

Chapter 2

Regulation of Phenazine Biosynthesis

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Abstract Microbiologists have historically been struck by both the beautiful pigmentation of phenazine-producing cultures and the high degree of variability in phenazine production among isolates, conditions, and even repeat experiments. Motivated by an interest in controlling phenazine biosynthesis, they have identified many of the factors that affect the regulation of this process. Phenazine production is controlled by complex regulatory networks. The variability of phenazine production can be explained in part by the effects of environmental conditions on these networks and by strain-specific differences in these networks. In this chapter, we describe the components of a common regulatory cascade that is represented in many phenazine-producing pseudomonads. Membrane sensor proteins and two component sensors control the activity of downstream regulators such as quorum sensing systems and RNA-binding proteins and small RNAs; these cytoplasmic regulators then control the production of phenazine biosynthetic proteins. We highlight examples from specific strains and cases where the mechanistic links may vary among them. We also discuss environmental parameters that have been shown to affect phenazine biosynthesis and compare their effects in different isolates. Ongoing work will further elaborate the details of the environmental sensing and regulatory responses that control production of these dramatically colored compounds. New findings have the potential to support enhanced application of phenazine-producing strains in agriculture, where they promote crop health, and the treatment of infections in which phenazines contribute to bacterial pathogenicity.

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2.1 Introduction

The dramatic coloration of phenazine-producing bacterial cultures has attracted researchers in many disciplines for over a century (Fordos 1859; Gessard 1894). It may have contributed to the early recognition and classification of *Pseudomonas chlororaphis* (Guignard and Sauvageau 1894), *P. aeruginosa*, and other bacteria whose species epithets derive from their pigmentation (Schroeter 1872). Furthermore, it has inspired researchers to ask many different types of questions about the biological relevance of phenazines, and a variety of physiological effects have been demonstrated for these compounds in both the organisms that produce them and the organisms exposed to them (see Chap. 3).

For microbiologists cultivating phenazine-producers, it is apparent that phenazine biosynthesis can vary unpredictably among cultures, suggesting that it is sensitive to subtle environmental variations (Fig. 2.1). Under many conditions, the precise molecular cues that interact with regulatory proteins to control phenazine biosynthetic gene expression are not known. However, downstream mechanisms controlling their expression have been identified in several species, and themes of phenazine regulation have emerged, including control by two component systems, quorum sensing (QS), and small noncoding RNAs (sRNAs) (Fig. 2.2). In this chapter, we will summarize the phylogenetic distribution of phenazine biosynthetic clusters and cite examples from phenazine-producing pseudomonads that illustrate specific regulatory mechanisms. In addition, we will discuss some of the environmental signals that control phenazine production in various isolates. We will focus on the regulation of phenazine biosynthesis in members of the genus *Pseudomonas*, where the bulk of studies on this topic have been conducted.

Fig. 2.1 *P. aeruginosa* PA14 grown on an agar plate containing a gradient of tryptone

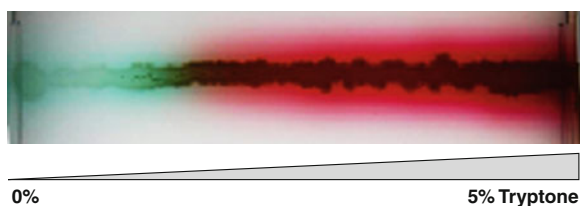
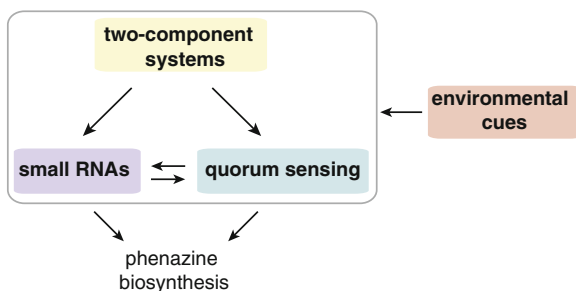


Fig. 2.2 Conceptual hierarchy of the phenazine regulation network



2.2 Phylogenetic Distribution and Mechanisms of Phenazine Biosynthesis

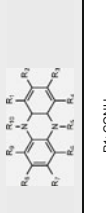
Phenazine-producing organisms have been identified that belong to the bacterial phyla Actinobacteria and Proteobacteria and the archaeal phylum Euryarcheota (Mavrodi et al. 2010). The gene cluster encoding biosynthetic enzymes for the archaeal electron carrier methanophenazine, produced by *Methanosarcina mazei*, and the conditions controlling its biosynthesis are unknown. In bacterial species for which phenazine production has been observed, variation exists at the level of the isolate, such that some strains within a species produce phenazines while others lack the biosynthetic genes. Bacterial phenazine biosynthetic pathways identified to date proceed via chorismate to the formation of the core phenazine structure and ultimately, to production of the common phenazine precursor phenazine-1-carboxylic acid (PCA) (Mentel et al. 2009; Seeger et al. 2011; see also Chap. 1 of this volume). Whether and how PCA is modified to produce other phenazines varies among organisms and depends to some extent on environmental conditions. A variety of functional groups can be added to the core structure to produce phenazines in a range of colors with diverse chemical properties (Turner and Messenger 1986; Laursen and Nielsen 2004; Mavrodi et al. 2006; Pierson and Pierson 2010) (Table 2.1). Many of the decorating enzymes responsible for PCA transformation have been identified and characterized, and regulation of their activities determines the complement of phenazines produced by a given strain under particular conditions.

The archetypical core phenazine operon is found in pseudomonads such as *P. chlororaphis*, *P. aeruginosa*, *Pseudomonas* sp. CMR12a, and *P. fluorescens* 2-79 and contains seven genes; the operon structure is more variable in other Proteobacteria and in Actinobacteria (Mavrodi et al. 2010). *P. aeruginosa* strains appear to be unique among these organisms in that their genomes contain two phenazine biosynthetic operons, which we will refer to as *phz1* and *phz2* and which are nearly identical at the DNA level. Although the contributions of the core *phz* operon products to phenazine biosynthesis are generally known, this is an area of active research (see Chap. 1 of this volume). Genes for decorating enzymes can lie adjacent to the core operon or elsewhere in the genome. In some cases, the products of adjacent genes play roles in regulation of the core operon or phenazine transport.

2.3 Mechanisms and Conditions Controlling Phenazine Biosynthesis in Pseudomonads

Researchers in disparate subdisciplines of microbiology have long been interested in elucidating the mechanisms that control phenazine biosynthesis. Phenazine production is critical for the biocontrol properties of certain agriculturally important root-colonizing pseudomonads that protect food crops from attack by

Table 2.1 Phenazine derivatives produced by *Pseudomonas* spp. and other bacteria

Phenazine derivative	References		actinobacteria	beta-proteo-bacteria	gamma-proteobacteria
1-phenazine-carboxamide	Lasseur (1911); Birkhofer (1947)	R1: CONH ₂			<i>P. aeruginosa</i> <i>P. chlororaphis</i>
5-methyl-phenazine-1-carboxylic acid	Byng, Eustice et al. (1979) Hanford and Holliman et al. (1972)	R1: COOH; R5: CH ₃			<i>P. aeruginosa</i>
Aeruginosin A	Holliman (1969)	R1: COOH; R5: CH ₃ ; R7: NH ₂			<i>P. aeruginosa</i>
Aeruginosin B	Herbert and Holliman (1969)	R1: COOH; R5: SO ₂ ; R6: CH ₃ ; R7: NH ₂			<i>P. aeruginosa</i>
4,9-dihydroxyphenazine-1,6-dicarboxylic acid dimethyl ester	Ballard, Pallaroni et al. (1970)	R1: COOCH ₃ ; R4: OH; R5: COOCH ₃ ; R6: OH	<i>Burkholderia cepacia</i>		
D-alanylgisculetinic acid	Giddens, Feng et al. (2002)	R1: COOH; R6: C O NH ₂ ; R8: OCH ₃			<i>Erwinia herbicola</i> <i>Vibrio</i> spp.
1-hydroxyphenazine	Gerber (1967); Schoental (1941)	R1: OH			<i>P. aeruginosa</i>
Pyocyanin	Fordis (1959); Von Saltza, Last et al. (1969)	R1: O; R5: CH ₃			<i>P. aeruginosa</i>
1-phenazine-carboxylic acid	Turner and Messenger (1986); Laursen and Nielsen (2004); Mentele, Ahuja et al. (2009)	R1: COOH	All phenazine-producing bacteria		
Iodinin	Clemo and Dalglish (1949); Gerber and Lochevalier (1964); Gerber and Lochevalier (1965); Lee and Gerber (1967); Tanabe and Otagaki (1971); Gerber (1967)	R1: OH; R5: O; R6: OH; R10: O	<i>Brevibacterium iodinum</i> <i>Micrococcus paraffinofiscus</i> <i>Actinomadura dassonvillei</i> <i>Amycolata hydrocarbonoxydans</i> <i>Microbospira</i> spp. <i>Comamonas</i> sp. <i>Coriobacter hydrocarbonoxazurum</i> <i>Arthrobacter paraffinifusus</i> <i>Nocardia hydrocarbonoxydans</i> <i>Streptomyces thiolobus</i>		
Iomolungin	Johnson and Dietz (1969); Tipton et al. (1970)	R1: COH; R2: OH; R4: O; R6: COOCH ₃ ; R9: OH	<i>Streptomyces lomodensis</i>		
Solphenzazine A	Rusman, Eppgaard et al. (2013)	R4, R5: C ₂ H ₅ O	<i>Streptomyces</i> sp.		
Endophenzazine A	Gebhardt et al. (2002); Haugen, Gluck et al. (2006)	R4: C ₂ H ₅ ; R6: COOH	<i>Streptomyces anulus</i> <i>Streptomyces crammionensis</i>		
1,6-phenazineimethanol	Choi, Kwon et al. (2009)	R1, R2: CH ₂ OH	<i>Brevibacterium</i> sp.		

