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REVIEW

Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics

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

Historically, microbiologists and chemists alike have categorized as 'secondary metabolites' a broad class of molecules produced at late stages of microbial growth in laboratory cultures. This nomenclature is, admittedly, pejorative, implying that these molecules are somehow less important than others to the cell that produces them. In particular, the traditional view is that secondary metabolites (i) do not contribute to the growth or survival of the producer (ii) are highly sensitive to the conditions stimulating their production (for example, medium composition) (iii) often have complex structures and (iv) have production rates that are decoupled from the doubling time of the cells[1,2]. Together, these leitmotifs present a conundrum: why would an organism limited for nutrients begin excreting large amounts of complex organic molecules? One reasonable and commonly stated answer is that they function as antibiotics, and are produced in copious quantities at this stage of growth to protect the producer from competitors[3]. In recent years, however, the idea that 'secondary' metabolites might have other functions, ranging from controlling gene expression[4] to supporting growth or iron acquisition in microbial communities[5,6], has become increasingly compelling. This is due, in large part, to the recognition that microbes typically exist in nature in biofilm communities[7] and/or in a metabolically quiescent state[8]; because the 'rules of the game' for metabolism under these conditions are virtually unknown, a re-examination of the function of secondary metabolites is warranted.

To illustrate the idea that secondary metabolites have the potential to perform primary metabolic functions, we will focus this review on a class of compounds known as 'phenazines', which have been of great interest to pharmaceutical and clinical research groups for the last 50 years[9]. Phenazines are heterocyclic compounds that are produced naturally and substituted at different points around their rings by different bacterial species (**Table 1**). Small modifications of the core phenazine structure give rise to a full spectrum of colors, ranging from the deep red of 5-methyl-7-amino-1-carboxyphenazinium betaine (aeruginosin A, **1**) to the lemon yellow of phenazine-1-carboxylic acid (PCA, **2**), to the bright blue of 1-hydroxy-5-methylphenazine (pyocyanin, **3**) (**Fig. 1**). The combination and variety of functional groups added also determine the redox potential and solubility of these compounds, thus affecting their biological activity[9–11].

The antagonistic effects of almost all of these derivatives are usually attributed to one general characteristic: redox activity. The 2-hydroxyphenazine-1-carboxylic acid (2-OHPCA, **4**) produced by *Pseudomonas aureofaciens* is thought to kill off competing fungi through the production of reactive oxygen species[12]. Many of the effects of pyocyanin (PYO) and PCA on a diversity of eukaryotic hosts as well as bacteria are thought to result from oxidative activity or the inactivation of proteins important in the oxidative stress response[13,14]. Regardless of whether they are acting as antibiotics in the soil, or virulence factors during infection, the redox transformations of phenazines strongly influence their physiological effects in other organisms. A more detailed understanding of phenazine metabolism in competing or host cells is emerging, as very recently, researchers have begun to recognize that small variations in the reactivity of phenazines can give rise to differences in their elicited response[15].

Concomitant with the development of ideas about phenazine activity during competition and infection, *Pseudomonas aeruginosa* and other phenazine-excreting bacteria have become popular model organisms for the study of quorum sensing and biofilm formation, two of the most active areas of research in the field of microbiology[16–18]. Whereas pharmaceutical and clinical groups have been focused on the physiological effects of these compounds in non-producing organisms, microbial physiologists and geneticists have typically viewed phenazines as metabolites that perform only secondary functions. As a result, despite research interest in both the bio-

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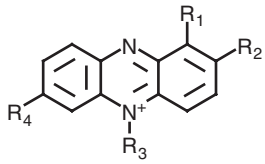


No	Name	R_1	R_2	R_3	R_4	K_{OW} ox	K_{OW} red	$E^{o\prime}$ (mV)
1	Aeruginosin A	COOH		CH_3	NH_2	–0.71	0.46	NA
2	Phenazine-1-carboxylic acid (PCA)	COOH				2.17	3.72	–177
3	Pyocyanin (PYO)	OH		CH_3		1.60	2.89	–34
4	2-Hydroxyphenazine-1-carboxylic acid (2-OHPCA)	COOH	OH			2.54	3.32	NA
5	Phenazine-1-carboxamide (PCN)	$CONH_2$				1.04	2.19	–115
6	1-Hydroxyphenazine (1-OHPHZ)	OH				1.81	2.35	–172

