to 1% by the wild type or PCA-only strain after 45 min.). Additionally, the 2OHPCA overproducer formed thicker biofilms than the wild type, but had a cell bio-volume similar to the wild type. In contrast, the PCA-only producer had a thicker biofilm with four-fold higher bio-volume of cells than either the wild type or 2OHPCA overproducer. Dispersion from the biofilm also was reduced in the 2OHPCA overproducer. These results suggest that 2OHPCA may facilitate cellular adhesion, whereas PCA may facilitate growth within the biofilm, possibly as an electron shuttle within the micro-aerophilic community. The idea that the phenazines produced by *P. chlororaphis* 30-84 may play different roles in biofilm structure and function is intriguing given that

early work showed that 2OHPCA had a greater ability to inhibit the growth of some microorganisms than PCA (Toohey et al. 1965). Moreover, *P. chlororaphis* 30-84 was found to be more effective against the fungal plant pathogen *Gaeumannomyces graminis* var. *tritici* than strains that produce PCA alone. These results suggest that bacteria may produce different phenazine structural derivative in specific concentrations due to the diverse roles they serve for the population.

Phenazines as signals

The observation that alterations in the levels of specific phenazine structures had significant impacts on cell adhesion and biofilm architecture raises an interesting question as to the mechanism(s) involved in these effects. One possibility is that phenazines, regulated by QS signals, are themselves signals capable of altering patterns of gene expression. Recently, experimental support for this hypothesis was established using *P. aeruginosa* PAO1 (Dietrich et al. 2006). A mutant was constructed in which both sets of phenazine biosynthetic genes were deleted. Transcriptome analysis of the mutant strain, grown with or without 0.2 mM purified PYO, revealed altered expression of 51 genes. Of these, 8 of the 22 genes up-regulated by PYO addition were efflux transporters, while 7 of the 29 genes down-regulated were involved in ferric iron uptake. This work demonstrated that PYO was itself a signal that modulated the physiology of *P. aeruginosa*.

The effects of PYO were shown subsequently to be dependent on two mechanisms, one involving a homologue of *E. coli* SoxR and a second dependent on another unknown regulator (Kobayashi and Tagawa 2004). In *E. coli*, SoxR regulates the expression of superoxide stress response regulons in conjunction with a second regulator, SoxS. Dietrich et al. (2008) used bioinformatic and gene expression analysis to study the role of SoxR regulons in *P. aeruginosa* and *Streptomyces coelicolor*. In *P. aeruginosa* and *S. coelicolor*, many of the SoxR regulons identified lacked genes involved in superoxide stress response, but contained genes involved in the transport of small molecules. One of the products under SoxR control in *P. aeruginosa* PA14 is the MexGHI-OpmD efflux system involved in transport of PCA and a red phenazine. Interestingly, PYO stimulated the expression of this efflux system, although it was not dependent on it for release from the cell. Comparison of wild type PA14, a *soxR* deletion mutant, and a PYO-overproducing strain indicated that the presence of higher levels of PYO resulted in the maintenance of a smooth colony morphology as compared to the wild type PA14. The higher levels of PYO in the *soxR* deletion and PYO-overproducing mutants is presumably

due to the inability of the *soxR* mutant to transport phenazines out of the cell and the overproducing strain having more phenazine produced as compared to the wild type. These results suggested that the diffusibility of phenazines may influence colony phenotype over distance.

In *P. chlororaphis*, transcriptome analysis was used to compare a phenazine structural mutant to the wild type strain. In this experiment, phenazine production occurred naturally under QS regulation. In preliminary experiments, 63 (1.1%) and 41 (0.8%) *P. chlororaphis* genes were up- or down-regulated by phenazine production, respectively (Pierson et al. unpub). In contrast to *P. aeruginosa*, many of the genes that appeared to be activated encode products associated with cell adhesion and biofilm development, including fimbrial and LPS genes. Although it is clear that phenazines can serve as signals in both *Pseudomonas* species, the effects of this signaling may not be the same for each species. Further, in cases where multiple phenazines are produced, distinct phenazines may effect the expression of different or overlapping gene sets. These differences may be useful tools in understanding the evolutionary selection experienced by each species.

Biotechnological applications

The ability of phenazines to promote electron transfer has many realized and potential biotechnological applications. Phenazines long have been used as colorimetric redox indicators. The pH indicator neutral red is among the best known. More recently, phenazines have been utilized for the development of sensors and in nanotechnology. For example, a phenazine derivative was used to develop a luminescence-based pH sensor (Ryazanova et al. 2007) and an amperometric sensor for hydrogen peroxide determination utilizing neutral red attached to multiwalled carbon nanotubes was developed (Jeykumari and Narayanan 2007).