In bacteria where the phenazine biosynthetic pathway has been examined, phenazine-1-carboxylic acid is the precursor for all the other phenazine derivatives. The carboxyl group can be replaced or removed, and a wide variety of functional groups can be added at different positions on the phenazine core structure. For more comprehensive collections of identified phenazines, see (Turner and Messenger 1986, Laursen and Nielsen 2004, Mavrodi et al. 2006)

pathogenic fungi (Chin-A-Woeng et al. 2003; Haas and Défago 2005; Mavrodi et al. 2006; Mavrodi et al. 2012). In the clinical setting, phenazine production contributes to virulence during acute and chronic *P. aeruginosa* infections (Lau

et al. 2004; Caldwell et al. 2009; Hunter et al. 2012). Regulation of phenazine biosynthesis has therefore been studied in diverse *Pseudomonas* isolates. Although the precise linkages between regulatory mechanisms may vary among species and even strains, general mechanisms and their overall hierarchy are often shared. We will highlight these commonalities and focus on specific systems that exemplify general principles. Figure 2.3 summarizes the main regulatory systems and



Fig. 2.3 Proteins and sRNAs that control phenazine production, with targets indicated. X indicates which pseudomonad contains a particular element or in which an environmental effect on phenazine production has been observed. Shading in the table indicates the location of each type of element in regulatory cascades; a sample cascade is shown. *Only found in *P. aeruginosa*

molecules that ultimately control phenazine production: two component systems, QS, sRNAs and environmental cues. These mechanisms and cues can act indirectly by influencing activities far upstream of phenazine biosynthetic gene expression or RNA translation, or they can directly control these processes. Additional details regarding the complex relationships between and within these regulatory systems can be found in recent reviews that summarize the literature (Mavrodi et al. 2006; Williams and Camara 2009; Pierson and Pierson 2010; Sonnleitner and Haas 2011; Balasubramanian et al. 2013).

2.3.1 Two Component Systems

In both biocontrol and pathogenic pseudomonads, two component systems were among the first regulatory mechanisms identified that play critical roles in phenazine regulation (Reimann et al. 1997; Chancey et al. 1999; van den Broek et al. 2003; De Maeyer et al. 2011). They lie conceptually at the top of signaling hierarchies because they have the potential to directly sense environmental cues and then modulate the activities of downstream regulatory mechanisms or directly control gene expression (Fig. 2.4). Such systems typically consist of a membrane-bound sensor protein and a cytoplasmic regulatory protein. The phosphorylation status of the sensor protein is altered through binding of a small molecule or other

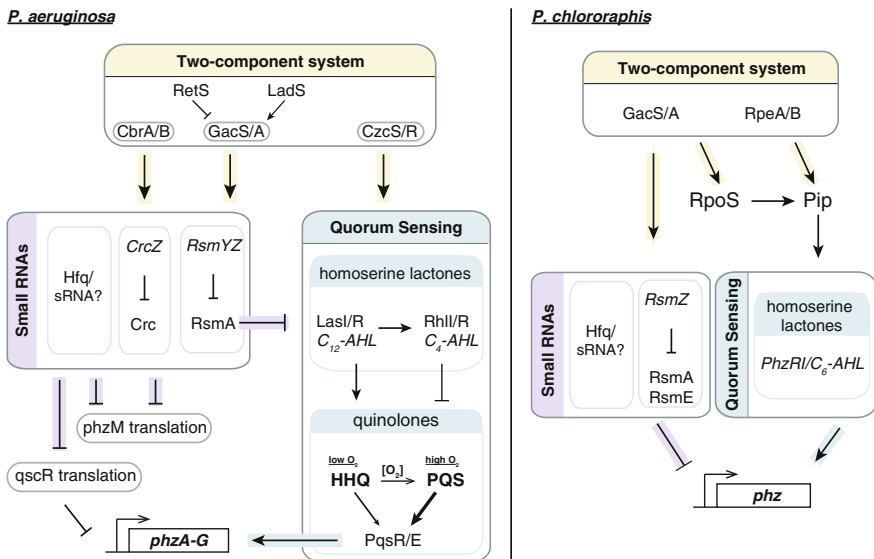


Fig. 2.4 Summary of two component, quorum sensing, and sRNA systems that control phenazine production in *P. aeruginosa* and *P. chlororaphis*. The *P. fluorescens* regulatory system is likely similar to that of *P. chlororaphis*

environmental cue that triggers conformational changes and affects activity. The phosphate group is then transferred to and activates the response regulator protein (Bourret and Silversmith 2010).

GacS/GacA, which is required for wild-type phenazine production in many isolates, is the best-characterized two component system controlling this process (Heeb and Haas 2001; Haas and Defago 2005). In phenazine-producing species, it occupies a position between environmental cues and downstream, intracellular regulatory mechanisms such as those dependent on sRNAs. In describing this system and others below, we will include references to the regulatory cascade in *P. fluorescens* strains that do not produce phenazines in cases where it is possible that the same cascade operates and affects *phz* gene expression in *P. fluorescens* 2-79. In addition, we note here that we use ORF numbers from *P. aeruginosa* PAO1 (starting with “PA”) for some of the proteins described below.

The cue that activates GacS has not been identified, but additional membrane proteins that control GacS activity in some isolates are known. These include RetS and LadS, hybrid sensor kinases that contain the unusual arrangement of periplasmic sensor domains linked to cytoplasmic histidine kinase and response regulator receiver domains (Goodman et al. 2004; Ventre et al. 2006). In strains of *P. fluorescens* and *P. aeruginosa*, RetS interacts with GacS and inhibits the phosphorelay (Goodman et al. 2009; Workentine et al. 2009; Vincent et al. 2010). A physical interaction between LadS and GacS is also predicted, however, this hybrid sensor potentiates phosphotransfer from GacS to GacA (Workentine et al. 2009). Although the GacS/GacA system does not directly control expression of *phz* genes, it does modulate the activities of sRNA- and QS-dependent regulatory mechanisms, which can then directly interact with *phz* gene promoters or transcripts (Fig. 2.4). These systems are discussed in further detail below.

The two component system CzcS/CzcR was recently implicated in regulation of *phz* gene expression in *P. aeruginosa* PAO1 (Dieppois et al. 2012). CzcS/CzcR is activated by zinc, cadmium, and cobalt and induces expression of an efflux pump that confers resistance to these metals. Dieppois et al. (2012) observed that mutants lacking functional CzcS/CzcR overproduce the phenazine pyocyanin (5-*N*-methyl-1-hydroxyphenazine, PYO), despite the fact that this two component system positively regulates QS. Chromatin immunoprecipitation assays suggest that CzcR binds to the *phzI* promoter when the system is activated by zinc. In this way, CzcR could inhibit *phzI* expression directly, negating the positive control of this operon by QS. This mechanism would constitute an unusual example of a direct link between a two component system and transcriptional control of *phz* genes.

Several other two component systems have been shown to affect phenazine production in various isolates, and evidence reported thus far indicates that this regulation is mediated via additional proteins and mechanisms. The RpeB/RpeA system positively regulates phenazine production in *P. chlororaphis* 30-84, and homologues to this system are present in other biocontrol strains but not in *P. aeruginosa* (Wang et al. 2012a). Proteins identified that positively regulate, or would be expected to regulate, phenazine production or *phz* genes in *P. aeruginosa* include the sensor/regulator pair BfiS/BfiR (acting via an indirect effect on levels

of the sRNA RsmZ) (Petrova and Sauer 2010); the sensor/regulator pair CbrA/CbrB, which induces the expression of sRNA CrcZ in response to nonpreferred carbon sources (Sonnleitner et al. 2009); the sensor PA2573, which affects PYO production through an unknown regulator (McLaughlin et al. 2012); and the individual sensors PA1611, PA1976, and PA2824, which can all control the activation state of the response regulator HptB (Hsu et al. 2008). HptB would be expected to affect phenazine production indirectly through a complex regulatory cascade that ultimately controls expression of the sRNA RsmY. The positions of RsmZ and RsmY in the regulatory network controlling phenazine production are discussed further below.

2.3.2 Quorum Sensing

Phenazine production in liquid batch cultures typically occurs after the period of most rapid growth, and phenazines accumulate in the culture in stationary phase. This is in part due to the regulation of *phz* gene expression by QS. In QS, bacteria excrete small molecule or peptide signals into the environment which can then affect gene expression in the producer. Their regulatory effects become apparent after they have reached a minimum concentration, often after a decrease in culture growth rate. Molecules with diverse structures have been implicated in this behavior, but acyl homoserine lactones (AHLs) and quinolone derivatives in particular are most relevant for *phz* gene expression.