Table 1 Characteristics of some phenazines excreted by pseudomonads[90–92]. Octanol-water partition coefficients (K_{ow}) were calculated using the KOWWIN demo program available at http://www.syrres.com/esc/est_kowdemo.htm. $E^{o\prime}$, standard redox potential, pH 7; NA, not available.

logical activity of the compounds themselves and in the physiology of their producers, the primary functions of phenazines for producing organisms such as the pseudomonads are still unknown. This is surprising, especially given that phenazine production and reduction is evident in many of the *Pseudomonas* cultures that microbiologists prepare for their work (**Fig. 2**) and that the mechanisms thought to be responsible for phenazine metabolism in non-producers (for example, reduction by NADH or glutathione, interaction with the respiratory chain) are present in most organisms[19,20]. That phenazines and other excreted compounds (i) react with common primary metabolites (ii) are potentially transformed by enzymes active in central metabolic pathways and (iii) induce gene expression calls into question their categorization as secondary metabolites. We will discuss here the recent discoveries that lead to new hypotheses about the relevance of phenazine metabolism in the context of the lifestyles of their producers.

Occurrence of phenazine production

Phenazines are first mentioned in the literature as early as the 1860s, when French researchers and clinicians noticed a blue coloration in the pus and sputum, or respiratory secretion, of infected patients. Carle Gessard and others examined the pus microscopically and identified a rod-shaped bacterium residing in these wounds, and upon isolating the organism discovered that it was responsible for the bluish tint. For this trait, they named the species *Bacillus pyocyaneus*, and it has since been renamed *Pseudomonas aeruginosa*, for the Latin *aerugo*, which refers to the blue-green rust of copper[21]. *P. aeruginosa* is widespread in terrestrial habitats, can grow in both marine and freshwater environments, and is known for its ability to infect a diversity of hosts, ranging from plants to humans[21–23]. The *P. aeruginosa* laboratory strains PAO1 and PA14 are capable of producing at least four different phenazine derivatives[24].

Several other *Pseudomonas* species are also phenazine producers and are known for their potential in biocontrol applications, in which an organism that inhibits the growth of crop pathogens is enriched in the soil to enhance crop yields. Representatives include the strains *P. aureofaciens* 30-84, *P. fluorescens* 2-79 and *P. chlororaphis* PCL1391. Along with *P. aeruginosa*, these isolates all produce one or more phenazines and differ in their biosynthetic capabilities with respect to phenazine derivatization. *P. aureofaciens*, for example, possesses the mono-oxygenase PhzO, which converts the common pseudomonad phenazine precursor PCA into 2-OHPCA, a bright orange pigment[25]. *P. chlororaphis*, on the other hand, expresses PhzH, a transamidase which converts this precursor into PCN, a green, sparingly soluble pigment that precipitates out of culture media[26].

Phenazine biosynthesis also has been observed in other bacterial genera, including other proteobacteria, such as *Brevibacterium*, *Burkholderia* and *Xanthomonas*, as well as the Gram-positive genus *Streptomyces* and even the archaeal genus *Methanosarcina*[22,27,28]. This review will focus on the pseudomonad phenazines, because they are the best studied with respect to biosynthesis, but will also include a discussion of the methanophenazine produced by *Methanosarcina mazei* Gö1, because it is the only phenazine thus far that has been unequivocally shown to have an important role in catabolism[27].

Roles in eukaryotic physiology and pseudomonad persistence

Phenazines in infection. Some of the most thorough studies that have been conducted to investigate the physiological consequences of phenazine exposure are those of Britigan and colleagues, who have reported the many effects of phenazines produced by *P. aeruginosa* during infection of the human lung[29]. Phenazine production plays an important role in both acute and chronic *P. aeruginosa* lung infections, which are frequent causes of mortality in patients who have cystic fibrosis or otherwise impaired lung function[21]. PYO has been detected in the sputum of patients with chronic *P. aeruginosa* infections at concentrations as high as 27 μg ml^{-1} (ref. 30), and the administration of purified PYO at comparable concentrations in laboratory mice has been shown to induce neutrophil influx in lung tissue[31]. Phenazine production is a common trait in strains of *P. aeruginosa* isolated from patients with cystic fibrosis[32], and mutant versions of the *P. aeruginosa* strains PAO1 and PA14 that are unable to synthesize PYO are attenuated in both acute and chronic mouse lung infection models[33].