Microbial fuel cells (MFC) use microorganisms to catalyze the conversion of chemical energy into electrical energy (Torres et al. 2010). An ongoing issue with MFCs is that the slow rate of electron transfer from the microorganism to the anodic electrode limits MFC efficiency. Early work demonstrated that phenazine methosulfate or phenazine ethosulfate served as good electron acceptors in photoelectrochemical cells (Sanderson et al. 1987). More recently, it was observed that other phenazines also contributed to the rates of electron transfer in MFCs. The addition of pyocyanin to MFC-containing *Brevibacillus* sp. PTH1 doubled the rate of electron transfer (Rabaey et al. 2005). The addition of a PCA-producing *P. chlororaphis* or a derivative that produces high levels of PCN to a mixed MFC also resulted in higher electron transfer rates (Pham et

al. 2008). A strain of *P. aeruginosa* was isolated from MFC maintained in a batch mode for over a year that supported 352 mV using 1500 mg/l glucose as fuel (Luo et al. 2009).

Phenazines conjugated to other compounds offer potential as components of organic light emitting devices (OLED), such as a phenanthroline-fused phenazine (Chen and Xiao-Chang 2004). OLEDs are gaining popularity due to their low voltage requirements, wide color range, and light weight. OLEDs are organic semiconductors containing an emissive layer placed between a transparent anode (e.g., transparent indium tin oxide) and a metal cathode (e.g., Mg, Al, Ag). When a bias is applied across the electrodes, the ‘holes’ (areas lacking electrons) and electrons combine in the emissive layer resulting in light emission (Li et al. 2009).

Phenazines are associated with antitumor activities (Laursen and Nielsen 2004; Mavrodi et al. 2006). Cells that are actively respiring, such as tumor cells, appear to be more susceptible to respiratory interference and ROS generation caused by phenazine compounds. Additionally, phenazine derivatives known to interfere with topoisomerase I and II activities in eukaryotic cells have been identified. Cancer cells, having high levels of both topoisomerases, are more susceptible to this interference. For example, active proliferation of human lymphocytes was inhibited by pyocyanin (Sorensen et al. 1983). The development of synthetic anticancer phenazine derivatives is an ongoing area of research aimed at combining known phenazine biological activities with increased target specificity towards cancer cells (Nakaike et al. 2005; Hari et al. 2009).

Concluding remarks

The roles of phenazines in biotechnology appear limitless based on their ability to shuttle electrons and new applications based on other properties are likely to be identified in the future. In natural systems, phenazine-producing bacteria often produce multiple derivatives and even individual phenazines appear to play multiple roles in bacterial interactions and behaviors. Future research needs to address the importance of the relative quantities of phenazine derivatives produced and how this balance is maintained. For example, how does changing the amount of one phenazine derivative produced relative to another affect bacterial fitness? Understanding the patterns of gene expression regulating the timing and levels of phenazines produced may lead to greater appreciation of the reasons bacteria produce diverse phenazine structural derivatives at different levels and perhaps a better understanding of the complex roles these phenazines serve in the lifestyles and behavior of bacteria.

Summary

- Phenazines constitute a large group of nitrogen-containing heterocyclic compounds that differ in their chemical and physical properties based on the type and position of the functional groups present.
- Most studies on phenazines have focused on Gram-negative bacteria such as *Pseudomonas* spp. The recognition that phenazines are produced by a wide variety of Eubacteria, and some Archaea, indicates a need for research on the regulation and functions of phenazines in other classes of bacteria.
- Many phenazine-producing bacteria commonly form associations with different hosts, although this may reflect a bias in how phenazine producing bacteria have been selected.
- Phenazine derivatives with chemical modifications at one or more positions of the aromatic ring structures have been identified, and most are due to one or a few terminal modifying enzymes. These differences in phenazine structure impact their biological functions, and therefore these modifications may determine the ecological niche the bacterium occupies.
- Phenazine production is controlled by regulatory networks that are organized differently in each species. Although phenazines serve multiple roles for the producing organism, the roles phenazines play for each producers may not be the same. Therefore, conclusions based on one or two experimental systems may provide only partial insights into their importance.
- Phenazines serve as signals, altering patterns of gene expression in the producing bacterium. However, the specific genes may be different in each bacterial species. Future work will determine if different phenazine structural derivatives affect the expression of a conserved or overlapping groups of genes, and how these genes affect bacterial behaviors.
- In order to gain a more complete understanding of the impacts of phenazines on bacterial behavior and fitness, future studies should address:
 - why strains produce specific and often more than one phenazine derivative,
 - why these derivatives are produced in distinct quantities or ratios, and
 - how these ratios are “fine-tuned.”
- Based on the knowledge gained from these studies, future biotechnological advances may come from the use of finely-tuned ratios of phenazines rather than relying on single derivatives.

Acknowledgements The authors wish to thank Dr. Dianne Newman for unpublished results. We also thank Dr. Martha Hawes and Dr. Anne Estes for their review comments. Parts of this work were supported by NSF Grant No. 0545804 and by USDA-NRI Grant No. 2008-00657.

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