Systems that support AHL-dependent QS control of *phz* gene expression contain homologues of LuxR, a DNA binding sensory protein whose activity is controlled by AHL, and, typically, homologues of LuxI, an AHL synthase. These proteins were first identified in the recently reclassified luminescent bacterium *Aliivibrio fischeri* (Meighen 1991) and their homologues have since been characterized in a broad diversity of species. LuxR homologues vary in their specificity for AHL derivatives, with some proteins requiring a single signal for activation and others responding to several variations on a core structure. In *P. chlororaphis* strains 30-84 and PCL1391, *P. fluorescens* 2-79, and *Pseudomonas* sp. CMR12a, the LuxR/I homologues PhzR and PhzI are encoded by ORFs that lie adjacent to the phenazine biosynthetic genes but are each individually transcribed (Pierson et al. 1994; Wood and Pierson 1996; Chin-A-Woeng et al. 2001; Khan et al. 2005; De Maeyer et al. 2011). The PhzR/I systems in these strains produce and are controlled by *N*-(3-hydroxy-hexanoyl)-HSL (3-OH-C₆-HSL). Although additional LuxR/I type systems in some of these strains produce and respond to other AHLs, 3-OH-C₆-HSL is the main such signal relevant for *phz* gene regulation (Khan et al. 2007). The promoter regions of the *phz* operons in the root-colonizing strains that have *phzR* and *phzI* contain canonical binding sites for LuxR-type regulators; these are near-perfect inverted repeats that can be referred to as *lux*, *las/rhl*, or *phz* boxes (Egland and Greenberg 1999; Chin-A-Woeng et al. 2001; Khan et al. 2005).

The *P. aeruginosa* genome encodes at least three LuxR homologues called LasR, RhlR, and QscR. The cognate AHL synthases for LasR and RhlR produce *N*-(3-oxododecanoyl) homoserine lactone (3-O-C₁₂-HSL) and *N*-butanoyl-*L*-homoserine lactone (C₄-HSL), respectively (Pearson et al. 1994; Pearson et al. 1995). Interestingly, QscR has no obvious cognate AHL synthase, but it responds most effectively to 3-O-C₁₂-HSL (Lee et al. 2006). Whether these LuxR homologues activate or repress gene expression depends on the location of the binding site relative to the transcription start site of the target gene. In contrast to the QS circuits in root-colonizing pseudomonads that control phenazine production, which are not known to regulate loci other than the *phz* operons, the AHL-controlled regulatory networks in *P. aeruginosa* affect expression of countless targets (Whiteley et al. 1999; Wagner et al. 2003).

Although their genomes share high sequence similarity, the *P. aeruginosa* strains PAO1, PA14, and M18 exhibit strain-dependent differences with respect to QS-dependent regulation of *phz* gene expression, and in many cases the mechanisms underlying these activities have not been thoroughly characterized. Often, the PCA derivative PYO is used as an indicator molecule in studies evaluating phenazine production because it is easier to detect than the other *P. aeruginosa* phenazines. In PAO1 and PA14, Las- and Rhl-defective mutant strains lose the ability to produce PYO, while in M18, the Las and Rhl systems are apparently negative regulators of phenazine production. Recently, Wurtzel et al. (2012) used gel mobility shift assays to confirm the presence of a LasR/RhlR binding site in the promoter region of *phz1* in PA14. This binding site also influences expression of *phzM*, which encodes an enzyme that catalyzes the first step in the transformation of PCA to PYO, and is divergently transcribed from the *phz1* operon. No *las/rhl* box has been identified in the promoter region of *phz2*, although interestingly, the gene encoding QscR lies adjacent to this operon. QscR is a negative regulator of *phz1* and *phz2* expression and appears to act through repression of *lasI* (Chugani et al. 2001; Ledgham et al. 2003).

Many additional regulators have been identified that affect QS, thereby altering phenazine production; in some cases they may affect phenazine production both indirectly through QS and through direct regulation of *phz* gene expression (Beatson et al. 2002; Juhas et al. 2004; Xu et al. 2005; Liang et al. 2009; Rampioni et al. 2009; Siehnel et al. 2010). An important class of regulators that can influence transcription are sigma factors, which associate with RNA polymerase and control preferences for specific promoters. The sigma factors σ^S (RpoS) and σ^{54} (RpoN) both affect QS-dependent regulation. In *P. aeruginosa*, σ^S participates in mutual regulation with AHL-dependent QS, in which σ^S stimulates a moderate induction of *lasR* and *rhlR*, and these QS systems subtly induce *rpoS* (Schuster et al. 2004). Despite this, *P. aeruginosa* PAO1 *rpoS* mutants overproduce PYO, suggesting that σ^S also acts independently of AHL to modulate phenazine biosynthesis (Suh et al. 1999). In *P. chlororaphis* 30-84, σ^S is positively regulated by the GacS/GacA two component system and activates phenazine inducing protein (Pip). Pip positively regulates the PhzR/I QS system, which ultimately upregulates the *P. chlororaphis* phenazine operon, making σ^S a positive regulator of phenazine production in this

strain (Girard et al. 2006a). Although the downstream effects of σ^S on phenazine production differ in *P. aeruginosa* and *P. chlororaphis*, the regulator PqsA positively controls σ^S activity in both strains (Kojic and Venturi 2001; Girard et al. 2006b). Conflicting results have been reported regarding the effects of σ^{54} on the Rhl QS system in *P. aeruginosa* (Heurlier et al. 2003; Thompson et al. 2003). PA14 mutants lacking functional σ^{54} produce less PYO (Hendrickson et al. 2001); this may be because the expression of the CrcZ sRNA (discussed below) is σ^{54} -dependent (Abdou et al. 2011).

One important target of the AHL-controlled regulatory network in *P. aeruginosa* is the operon *pqsABCDE*. This locus is required for the production of another class of chemical signaling molecules called quinolones, and together, the AHL and quinolone signaling pathways form the core of the *P. aeruginosa* QS signaling cascade (Pesci et al. 1999). *pqsA-D* encode biosynthetic enzymes that are required for production of 2-heptyl-4-quinolone (HHQ). PqsH, encoded elsewhere in the genome, is a monooxygenase that converts HHQ to *Pseudomonas* Quinolone Signal (PQS) (Deziel et al. 2004). Both HHQ and PQS activate the transcriptional regulator PqsR (also known as MvfR), but PQS does so with greater efficiency (Xiao et al. 2006; Diggle et al. 2007). PqsR itself activates expression of *pqsABCDE*; therefore, HHQ/PQS and PqsR participate in an autoregulatory positive feedback loop in which the quinolones potentiate their own production (Fig. 2.5).

pqsE encodes a putative metallo- β -hydrolase of unknown function (Yu et al. 2009) that appears to be “caught” in the positive feedback loop controlling HHQ production: it is induced as a result of this mechanism but is not required for HHQ synthesis. Nevertheless, PqsE is required for phenazine production in *P. aeruginosa* PAO1 and PA14 (Gallagher et al. 2002; Recinos et al. 2012). Constitutive expression of PqsE in a *pqsA* mutant background is sufficient to promote phenazine production (Farrow et al. 2008), suggesting that, in the context of *phz* operon expression, the positive feedback loop that promotes HHQ production serves the

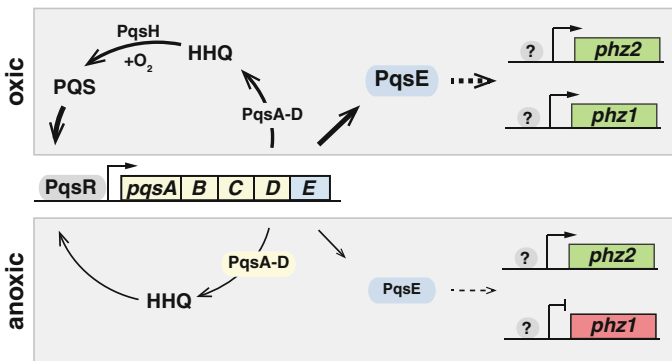


Fig. 2.5 Quinolone-mediated expression of the *pqs* operon and its influence on transcription of the two *phz* operons in the presence and absence of oxygen

sole purpose of tangentially upregulating *pqsE* (Williams and Camara 2009). The mechanism whereby PqsE promotes *phz* operon expression remains completely undefined, as PqsE itself does not contain a DNA binding domain. PqsE may be involved in the transformation of an unknown signal (Yu et al. 2009). We hypothesize that this signal controls the activity of an unidentified regulator of *phz* gene expression.