The oxidative activity of phenazines in particular has been shown to be important in pathogenesis during *P. aeruginosa* lung infection[14]. Both PYO and PCA can increase oxidant formation in human airway epithelial cells through a number of mechanisms including the oxidation of glutathione and NADH and inhibition of antioxidant enzymes[13,15,34]. Once it is reduced, PYO can then react with oxygen, forming superoxide radical and hydrogen peroxide[35]. Pyocyanin radical is also formed as an intermediate during its redox cycling, and can further contribute to the formation of reactive oxygen species[36–39]. These insults to the host cell's internal redox balance may lead to increased secretion and thereby contribute to the generation of sputum, the physical and nutritional substrate for *P. aeruginosa* in the lungs of individuals with cystic fibrosis[40,41]. The generation of radical species (of phenazines and oxygen)

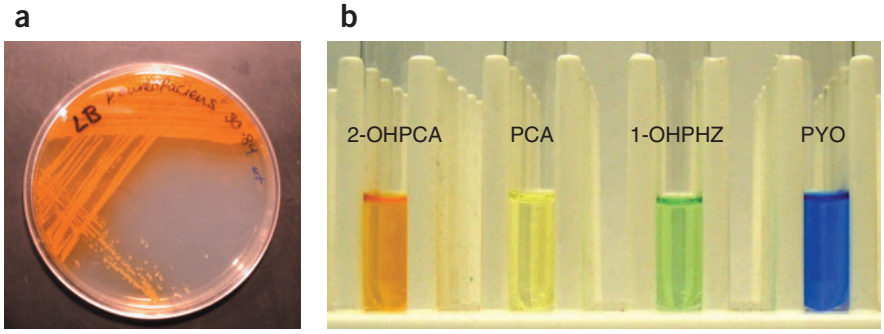


Figure 1 Phenazines are colorful, diffusible bacterial metabolites. (**a**) Streak plate of the biocontrol strain *P. aureofaciens* 30-84. The phenazine 2-OHPCA turns the agar bright orange. (**b**) Aqueous solutions of some of the phenazines produced by various *Pseudomonas* strains.

is potentially harmful to other microbes competing for resources in the lung, such as *Staphylococcus aureus*, and may help *P. aeruginosa* to persist in this environment[42]. However, recent work has demonstrated that phenazine production is beneficial to the growth or survival of *P. aeruginosa* in mouse infection models even in the absence of competing organisms, implying that these compounds may additionally provide a physiological benefit to their producers during infection[33].

Phenazines in soil ecosystems. The other well-studied niche for phenazine-producing pseudomonads is the rhizosphere, the zone surrounding the roots of plants. As is the case for *P. aeruginosa* in an infected lung, species such as *P. aureofaciens*, *P. fluorescens* and *P. chlororaphis* compete in this ecosystem with other organisms for resources. More importantly, they compete for colonization sites on the roots of agriculturally important crops, where they thrive as microcolonies (biofilms) and protect the plants from pathogenic fungi. Phenazines are thought to be important in this competition, and consistent with this, phenazine-producing strains of *P. aureofaciens* and *P. fluorescens* are better able to colonize the roots of wheat plants and persist in the rhizosphere than are phenazine-lacking mutants[43].

The toxicity of phenazines to bacteria and fungi typically present in the rhizosphere has been demonstrated, and again is thought to be mostly due to the generation of reactive oxygen species[12]. If biocontrol strains did use phenazine toxicity as a weapon to compete with indigenous soil populations for resources, one would expect the composition of rhizosphere communities to change after exposure to phenazines; however, this does not occur. The overall number of organisms competing with *P. fluorescens* for resources does not decline after this strain has colonized the root[44]. This implies that it is not just the antibiotic activity of phenazines that is important for the ability of their producers to compete in the soil. As is the case for phenazines produced by *P. aeruginosa*, there is evidence indicating that phenazines have roles in the physiology and thus the ecological competence of the biocontrol pseudomonads.

Biosynthesis of phenazine derivatives

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

In *Pseudomonas* spp., the phenazine biosynthetic pathway branches off from the shikimic acid pathway, which is also the source for metabolites such as the aromatic amino acids, siderophores and quinones (**Scheme 1**)[45–47]. Genes encoding the phenazine biosynthetic enzymes are arranged in one core operon, *phzABCDEFG*, in most phenazine-producing pseudomonads[25,48]. Such an operon exists in the genome of *P. aeruginosa* in duplicate, and the expression of the two copies of the operon is differentially regulated[24]. In many strains, additional genes involved in phenazine decoration, such as *phzM*, *phzS*, *phzO* and *phzH*, are present in single copy and can be located proximally to the core operon or elsewhere in the genome[26]; currently, little is known about how these genes are regulated.

Expression of phenazine biosynthetic genes is regulated by multiple mechanisms, which are strongly influenced by environmental conditions. One of the primary factors governing phenazine production is population density, and in *P. aeruginosa* this dependency is effected by at least three quorum-sensing systems[49,50]. Bacteria participating in quorum sensing release intercellular signals such as *N*-acyl-L-homoserine lactones (AHLs, **7** and **8**) and 2-heptyl-3-hydroxy-*R*-quinolone (the *Pseudomonas* quinolone signal, PQS, **9**) into the environment, where they can be taken up by neighboring cells of the same or different species (**Fig. 3**). Inside the cell, these compounds induce the expression of genes for their own biosynthesis—as well as many other genes important in virulence, competition and behavior—when they accumulate to a threshold concentration[18]. The dependence of phenazine biosynthesis on cell density has also been demonstrated for biocontrol pseudomonads, and in these species is mediated by a seemingly less complex quorum-sensing network[51–53].

In addition to cell density, numerous environmental factors have been identified that affect the regulation of phenazine biosynthesis, including

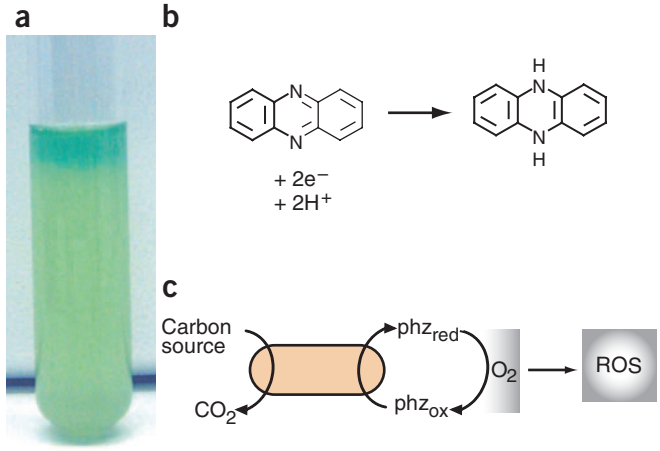
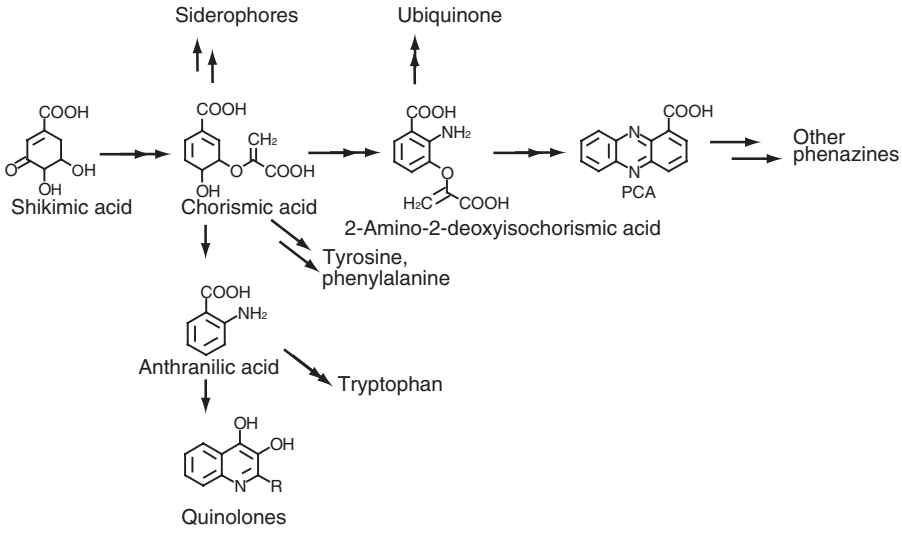