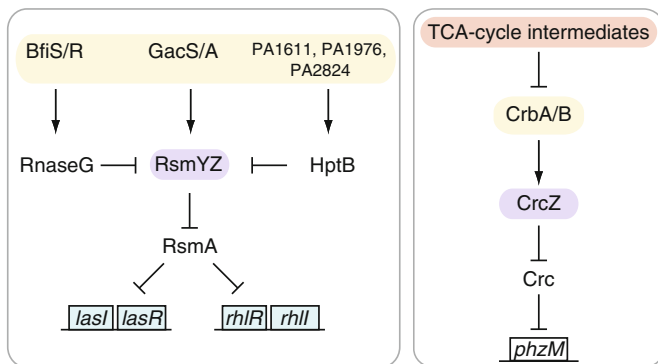
Until recently, studies examining the roles of quinolones in the regulation of *phz* gene expression focused on aerobically grown, well-mixed planktonic cultures. Under this condition, PQS is required for *phz1* expression and *phz1* is a major contributor to phenazine biosynthesis. Our group has evaluated the relative contributions of *phz1* and *phz2* to phenazine production in aerobic liquid cultures and biofilms in *P. aeruginosa* PA14 (Recinos et al. 2012). We have reported that, although *phz1* is expressed at much higher levels than *phz2* in *P. aeruginosa* PAO1 grown in aerobic liquid cultures (Whiteley et al. 1999; Chugani et al. 2001), in strain PA14 both operons make significant contributions to phenazine production when it is grown under the same conditions.

Interestingly, when *P. aeruginosa* PA14 is grown as a colony biofilm on agar plates, *phz2* alone is sufficient for wild-type phenazine production. Furthermore, HHQ is sufficient to fully activate expression of *phz2* in this context. The observation that HHQ rather than PQS is the major regulator of *phz* gene expression in biofilms is intriguing when considered in the context of oxygen availability. The conversion of HHQ to PQS is catalyzed by PqsH and requires molecular oxygen (Schertzer et al. 2010). In biofilms, which become anoxic at depth due to limited diffusion and oxygen consumption by cells closer to the surface (Dietrich et al. 2013), HHQ is likely produced in greater abundance than PQS. *phz2* expression is mediated through PqsE and downstream regulators that are apparently specific for this operon. The mechanism whereby PqsE controls expression of *phz1* and *phz2*, and the mechanisms that confer differential, condition-dependent expression of *phz1* and *phz2* are currently under investigation but likely include AHL-dependent regulation (Farrow et al. 2008).

2.3.3 Post-transcriptional Regulation

Several regulatory mechanisms have been identified that control, or would be expected to control, pseudomonad phenazine production post-transcriptionally (Fig. 2.6). These mechanisms are diverse and include mRNA binding by proteins and mRNA base pairing with 5'-leader RNA sequences, both of which can affect translation (Sonnleitner and Haas 2011). Additional sRNAs can further modulate the binding of such proteins and *cis*-acting regulatory RNAs to mRNA. Expression of these protein and RNA regulators is often controlled by two component systems or QS. They can indirectly control *phz* gene expression by modulating earlier steps in the regulatory cascade, or directly control translation of *phz* mRNAs. Extensive characterization of post-transcriptional regulators has been conducted in

I. Protein sequestration



II. Base pairing

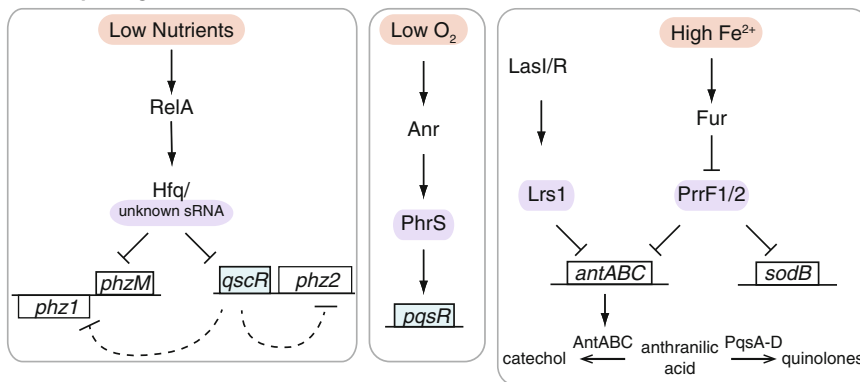


Fig. 2.6 sRNAs and proteins that regulate phenazine production post-transcriptionally in *P. aeruginosa*. Some sRNAs regulate target genes by base pairing with target mRNAs, while others sequester proteins that interact with mRNAs. Known environmental cues that regulate these elements are indicated

P. aeruginosa phenazine-producing strains, but also in *P. fluorescens* strains that do not contain *phz* operons. Our discussion includes references to these *P. fluorescens* strains as their post-transcriptional regulatory mechanisms may be relevant for the regulation of phenazine production in *P. fluorescens* 2-79.

The proteins RsmA and RsmE modulate secondary metabolism, QS-dependent activities and phenazine production in diverse pseudomonads. Both proteins are found in *P. fluorescens* and *P. chlororaphis*, while only RsmA is found in *P. aeruginosa* (Blumer et al. 1999; Reimmann et al. 2005). In *P. fluorescens*, RsmA and RsmE have been shown to interact with and inhibit the translation of target mRNAs that contain unpaired ANGA motifs near the ribosomal binding site; by stabilizing a stem loop that contains the ANGA motif, RsmA/E prevents the ribosome from binding (Lapouge et al. 2007). In *P. aeruginosa* strains, RsmA

expression increases with cell density and regulates the LasR/I and RhlR/I QS circuits in a post-transcriptional manner (Pessi et al. 2001). It is thought that RsmA binds to *lasR* and *rhlR* mRNAs, inhibiting their translation. Though RsmA and RsmE have not been studied in the phenazine-producer *P. fluorescens* 2-79 (containing the LuxR/I homologues PhzR/I), they may have a similar effect on QS in this strain.

As RsmA is a negative regulator of *lasR* and *rhlR* mRNAs, one would predict that mutations in *rsmA* would lead to phenazine overproduction (Reimann et al. 2005). Burrowes et al. (2006) found, however, that the phenotype of an *rsmA* mutant was condition-dependent: the mutant showed decreased PYO production in LB but increased PYO production in a defined medium containing glycerol and alanine (Burrowes et al. 2006). Furthermore, differing phenazine production phenotypes in *rsmA* mutants in strains PAO1 and M18 may indicate that temperature is an additional environmental parameter that affects this regulatory cascade. In M18, which is typically grown at 28 °C, RsmA is a positive regulator of phenazine production (Zhang et al. 2005). Interestingly, RsmA consensus sequences are present near the ribosomal binding sites of the *phzA1* and *phzA2* promoters, raising the possibility of direct control of the phenazine biosynthetic operons by RsmA. Preliminary evidence suggests that RsmA and RsmE also regulate expression of the *phz* operon in *P. chlororaphis*, although whether this occurs via direct interaction with *phz* operon mRNA, through regulation of the PhzR/I QS system, or both has not been reported (Wang et al. 2012a).

sRNAs containing the ANGA motif can compete with target mRNAs for binding sites on RsmA and RsmE, thereby controlling the extent to which these proteins repress their targets. In *P. aeruginosa*, two of these sRNAs, called RsmY (sometimes referred to as RsmB) and RsmZ, have been identified (Heurlier et al. 2004; Burrowes et al. 2005). *P. fluorescens* strains produce homologues of these plus an additional sRNA called RsmX (Heeb et al. 2002; Valverde et al. 2003; Kay et al. 2005). Evidence suggests that the genes encoding these sRNAs are directly regulated by GacA. When phosphorylated GacA activates transcription of *rsmX*, *rsmY*, and *rsmZ*, the sRNA products bind to and sequester the translational repressors RsmA and RsmE, allowing expression of RsmA/E target genes (Heurlier et al. 2004; Kay et al. 2006). In *P. aeruginosa*, additional regulators have been reported to control expression of the RsmY and RsmZ sRNAs. HptB indirectly represses RsmY expression through a complex regulatory cascade (Hsu et al. 2008; Bordi et al. 2010). Furthermore, the BfiS/BfiR two component system induces expression of RNaseG, which specifically degrades RsmZ (Petrova and Sauer 2010). Levels of RsmA and RsmE are also regulated by complicated networks involving two component sensors, sRNAs, and QS systems. These mechanisms further contribute to the complexity of regulation of *phz* gene expression, but their ultimate effects on phenazine production have not been measured.

The two component system CbrA/CbrB controls expression of *phzM* via a mechanism analogous to the network linking GacS/GacA to *phz* operon expression. When CbrB is activated, it induces expression of CrcZ, an sRNA that binds to and sequesters the translational repressor Crc. The CrcZ and *phzM* transcripts

both contain an A-rich motif that is recognized by Crc. CrcZ therefore limits the ability of Crc to inhibit *phzM* translation, and a *crc* mutant overproduces PYO due to increased PhzM levels (Huang et al. 2012).

In contrast to RsmX/Y/Z and CrcZ, which are controlled at the transcriptional level by two component systems, the sRNAs Lrs1 and Lrs2 are regulated by QS (Wurtzel et al. 2012). Using *P. aeruginosa* PA14 as a model strain, Wurtzel et al. identified *las* boxes in the promoter regions of the *lrs1* and *lrs2* genes and confirmed their regulation by LasR. In addition, they generated an *lrs1* deletion mutant and found that it was defective in PYO production. RNA-seq analysis revealed two major differences in transcript levels between this mutant and the wild-type parent: increased abundance of transcript from the *antABC* operon, and increased abundance of the PrrF1 and PrrF2 sRNAs (discussed further below). The authors hypothesized that the PYO production defect in the *lrs1* mutant arose from increased flux through an anthranilate-catechol conversation pathway (mediated by the products of the *antABC* operon). Anthranilate and phenazines are produced by pathways that branch from chorismate as a common precursor (Mentel et al. 2009). Notably, increased conversion of anthranilate to catechol also diverts it away from the quinolone biosynthetic pathway. Given that quinolones regulate *phz* operon expression, indirect Lrs1-dependent downregulation of anthranilate degradation may be important for wild-type levels of quinolone, and therefore phenazine production.