Figure 2 Pseudomonads stimulate phenazine reduction. (**a**) Characteristic gradient formed by standing cultures of *P. aeruginosa*. Bacterial respiration renders most of the culture anoxic. Phenazines (phz) are reduced and, in the case of PYO, become colorless. The darker blue at the top represents oxidized PYO. (**b**) Half-reaction representing generic two-electron phenazine reduction. (**c**) Schematic of phenazine reduction and auto-oxidation responsible for gradient formation in standing cultures. Reduced phenazines are oxidized abiotically by oxygen, generating reactive oxygen species (ROS).



Scheme 1 Phenazine biosynthesis and its relation to the shikimic acid pathway in pseudomonads[24,45,46,93].

oxygen, iron and phosphate concentration as well as the nature of the carbon sources available[54]. For many of these effects, it is not entirely clear whether they are above quorum sensing in the regulatory cascade (for example, iron limitation induces quorum-signal production, which in turn induces phenazine biosynthesis) or are the result of regulators acting independently of quorum sensing[55]. The GacA/S two-component system, which effects global changes in transcription, has been implicated in the control of phenazine biosynthesis and is thought to act by regulating quorum sensing, but there is also evidence that it affects phenazine gene expression through other mechanisms[56–58]. Repressors of phenazine biosynthesis have been identified in the plant symbionts *P. aureofaciens* and *P. chlororaphis*. Mutations in these repressors seem to override the quorum-sensing regulation of phenazine biosynthesis in these organisms, resulting in constitutive phenazine production[58,59].

Intercellular signaling: a regulatory role for phenazines

Recent work from our laboratory has contributed to understanding of the complexity of the *P. aeruginosa* quorum-sensing system and the place of phenazines in this cell density-dependent cascade. We have found that, in addition to being regulated by cell-cell communication, phenazines themselves can act as intercellular signals. Our work indicates that PYO is the physiological inducer of a set of genes previously identified as members of the quorum-sensing regulon. PYO acts downstream of PQS, which previously had been deemed the terminal signal in the *P. aeruginosa* quorum-sensing cascade. PYO's function as a quorum signal explains what was thought to be a delayed response in the expression of a specific set of genes in response to PQS[49]. We now understand that these genes are expressed later than those induced directly by PQS because PQS first has to upregulate the biosynthesis of phenazines so that PYO can subsequently induce its stimulon (ref. 60 and unpublished data) (**Fig. 3**).

The signaling function of PYO makes it the newest addition to the growing list of small molecules excreted by *P. aeruginosa* that have been shown to perform multiple functions[61]. PQS, like PYO, was also long recognized for its antibiotic and virulence properties before its role in signaling was elucidated[62]. Recent studies have demonstrated that certain AHL and quinolone derivatives chelate iron[63,64], raising the possibility that these metabolites facilitate iron uptake *in vivo*. The accumulating knowledge about the chemistry and biological activity of small molecules excreted by *Pseudomonas* spp. calls for a re-evaluation of our categorizations of these compounds. Rather than clearly performing one dedicated purpose, they seem to be capable of multiple roles. To determine the most important physiological effects of phenazines and other small metabolites in the environment and during infection, we will need to understand the physiological conditions allowing, requiring and regulating their activities. Elucidating their mechanisms of action at the molecular level may also provide indications of the conditions relevant for activity.

Other physiological roles

In addition to inducing gene expression, phenazines also act as substrates in intracellular redox transformations. This further metabolism of phenazines, subsequent to their biosynthesis, can be observed as a color change in pseudomonad cultures that have become limited for terminal electron acceptors. This is because pseudomonads reduce their own phenazines, and changing the oxidation state of a phenazine changes its absorbance spectrum. In cultures of the bacterium *P. aeruginosa*, this is observed as a loss of blue coloration, because the main phenazine produced by this organism, PYO, changes from blue to colorless upon reduction at neutral pH (**Fig. 2**)[65]. This activity has also been demonstrated in oxygen-limited cultures of the bacterium *P. chlororaphis*, which can use its phenazine product, phenazine-1-carboxamide (PCN, **5**) to reduce extracellular iron oxides[5]. Although a good deal of research effort has gone toward understanding phenazine reduction by mammalian cells, less work has been done to elucidate the mechanisms of the phenazine reduction that is readily observed in pseudomonad cultures. Here, we will discuss what is known about phenazine reduction and its physiological functions in phenazine-producing and non-producing prokaryotes.