The sRNAs PrrF1 and PrrF2 have been characterized in further detail in *P. aeruginosa* PAO1. PrrF1 and PrrF2 are encoded by adjacent loci and repressed by the iron-dependent regulator Fur when iron is abundant (Wilderman et al. 2004; Oglesby et al. 2008). They are expressed during iron limitation and base-pair with target mRNAs, preventing their translation. One such target is the transcript of *sodB*, which encodes superoxide dismutase and is, for unknown reasons, required for PYO production in *P. aeruginosa* PAO1 (Hassett et al. 1995). Also in this strain, a *prfF1/prfF2* mutant shows increased expression of the *antABC* operon, an effect that seems to contradict the simultaneous upregulation of *antABC* and *prfF1/prfF2* transcripts in the *lrs1* mutant of strain PA14 (Wurtzel et al. 2012). This may represent a strain-dependent difference in this branch of the *P. aeruginosa* sRNA-dependent regulatory network.

Expression of PhrS, an sRNA that positively controls translation of *pqsR* mRNA, is also controlled by a regulator that responds to an environmental cue: the oxygen-sensitive transcription factor ANR. In the absence of PhrS, *pqsR* mRNA adopts an intramolecular secondary structure in which an upstream open reading frame base-pairs with the *pqsR* transcript and inhibits translation. Under oxygen-limited conditions, ANR is activated and induces expression of PhrS. PhrS competes with the *pqsR* transcript for binding of its 5' untranslated region, and via this anti-antisense mechanism, exposes the *pqsR* mRNA to allow for ribosome binding and translation. This regulatory cascade was elucidated in *P. aeruginosa* PAO1, where a PhrS-overexpressing strain shows increased PYO production due to elevated PqsR levels and quinolone production (Sonnleitner et al. 2011).

Hfq, an abundant mRNA-binding protein found in diverse bacteria, also affects phenazine production through post-transcriptional mechanisms. In *P. aeruginosa*

M18, Hfq binds *qscR* and *phzM* mRNA transcripts via AU-rich sequences present in their 5'-leader sequences and inhibits their translation (Wang et al. 2012b). As *qscR* is a negative regulator of the *phz* operons, and *phzM* is required for the conversion of PCA to PYO, Hfq would be expected to enhance phenazine production overall but limit PYO production. In mutants lacking functional Hfq, Wang et al. observed increased PYO production and decreased PCA production, consistent with decreased production of the PhzA-G biosynthetic enzymes, but increased translation of *phzM*. Formation of the active, hexameric form of Hfq is promoted by the RelA enzyme, a critical regulator of the stringent response to amino acid starvation (Argaman et al. 2012). *P. aeruginosa* PAO1 *relA* mutants also overproduce PYO, suggesting that Hfq may regulate translation of the *phzM* transcript in this strain according to a mechanism similar to the one described for M18 (Erickson et al. 2004).

2.3.4 Environmental Signals and Conditions Affecting *phz* Gene Expression

Many studies characterizing the conditional dependence of phenazine production have revealed environmental cues that affect the regulation of this process and, in some cases, mechanisms linking the condition to the response. These studies have evaluated the effects of environmental parameters such as temperature, pH, salinity, and oxygen availability. They have also examined how phenazine production is influenced by the availability of carbon and nitrogen sources, phosphate, sulfate, iron, and magnesium. These environmental variables can affect phenazine production by indirectly or directly altering expression of Phz proteins (for example, through their effects on the production of signals upstream in the regulatory cascade (van Rij et al. 2004; Farrow and Pesci 2007)), or they can alter the availability of substrates and thus, flux through the relevant metabolic pathways that support phenazine biosynthesis.

The effect of temperature on phenazine production has been investigated in *P. chlororaphis* PCL1391, *P. fluorescens* 2-79, and multiple strains of *P. aeruginosa*. *P. chlororaphis* PCL1391 produces the PCA derivative phenazine-1-carboxamide (PCN) at comparable levels when grown at temperatures ranging from 21 to 31 °C, but production is almost undetectable when it is grown at 16 °C (van Rij et al. 2004). In *P. fluorescens* 2-79, PCA production was found to inversely correlate with temperature in a survey of temperatures ranging from 25 to 37 °C (Slininger and Shea-Wilbur 1995). In *P. aeruginosa* M18, transcription of *phz1* and *phz2* is elevated at 28 °C compared to 37 °C, and this correlates with a large increase in PCA production (Huang et al. 2009). In *P. aeruginosa* PA14, PYO production increases modestly when this strain is grown at 37 °C compared to 28 °C. Using RNA-seq, Wurtzel et al. (2012) found that the transcript abundances of both *phz1* and *phz2* are elevated at the higher temperature, with a larger effect on *phz1* than *phz2*. These results also indicated the presence of a temperature-

dependent transcriptional start site upstream of *phzB1*. The differential regulation of *phzA1* and *phzB1* is interesting because these two genes encode highly similar proteins that form heterodimers required for in vivo formation of the phenazine core. Temperature-dependent differences in expression may have consequences for PhzA/B dimerization (Ahuja et al. 2008).

Ambient oxygen levels also influence the production of different phenazine derivatives. In *P. aeruginosa*, PCA can be biosynthesized anaerobically (Dietrich et al. 2006; Mentel et al. 2009; Recinos et al. 2012). However, oxygen is required for the conversion of 5-methylphenazine-1-carboxylic acid (the product of PhzM, 5-MCA) to PYO by the PhzS monooxygenase. Therefore, PYO production is inhibited in the absence of oxygen. Interestingly, Holliman (1969) reported increased production of the red phenazines aeruginosin A and B in low oxygen conditions; inefficient conversion of 5-MCA to PYO may shunt the biosynthetic pathway toward the production of these alternative phenazines when oxygen is limited. An effect of oxygen limitation on phenazine biosynthesis has also been observed in *P. chlororaphis* PCL1391, where growth in low oxygen conditions leads to PCN overproduction (van Rij et al. 2004).

The effects of pH and salinity on phenazine production have been tested in biocontrol strains, where optimization of soil conditions could facilitate the application of such strains for crop growth promotion. *P. chlororaphis* PCL1391 produces PCN when grown at pH 7 or pH 8, but not at pH 6 (van Rij et al. 2004). For *P. fluorescens* 2-79, however, PCA production was maximized at pH 7, partially decreased but still substantial at pH 6, and abolished at pH 8 (Slininger and Shea-Wilbur 1995). Increasing concentrations of salts decreased PCN production in *P. chlororaphis* PCL1391, but this effect was specific to ionic solutes as xylose did not affect PCN production when introduced at isoosmotic levels, and osmoprotectants did not restore PCN production in a high-salt medium.

Variations in the availability of minerals and the compounds that provide the major elements for biomass can have dramatic effects on phenazine biosynthesis. In a survey of carbon sources for growth of *P. chlororaphis* PCL1391, van Rij et al. (2004) found that glucose, glycerol, and *L*-pyroglutamic acid gave rise to the highest levels of PCN production. The amount of PCN produced did not correlate with growth rate, and the most stimulatory carbon sources were not the most abundant organic compounds in the rhizosphere, where the organism is commonly found. Glucose and glycerol have also been found to stimulate PCA production in *P. fluorescens* 2-79. That glucose and glycerol promote the highest levels of phenazine production is surprising because they are not preferred carbon sources for pseudomonads; unlike *E. coli*, *Pseudomonas* species typically utilize organic acids such as succinate before utilizing sugars (Behrends et al. 2009; Rojo 2010; Valentini and Lapouge 2012).

Given that phenazine structures, and particularly that of PCN, contain multiple nitrogen atoms, one would predict that the type of nitrogen source provided would affect phenazine production. Generally, supplementation with amino acids stimulates phenazine production, but the effects of individual amino acids and inorganic nitrogen sources on phenazine production vary widely between species and

conditions. Increasing levels of nitrogen provided as ammonium sulfate stimulated PCN production in *P. chlororaphis* PCL1391, but did not stimulate PCA production in *P. fluorescens*. Although glutamine is used to form the carboxamide functional group in PCN, the addition of this amino acid to the medium did not stimulate PCN production any more than other individual amino acids such as leucine. All aromatic amino acids stimulate PCN production in *P. chlororaphis* PCL1391, whereas the effects of phenylalanine, tryptophan, and tyrosine on PYO production in *P. aeruginosa* appear to be strain- and condition-dependent (Burton et al. 1947, Palmer et al. 2007). The effect of tryptophan in particular is at least partially related to its ability to serve as a precursor for quinolone biosynthesis (Farrow and Pesci 2007).

In both *P. aeruginosa* and *P. chlororaphis* PCL1391, PYO and PCN production, respectively, are maximized when the medium contains an intermediate level of phosphate; this is apparently not an artifact of effects on growth (Burton et al. 1947; van Rij et al. 2004). Iron and magnesium supplementation at micromolar levels is required and optimal for growth and phenazine production by *P. aeruginosa* and *P. chlororaphis* PCL1391. Because iron and magnesium are often provided as sulfate salts, it can be difficult to decouple their effects from that of varying the sulfur source. The importance of sulfate has been thoroughly evaluated in *P. chlororaphis* PCL1391, however, where millimolar concentrations are required for maximum production of PCN (van Rij et al. 2004).

2.4 Regulation of Phenazine Biosynthesis in Other Genera

In addition to the *Pseudomonas* species we have discussed, many diverse species belonging to other genera also produce phenazines with highly derivatized chemical structures (Table 2.1). Relatively little is known about the regulation of phenazine biosynthesis in these species, but recent studies have identified regulators that affect the process in strains of *Burkholderia* and *Streptomyces* (Ramos et al. 2010; Saleh et al. 2012). In *Burkholderia cenocepacia* K56-2, wild-type phenazine production requires a regulator called phenazine biosynthesis regulator (Pbr), which is encoded by a gene that lies near *phzF* and *phzD* homologues on the chromosome (Ramos et al. 2010). Pbr binds to the promoter region of the *phzF-phzD* operon and is required for wild-type expression. In *Streptomyces anulatus* 9663, regulators of *phz* gene expression have been identified through characterization of a large gene cluster that includes all of the genes required for PCA biosynthesis and genes required for transformation of PCA to the prenylated phenazines endophenazines A and E (Saleh et al. 2012). One of these regulators, encoded by the gene *ppzV*, is similar to a putative TetR-family regulator called EpzV found in *S. cinnamonensis*, another phenazine-producer. Inactivating the *ppzV* gene in a strain expressing the large phenazine biosynthetic cluster led to loss of the ability to produce prenylated phenazines but an increase in the amount of unprenylated phenazines, suggesting that the *ppzV* product regulates PCA derivatization. The second regulator, encoded by *ppzY*, is similar to transcriptional

regulators of the LysR family. Inactivation of *ppzY* led to a nearly complete defect in all phenazine production, suggesting that the *ppzY* product is required for expression of PCA biosynthetic genes in *S. anulatus* 9663.

2.5 Conclusion

Characterization of the regulation of phenazine biosynthesis in diverse *Pseudomonas* isolates has revealed common mechanisms and hierarchies. As more of the mechanisms regulating phenazine biosynthesis in other genera are uncovered, it will be interesting to compare them to the *Pseudomonas* paradigm and evaluate their physiological relevance in these new contexts. The intricacy of the networks controlling phenazine production in *Pseudomonas* is becoming clear at a time when phenazines themselves are gaining recognition for their roles in bacterial physiology, which include intercellular signaling and redox balancing. The multilayered cascades that modulate phenazine biosynthesis are consistent with their new status as primary players in cellular metabolism and communication. Indications that not just the core genes for PCA synthesis, but also the genes for PCA modification, are regulated at multiple levels may suggest that different phenazines perform different physiological roles, consistent with their unique chemistries.

Although our understanding of the complicated networks controlling phenazine production is still developing, a hint at this complexity has long been evident in the variability of phenazine production that is apparent among species, isolates, and even repeat cultivations of the same strain. Differences in phenazine production among strains of the same species likely arise in part from subtle discrepancies in regulatory networks and sensing mechanisms. On the other hand, differences between repeat experiments imply that, although many of the conditions and regulators that affect phenazine production have been identified, unrecognized variables can still alter phenazine production in unpredictable ways. Elucidating the parameters and mechanisms that affect this process has the potential to facilitate the use of beneficial phenazine-producing pseudomonads in agriculture, support the development of therapeutics for patients suffering from *P. aeruginosa* infections, and allow us to learn new techniques for controlling antibiotic production in diverse species.

References

- Abdou L, Chou HT, Haas D et al (2011) Promoter recognition and activation by the global response regulator CbrB in *Pseudomonas aeruginosa*. *J Bacteriol* 193(11):2784–2792
- Ahuja EG, Janning P, Mentel M et al (2008) *PhzA/B* catalyzes the formation of the tricycle in phenazine biosynthesis. *J Am Chem Soc* 130(50):17053–17061
- Argaman L, Elgrably-Weiss M, Hershko T et al (2012) RelA protein stimulates the activity of RyhB small RNA by acting on RNA-binding protein Hfq. *Proc Natl Acad Sci USA* 109(12):4621–4626

- Balasubramanian D, Schnepfer L, Kumari H et al (2013) A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence. *Nucl Acids Res* 41(1):1–20
- Ballard RW, Palleroni NJ, Doudoroff M et al (1970) Taxonomy of the aerobic pseudomonads: *Pseudomonas cepacia*, *P. marginata*, *P. alliicola* and *P. caryophylli*. *J Gen Microbiol* 60(2): 199–214
- Beatson SA, Whitchurch CB, Sargent JL et al (2002) Differential regulation of twitching motility and elastase production by Vfr in *Pseudomonas aeruginosa*. *J Bacteriol* 184(13):3605–3613
- Behrends V, Ebbels TM, Williams HD et al (2009) Time-resolved metabolic footprinting for nonlinear modeling of bacterial substrate utilization. *Appl Environ Microbiol* 75(8):2453–2463
- Birkofer L (1947) Das Verhalten der Diazoketone bei der katalytischen Hydrierung; eine neue Synthese des Threonins. *Chem Berichte* 80(1):83–94
- Blumer C, Heeb S, Pessi G et al (1999) Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci USA* 96(24):14073–14078
- Bordi C, Lamy MC, Ventre I et al (2010) Regulatory RNAs and the HptB/RetS signalling pathways fine-tune *Pseudomonas aeruginosa* pathogenesis. *Mol Microbiol* 76(6):1427–1443
- Bourret RB, Silversmith RE (2010) Two-component signal transduction. *Curr Opin Microbiol* 13(2):113–115
- Burrowes E, Abbas A, O’Neill A et al (2005) Characterisation of the regulatory RNA RsmB from *Pseudomonas aeruginosa* PAO1. *Res Microbiol* 156(1):7–16
- Burrowes E, Baysse C, Adams C et al (2006) Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. *Microbiology* 152(Pt 2):405–418
- Burton MO, Eagles BA, Campbell JJ (1947) The amino acid requirements for pyocyanin production. *Can J Res* 25(4):121–128
- Byng GS, Eustice DC, Jensen RA (1979) Biosynthesis of phenazine pigments in mutant and wild-type cultures of *Pseudomonas aeruginosa*. *J Bacteriol* 138(3):846–852
- Caldwell CC, Chen Y, Goetzmann HS et al (2009) *Pseudomonas aeruginosa* exotoxin pyocyanin causes cystic fibrosis airway pathogenesis. *Am J Pathol* 175(6):2473–2488
- Chancey ST, Wood DW, Pierson LS 3rd (1999) Two-component transcriptional regulation of N-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Appl Environ Microbiol* 65(6):2294–2299
- Chin-A-Woeng TFC, van den Broek D, de Voer G et al (2001) Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium. *Mol Plant Microbe Interact* 14(8):969–979
- Chin-A-Woeng TFC, Bloemberg GV, Lugtenberg BJJ (2003) Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytol* 157:503–523
- Choi EJ, Kwon HC, Ham J et al (2009) 6-Hydroxymethyl-1-phenazine-carboxamide and 1,6-phenazinedimethanol from a marine bacterium, *Brevibacterium* sp. KMD 003, associated with marine purple vase sponge. *J Antibiot (Tokyo)* 62(11):621–624
- Chugani SA, Whiteley M, Lee KM et al (2001) QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 98(5): 2752–2757
- Clemo GR, Daghli AF (1948) Structure of the pigment of *Chromobacterium iodinum*. *Nature* 162(4124):776
- De Maeyer K, D’Aes J, Hua GK et al (2011) N-Acylhomoserine lactone quorum-sensing signalling in antagonistic phenazine-producing *Pseudomonas* isolates from the red cocoyam rhizosphere. *Microbiology* 157(Pt 2):459–472
- Deziel E, Lepine F, Milot S et al (2004) Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc Natl Acad Sci USA* 101(5):1339–1344
- Diepoois G, Ducret V, Caille O et al (2012) The transcriptional regulator CzcR modulates antibiotic resistance and quorum sensing in *Pseudomonas aeruginosa*. *PLoS ONE* 7(5): e38148