Phenazines as 'respiratory pigments.' Based on the redox potentials, metabolism and solubilities of phenazines, it has been proposed that phenazines act as electron acceptors in cellular energy generation or in the maintenance of the intracellular redox balance[66]. Studies conducted by Ernst Friedheim in the 1930s, in which he observed that PYO increased the oxygen consumption of cell suspensions of *P. aeruginosa*[67], support this idea. Several reports on the interactions of PYO and 1-hydroxyphenazine (1-OHPHZ, **6**) with the mammalian respiratory chain were published in the decades that followed. 1-OHPHZ, but not PYO, was shown to inhibit respiration at the level of ubiquinone in the electron transport chain of mammalian cells. It was concluded, based on measurements of oxygen depletion (which did not decrease in the presence of PYO) that PYO did not inhibit mammalian cell respiration[19,68]. We question this interpretation, however, given that PYO can accept electrons from NADH and transfer them to oxygen; accordingly, what was thought to be normal respiration may actually have been short-circuiting of the electron transport chain by PYO. In contrast, it makes sense that oxygen depletion was not observed in the presence of 1-OHPHZ given that reduced 1-OHPHZ does not react with oxygen at appreciable rates[69].

Notably, the interactions of phenazines with the pseudomonad respiratory chain are largely unknown. Numerous groups have observed that both synthetic and natural phenazines are reduced by prokaryotes, but in most cases the physiological effect of this reduction has not been evaluated[70,71]. One exception is the role of phenazine reduction in the

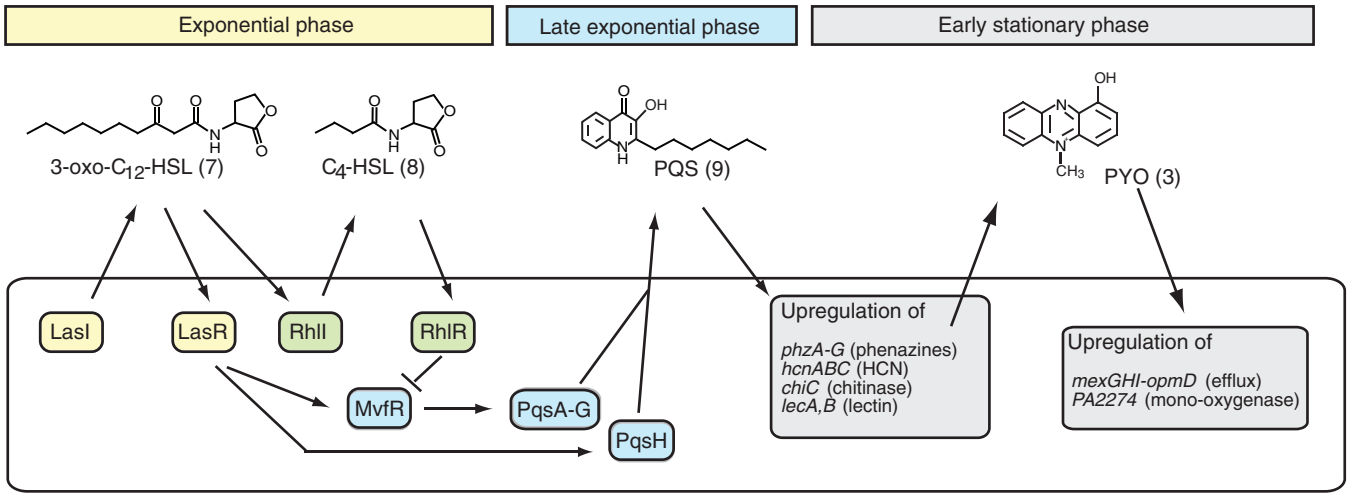


Figure 3 Model of the quorum-sensing network in *Pseudomonas aeruginosa*. The quorum-sensing network in *Pseudomonas aeruginosa* comprises a cascade of three types of signaling molecules that function in a growth stage–dependent manner. The AHLs 3-oxo-C_{12}-HSL and C_4-HSL are released in exponential phase and control the production of the quinolone PQS. PQS accumulates in late exponential phase and is required for the synthesis of phenazines. Recent findings from our laboratory show that the phenazine PYO upregulates genes that have previously been demonstrated to be controlled by quorum sensing, establishing PYO as a signaling molecule[50,94].

respiratory chain of *M. mazei* Gö1 (ref. 72). This archaeon produces methanophenazine, a phenazine derivative with a pentaisoprenoid side chain, and can utilize phenazines in lieu of quinones in electron transport. *In vitro*, methanophenazine has been shown to accept electrons from hydrogen or a reduced coenzyme by means of the activity of either of two membrane-bound dehydrogenases, one of which is homologous to the NADH dehydrogenase found in bacteria and mitochondria. Reduced methanophenazine can then donate electrons to another cofactor in a reaction catalyzed by a membrane-bound heterodisulfide reductase[73]. *In vivo*, these respiratory enzymes couple electron transport to the translocation of protons, generating a proton gradient that can be used to make ATP[74]. In *M. mazei*, therefore, phenazine reduction is not only crucial to energy metabolism in that it reoxidizes the NADH analog found in methanogens, but it is presumably also required for ATP synthesis[72,75]. Whether phenazine reduction can be similarly used by phenazine-producing pseudomonads to reoxidize NADH or generate ATP is still an open question.

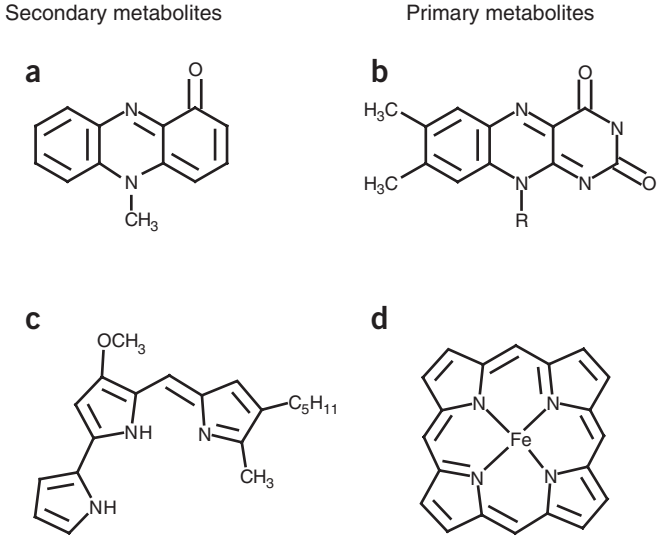


Figure 4 Key structural elements of secondary metabolites resemble those of cofactors that have critical roles in energy metabolism. (**a**) Pyocyanin. (**b**) Generic flavin. (**c**) Prodigiosin, an antibiotic produced by *Serratia marcescens*. (**d**) Generic heme[95,96].

Phenazines in redox homeostasis and iron acquisition Advances in our understanding of bacterial communities have provided an environmental context for the hypothesis that pseudomonads benefit by reducing phenazines. It has been proposed that the reduction of diffusible small molecules is advantageous during growth in a biofilm, a surface-attached population of bacteria suspended in an excreted matrix[66]. The diffusion rate of oxygen through a biofilm is thought to be slow, and cells at the base of an aerobic biofilm become limited for oxidants[76,77]. Under this condition, phenazines could allow bacteria to generate energy for growth or help maintain redox homeostasis by acting as electron acceptors for the reoxidation of accumulating NADH. Indeed, maintaining a proper redox balance in the pyridine nucleotide pool is essential for metabolism[78], and recent work from our lab indicates that P. aeruginosa phenazine-negative mutants have higher intracellular NADH/NAD^+ ratios in stationary phase than does the parent strain in planktonic cultures(unpublished data). This suggests that an important role for phenazines could be to serve as intracellular redox 'buffers'.