- Dietrich LE, Price-Whelan A, Petersen A et al (2006) The phenazine pyocyanin is a terminal signaling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol Microbiol* 61(5):1308–1321
- Dietrich LE, Okegbe C, Price-Whelan A et al (2013) Bacterial community morphogenesis is intimately linked to the intracellular redox state. *J Bacteriol*. doi:[10.1128/JB.02273-12](https://doi.org/10.1128/JB.02273-12)
- Diggie SP, Matthijs S, Wright VJ et al (2007) The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem Biol* 14(1):87–96
- Egland KA, Greenberg EP (1999) Quorum sensing in *Vibrio fischeri*: elements of the *luxI* promoter. *Mol Microbiol* 31(4):1197–1204
- Erickson DL, Lines JL, Pesci EC et al (2004) *Pseudomonas aeruginosa* *relA* contributes to virulence in *Drosophila melanogaster*. *Infect Immun* 72(10):5638–5645
- Farrow JM 3rd, Pesci EC (2007) Two distinct pathways supply anthranilate as a precursor of the *Pseudomonas* quinolone signal. *J Bacteriol* 189(9):3425–3433
- Farrow JM 3rd, Sund ZM, Ellison ML et al (2008) PqsE functions independently of PqsR-*Pseudomonas* quinolone signal and enhances the *rhl* quorum-sensing system. *J Bacteriol* 190(21):7043–7051
- Fordos MJ (1859) Recherches sur la matière colorante des suppurations bleues: pyocyanine. *Rec Trav Soc d'Émul Sci Pharm* 3:30
- Gallagher LA, McKnight SL, Kuznetsova MS et al (2002) Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J Bacteriol* 184(23):6472–6480
- Gebhardt K, Schimana J, Krastel P et al (2002) Endophenazines A-D, new phenazine antibiotics from the arthropod associated endosymbiont *Streptomyces anulatus*. I. Taxonomy, fermentation, isolation and biological activities. *J Antibiot (Tokyo)* 55(9):794–800
- Gerber NN (1967) Phenazines, phenoxazinones, and dioxopiperazines from *Streptomyces thioluteus*. *J Org Chem* 32(12):4055–4057
- Gerber NN, Lechevalier MP (1964) Phenazines and phenoxazinones from *Waksmania aerata* sp. nov. and *Pseudomonas iodina*. *Biochemistry* 3:598–602
- Gerber NN, Lechevalier MP (1965) 1,6-Phenazinediol-5-oxide from microorganisms. *Biochemistry* 4:176–180
- Gessard C (1894) On the blue and green coloration that appears on bandages. *Rev Inf Dis* 6:S775–S776
- Giddens SR, Feng Y, Mahanty HK (2002) Characterization of a novel phenazine antibiotic gene cluster in *Erwinia herbicola* Eh1087. *Mol Microbiol* 45(3):769–783
- Girard G, Barends S, Rigali S et al (2006a) Pip, a novel activator of phenazine biosynthesis in *Pseudomonas chlororaphis* PCL1391. *J Bacteriol* 188(23):8283–8293
- Girard G, van Rij ET, Lugtenberg BJJ et al (2006b) Regulatory roles of *psrA* and *rpoS* in phenazine-1-carboxamide synthesis by *Pseudomonas chlororaphis* PCL1391. *Microbiology* 152(Pt 1):43–58
- Goodman AL, Kulasekara B, Rietsch A et al (2004) A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* 7(5):745–754
- Goodman AL, Merighi M, Hyodo M et al (2009) Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. *Genes and Dev* 23(2):249–259
- Haagen Y, Gluck K, Fay K et al (2006) A gene cluster for prenylated naphthoquinone and prenylated phenazine biosynthesis in *Streptomyces cinnamomensis* DSM 1042. *ChemBioChem* 7(12):2016–2027
- Haas D, Defago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* 3(4):307–319
- Hansford GS, Holliman FG, Herbert RB (1972) Pigments of *Pseudomonas* species. IV. In vitro and in vivo conversion of 5-methylphenazinium-1-carboxylate into aeruginosin A. *J Chem Soc Perkin Trans I* 1:103–105

- Hassett DJ, Schweizer HP, Ohman DE (1995) *Pseudomonas aeruginosa* *sodA* and *sodB* mutants defective in manganese- and iron-cofactored superoxide dismutase activity demonstrate the importance of the iron-cofactored form in aerobic metabolism. *J Bacteriol* 177(22):6330–6337
- Heeb S, Haas D (2001) Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. *Mol Plant Microbe Interact* 14(12):1351–1363
- Heeb S, Blumer C, Haas D (2002) Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol* 184(4):1046–1056
- Hendrickson EL, Plotnikova J, Mahajan-Miklos S (2001) Differential roles of the *Pseudomonas aeruginosa* PA14 *rpoN* gene in pathogenicity in plants, nematodes, insects, and mice. *J Bacteriol* 183(24):7126–7134
- Herbert RB, Holliman FG (1969) Pigments of *Pseudomonas* species. II. Structure of aeruginosin B. *J Chem Soc Perkin* 1(18):2517–2520
- Heurlier K, Denervaud V, Pessi G et al (2003) Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 185(7):2227–2235
- Heurlier K, Williams F, Heeb S et al (2004) Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 186(10):2936–2945
- Holliman FG (1969) Pigments of *Pseudomonas* species. I. Structure and synthesis of aeruginosin A. *J Chem Soc Perkin* 1(18):2514–2516
- Hsu JL, Chen HC, Peng HL et al (2008) Characterization of the histidine-containing phosphotransfer protein B-mediated multistep phosphorelay system in *Pseudomonas aeruginosa* PAO1. *J Biol Chem* 283(15):9933–9944
- Huang J, Xu Y, Zhang H, Li Y, Huang X, Ren B, Zhang X (2009) Temperature-dependent expression of *phzM* and its regulatory genes *lasI* and *ptsP* in rhizosphere isolate *Pseudomonas* sp. strain M18. *Appl Environ Microbiol* 75(20):6568–6580
- Huang J, Sonnleitner E, Ren B et al (2012) Catabolite repression control of pyocyanin biosynthesis at an intersection of primary and secondary metabolism in *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 78(14):5016–5020
- Hunter RC, Klepac-Ceraj V, Lorenzi MM et al (2012) Phenazine content in the cystic fibrosis respiratory tract negatively correlates with lung function and microbial complexity. *Am J Respir Cell Mol Biol* 47(6):738–745
- Johnson LE, Dietz A (1969) Lomofungin, a new antibiotic produced by *Streptomyces lomondensis* sp. n. *Appl Microbiol* 17(5):755–759
- Juhas M, Wiehlmann L, Huber B et al (2004) Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. *Microbiology* 150(Pt 4):831–841
- Kay E, Dubuis C, Haas D (2005) Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0. *Proc Natl Acad Sci USA* 102(47):17136–17141
- Kay E, Humair B, Denervaud V et al (2006) Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* 188(16):6026–6033
- Khan SR, Mavrodi DV, Jog GJ et al (2005) Activation of the *phz* operon of *Pseudomonas fluorescens* 2-79 requires the LuxR homolog PhzR, N-(3-OH-hexanoyl)-L-homoserine lactone produced by the LuxI homolog PhzI, and a cis-acting *phz* box. *J Bacteriol* 187(18):6517–6527
- Khan SR, Herman J, Krank J et al (2007) N-(3-hydroxyhexanoyl)-l-homoserine lactone is the biologically relevant quorumone that regulates the *phz* operon of *Pseudomonas chlororaphis* strain 30-84. *Appl Environ Microbiol* 73(22):7443–7455
- Kojic M, Venturi V (2001) Regulation of *rpoS* gene expression in *Pseudomonas*: involvement of a TetR family regulator. *J Bacteriol* 183(12):3712–3720
- Lapouge K, Sineva E, Lindell M et al (2007) Mechanism of *hcnA* mRNA recognition in the Gac/Rsm signal transduction pathway of *Pseudomonas fluorescens*. *Mol Microbiol* 66(2):341–356
- Lasseur A (1911) These de Faculte des Sciences de l'Universite de Nancy
- Lau GW, Ran H, Kong F et al (2004) *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. *Infect Immun* 72(7):4275–4278