Various research groups have recently become more interested in phenazine reduction by biofilm-forming bacteria because phenazines make excellent electron transfer mediators to electrodes in biological fuel cells[79]. In biological fuel cells deployed in the environment as well as those set up in laboratories, bacteria often grow as biofilms attached to the electrode surface[80,81]. That phenazines facilitate electron transfer to electrodes has been demonstrated by Zeikus and colleagues, who investigated the ability of *Escherichia coli* to reduce the synthetic phenazine neutral red. They showed that *E. coli* is able to use this synthetic phenazine as an electron transfer mediator in the reduction of iron oxide, and presented evidence indicates that hydrogenase is at least partially responsible for this

capability[71]. Our group as well as others have shown that other synthetic dyes, with structures resembling those of phenazines, are reduced by *Bacillus*, *Lactococcus* and *Shewanella* species[5,70,82]. The Verstraete group has shown that phenazine production enhances power output from microbial fuel cells, and that biofuel cells enrich for phenazine-producing organisms; whether phenazine production influences the growth or survival of these bacteria in this context remains unclear[83,84].

Aside from these proposed roles in energy generation, it has been suggested that phenazine reduction could act to make iron more available to the producing organism. PYO may assist infectious *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[85]. As mentioned above, *P. chlororaphis* has been shown to reduce iron oxides through electron transfer to PCN, and it is thought that this ability may be important in the rhizosphere, where iron is present predominantly in an insoluble form[5]. An examination of the relationship between iron availability and the regulation of phenazine biosynthesis, however, presents a complicated picture that neither refutes nor supports a role for these compounds in iron acquisition. Although in many cases it has been reported that phenazine production is enhanced in iron-deprived cultures, other studies have demonstrated a requirement for iron in media optimized for phenazine biosynthesis[54,85,86]. These differences probably arise from the high degree of variability with respect to other parameters, such as carbon source and the concentrations of oxygen and various salts. Perhaps the best way to ascertain whether or not iron availability has relevance to phenazine production will be to observe its effects under conditions that imitate the most common habitats for phenazine-producing pseudomonads[41].

How are phenazines reduced? Although we are beginning to recognize the potential physiological importance of phenazine redox cycling, we have yet to identify the mechanisms by which pseudomonads catalyze the reduction of these compounds. On the basis of their low redox potentials and the mechanisms for phenazine reduction identified in eukaryotic cells, we might predict that NADH or glutathione would act as electron donors in these reactions. In the case of PYO, an enzyme may not be required, given that this compound reacts with these electron donors spontaneously. However, the relatively rapid reduction of other phenazines by bacterial suspensions is thought to require one or more enzymes[71]. The flavin-like structures of phenazines lead us to hypothesize that flavin-cofactored enzymes, capable of accepting electrons from low-potential donors such as NADH, might be responsible for catalyzing these transformations. If such an enzyme were associated with the respiratory chain, pseudomonads might be able to couple the reduction of their own excreted metabolites to the generation of a proton-motive force; in the absence of a better electron acceptor, they could utilize phenazines to generate ATP. Alternatively, if the enzyme were not associated with respiration, or were not a coupling site for proton transfer, phenazine reduction could still serve the valuable function of reoxidizing NADH under conditions in which the intracellular NADH/NAD^+ pool had shifted toward reducing.

Conclusions

As more is learned about the chemistry and biological activity of phenazines, we begin to question their categorization as 'secondary' metabolites. This compels us to rethink secondary metabolism as a phenomenon more generally. It is striking that the conditions under which secondary metabolites are produced in laboratory cultures (that is, stationary phase) are effectively the same as those that define many microbial habitats in nature[8]. Consistent with this, gene expression and physiological attributes seem to be very similar in stationary-phase planktonic cultures and biofilms[87,88]. As we have discussed for phenazines, stationary-phase metabolites can allow bacteria to sense the conditions of their surroundings and induce appropriate changes in gene expression; moreover, they can facilitate extracellular electron transfer to oxidants such as insoluble iron[5,24] and have the potential to play a role in redox homeostasis. Notably, phenazines are only one class among the myriad natural products made by microorganisms[89], many of which bear intriguing structural resemblances to cofactors that have important roles in primary metabolism (**Fig. 4**). Now that we are beginning to understand stationary-phase physiology and its ecological relevance, it is time to revisit the roles of these compounds in gene expression and survival. We suspect that such studies will further blur the line between primary and secondary metabolism and will lead to a more complete picture of the mechanisms allowing organisms to persist in dynamic environments.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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