- Laursen JB, Nielsen J (2004) Phenazine natural products: biosynthesis, synthetic analogues, and biological activity. *Chem Rev* 104(3):1663–1686
- Lechevalier H, Lechevalier MP (1965) Classification of aerobic actinomycetes based on their morphology and their chemical composition. *Ann Inst Pasteur (Paris)* 108(5):662–673
- Ledgham F, Ventre I, Soscia C et al (2003) Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhlR. *Mol Microbiol* 48(1):199–210
- Lee JH, Lequette Y, Greenberg EP (2006) Activity of purified QscR, a *Pseudomonas aeruginosa* orphan quorum-sensing transcription factor. *Mol Microbiol* 59(2):602–609
- Liang H, Li L, Kong W et al (2009) Identification of a novel regulator of the quorum-sensing systems in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 293(2):196–204
- Mavrodi DV, Blankenfeldt W, Thomashow LS (2006) Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. *Annu Rev Phytopathol* 44:417–445
- Mavrodi DV, Peever TL, Mavrodi OV et al (2010) Diversity and evolution of the phenazine biosynthesis pathway. *Appl Environ Microbiol* 76:866–879
- Mavrodi DV, Parejko JA, Mavrodi OV et al (2012) Recent insights into the diversity, frequency and ecological roles of phenazines in fluorescent *Pseudomonas* spp. *Environ Microbiol*:doi:doi:10.1111/j.1462-2920.2012.02846.x
- McLaughlin HP, Caly DL, McCarthy Y et al (2012) An orphan chemotaxis sensor regulates virulence and antibiotic tolerance in the human pathogen *Pseudomonas aeruginosa*. *PLoS ONE* 7(8):e42205
- Meighen EA (1991) Molecular biology of bacterial bioluminescence. *Microbiol Rev* 55(1):123–142
- Mentel M, Ahuja EG, Mavrodi DV et al (2009) Of two make one: the biosynthesis of phenazines. *ChemBioChem* 10:2295–2304
- Oglesby AG, Farrow JM 3rd, Lee JH et al (2008) The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing. *J Biol Chem* 283(23):15558–15567
- Palmer KL, Aye LM, Whiteley M (2007) Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. *J Bacteriol* 189(22):8079–8087
- Pearson JP, Gray KM, Passador L et al (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* 91(1):197–201
- Pearson JP, Passador L, Iglewski BH et al (1995) A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 92(5):1490–1494
- Pesci EC, Milbank JB, Pearson JP et al (1999) Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 96(20):11229–11234
- Pessi G, Williams F, Hindle Z, Heurlier K et al (2001) The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J Bacteriol* 183(22):6676–6683
- Petrova OE, Sauer K (2010) The novel two-component regulatory system BfiSR regulates biofilm development by controlling the small RNA *rsmZ* through CafA. *J Bacteriol* 192(20):5275–5288
- Pierson LS 3rd, Pierson EA (2010) Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Appl Microbiol Biotechnol* 86:1659–1670
- Pierson LS 3rd, Keppenne VD, Wood DW (1994) Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by *PhzR* in response to cell density. *J Bacteriol* 176(13):3966–3974
- Ramos CG, Sousa SA, Grilo AM et al (2010) The *Burkholderia cenocepacia* K56-2 pleiotropic regulator Pbr is required for stress resistance and virulence. *Microb Pathog* 48(5):168–177
- Rampioni G, Schuster M, Greenberg EP et al (2009) Contribution of the RsaL global regulator to *Pseudomonas aeruginosa* virulence and biofilm formation. *FEMS Microbiol Lett* 301(2):210–217

- Recinos DA, Sekedat MD, Hernandez A et al (2012) Redundant phenazine operons in *Pseudomonas aeruginosa* exhibit environment-dependent expression and differential roles in pathogenicity. *Proc Natl Acad Sci USA* 109:19420–19425
- Reimmann C, Beyeler M, Latifi A et al (1997) The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* 24(2):309–319
- Reimmann C, Valverde C, Kay E et al (2005) Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain *Pseudomonas fluorescens* CHA0. *J Bacteriol* 187(1):276–285
- Rojo F (2010) Carbon catabolite repression in *Pseudomonas*: optimizing metabolic versatility and interactions with the environment. *FEMS Microbiol Rev* 34(5):658–684
- Rusman Y, Opegard LM, Hiasa H et al (2013) Solphenazines A-F, glycosylated phenazines from *Streptomyces* sp. strain DL-93. *J Nat Prod* 76(1):91–96
- Saleh O, Flinspach K, Westrich L et al (2012) Mutational analysis of a phenazine biosynthetic gene cluster in *Streptomyces anulatus* 9663. *Beilstein J Org Chem* 8:501–513
- Schertzer JW, Brown SA, Whiteley M (2010) Oxygen levels rapidly modulate *Pseudomonas aeruginosa* social behaviours via substrate limitation of PqsH. *Mol Microbiol* 77(6):1527–1538
- Schoental R (1941) The nature of the antibacterial agents present in *Pseudomonas pyocyanea* cultures. *Brit J Exp Pathol* 22(3):37–147
- Schroeter J (1872) Ueber einige durch Bacterien gebildete Pigmente. Beiträge zur Biologie der Pflanzen Band 1(Zweites Heft):109–126
- Schuster M, Hawkins AC, Harwood CS et al (2004) The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* 51(4):973–985
- Seeger K, Flinspach K, Haug-Schiffedercker E et al (2011) The biosynthetic genes for prenylated phenazines are located at two different chromosomal loci of *Streptomyces cinnamonensis* DSM 1042. *Microb Biotechnol* 4(2):252–262
- Siehnel R, Traxler B, An DD et al (2010) A unique regulator controls the activation threshold of quorum-regulated genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 107(17):7916–7921
- Slininger PJ, Shea-Wilbur MA (1995) Liquid-culture pH, temperature, and carbon (not nitrogen) source regulate phenazine productivity of the take-all biocontrol agent *Pseudomonas fluorescens* 2-79. *Appl Microbiol Biotechnol* 43(5):794–800
- Sonnleitner E, Haas D (2011) Small RNAs as regulators of primary and secondary metabolism in *Pseudomonas* species. *Appl Microbiol Biotechnol* 91(1):63–79
- Sonnleitner E, Abdou L, Haas D (2009) Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 106(51):21866–21871
- Sonnleitner E, Gonzalez N, Sorger-Domenigg T et al (2011) The small RNA PhrS stimulates synthesis of the *Pseudomonas aeruginosa* quinolone signal. *Mol Microbiol* 80(4):868–885
- Suh SJ, Silo-Suh L, Woods DE et al (1999) Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* 181(13):3890–3897
- Tanabe I, Obayashi A (1971) Cultivation of *Brevibacterium stationis* var. *iodinofaciens* and its iodinin production. *Memoirs of the Faculty of Agriculture, Kagoshima University* 8(1):373–389
- Thompson LS, Webb JS, Rice SA et al (2003) The alternative sigma factor RpoN regulates the quorum sensing gene *rhlI* in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 220(2):187–195
- Tipton CD, Rinehart KL Jr (1970) Lomofungin. I. Degradative studies of a new phenazine antibiotic. *J Am Chem Soc* 92(5):1425–1426
- Turner JM, Messenger AJ (1986) Occurrence, biochemistry and physiology of phenazine pigment production. *Adv Microb Physiol* 27:211–275
- Valentini M, Lapouge K (2012) Catabolite repression in *Pseudomonas aeruginosa* PAO1 regulates the uptake of C(4) -dicarboxylates depending on succinate concentration. *Environ Microbiol*. doi:10.1111/1462-2920.12056

- Valverde C, Heeb S, Keel C et al (2003) RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. *Mol Microbiol* 50(4):1361–1379
- van den Broek D, Chin-A-Woeng TFC, Eijkemans K et al (2003) Biocontrol traits of *Pseudomonas* spp. are regulated by phase variation. *Mol Plant Microbe Interact* 16(11):1003–1012
- van Rij ET, Wesselink M, Chin-A-Woeng TFC et al (2004) Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* PCL1391. *Mol Plant Microbe Interact* 17(5):557–566
- Ventre I, Goodman AL, Vallet-Gely I et al (2006) Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci USA* 103(1):171–176
- Vincent F, Round A, Reynaud A et al (2010) Distinct oligomeric forms of the *Pseudomonas aeruginosa* RetS sensor domain modulate accessibility to the ligand binding site. *Environ Microbiol* 12(6):1775–1786
- Von Saltza MH, Last JA, Stapleton PG et al (1969) Cyanomycin, its identity with pyocyanine. *J Antibiot (Tokyo)* 22(2):49–54
- Wagner VE, Bushnell D, Passador L et al (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* 185(7):2080–2095
- Wang D, Yu JM, Pierson LS 3rd et al (2012a) Differential regulation of phenazine biosynthesis by RpeA and RpeB in *Pseudomonas chlororaphis* 30-84. *Microbiology* 158(Pt 7):1745–1757
- Wang G, Huang X, Li S et al (2012b) The RNA chaperone Hfq regulates antibiotic biosynthesis in the rhizobacterium *Pseudomonas aeruginosa* M18. *J Bacteriol* 194(10):2443–2457
- Whiteley M, Lee KM, Greenberg EP (1999) Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 96(24):13904–13909
- Wilderman PJ, Sowa NA, FitzGerald DJ et al (2004) Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. *Proc Natl Acad Sci USA* 101(26):9792–9797
- Williams P, Camara M (2009) Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. *Curr Opin Microbiol* 12(2):182–191
- Wood DW, Pierson LS 3rd (1996) The *phzI* gene of *Pseudomonas aureofaciens* 30–84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* 168(1):49–53
- Workentine ML, Chang L, Ceri H et al (2009) The GacS-GacA two-component regulatory system of *Pseudomonas fluorescens*: a bacterial two-hybrid analysis. *FEMS Microbiol Lett* 292(1):50–56
- Wurtzel O, Yoder-Himes DR, Han K et al (2012) The single-nucleotide resolution transcriptome of *Pseudomonas aeruginosa* grown in body temperature. *PLoS Pathog* 8(9):e1002945
- Xiao G, Deziel E, He J et al (2006) MvfR, a key *Pseudomonas aeruginosa* pathogenicity LTTR-class regulatory protein, has dual ligands. *Mol Microbiol* 62(6):1689–1699
- Xu H, Lin W, Xia H et al (2005) Influence of *ptsP* gene on pyocyanin production in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 253(1):103–109
- Yu S, Jensen V, Seeliger J et al (2009) Structure elucidation and preliminary assessment of hydrolase activity of PqsE, the *Pseudomonas* quinolone signal (PQS) response protein. *Biochemistry* 48(43):10298–10307
- Zhang X, Wang S, Geng H et al (2005) Differential regulation of *rsmA* gene on biosynthesis of pyoluteorin and phenazine-1-carboxylic acid in *Pseudomonas* sp. M18. *World J Microbiol Biotechnol* 21(6–7):883–889