Sudhir Chincholkar
Linda Thomashow *Editors*

Microbial Phenazines

Biosynthesis, Agriculture and Health

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Preface

Phenazines, a large class of nitrogen-containing redox-active heterocyclomers of biological and chemical origin, have attracted scientific interest since historical times because of their colorful pigmentation. More than 6,000 phenazine derivatives with wide-ranging bioactivities are now known, of which the hundred or so produced by bacteria currently are the most studied because of their importance in interactions not only with other microorganisms, but also with plants and animals. In this work we have attempted to bring together several major aspects of phenazine research with the hope that these chapters will lead to further advances in our knowledge of the biological origins and activities of these fascinating and versatile molecules as well their future employment in applications ranging from agriculture to human health.

In this volume, authors from all over the world have shared insights on phenazine biosynthesis, biochemistry, and physiological properties, the ecological distribution and antibiotic activity of these compounds, and their current and emerging agricultural and medicinal applications. Chapters highlight the long-sought pathway underpinning the synthesis of the phenazine tricycle, the diversity and complexity of the genetic regulatory mechanisms controlling expression of the biosynthetic genes, and the physiological consequences of phenazine gene expression that extend well beyond the producing bacteria to other organisms in the immediate environment. The involvement of phenazines in such fundamental biological processes as quorum sensing and biofilm formation is recognized, as is the emerging picture of the widespread distribution in nature and the wealth of structural variation and biological activity among these intriguing natural products. Several chapters review the roots of phenazine research in biological control, culminating in description of the commercial production of phenazine-1-carboxylic acid as a natural pesticide for the control of a variety of plant pathogens. Commercialization of a natural product, whether for agricultural or medicinal application, requires scale-up and purification, and these topics, as well as emerging research on the use of phenazines to treat cancer, also are presented.

The editors are much indebted to all of the authors for their contributions to this book. We are greatly obliged to Dr. Jutta Lindenborn of Springer, Heidelberg, Germany for making this volume possible, and for her support and patience during its preparation. Sudhir Chincholkar is particularly grateful to his former Vice Chancellor, Dr. K. B. Patil, and his present Vice Chancellor, Dr. S. U. Meshram,

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Jalgaon, India — Sudhir Chincholkar
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Contents

Chapter 1
The Biosynthesis of Phenazines

Wulf Blankenfeldt

Abstract The phenazines are nitrogen-containing colored aromatic secondary metabolites that many bacterial species produce and excrete into the environment, sometimes in such large quantities that they are visible to the naked eye. Phenazines act as broad-specificity antibiotics and as virulence as well as survival factors in infectious disease, which is in general a consequence of their redox activity. This chapter gives a historical perspective of research that led to our current understanding of phenazine biosynthesis, starting with the isolation of the first phenazine derivative pyocyanin in 1859. The focus is on recent biochemical and structural studies of the enzymes PhzE, PhzD, PhzF, PhzB, and PhzG, which convert chorismic acid via 2-amino-2-desoxyisochorismic acid (ADIC), *trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHHA), 6-amino-5-oxocyclohex-2-ene-1-carboxylic acid (AOCHC), hexahydro-phenazine-1,6-dicarboxylate (HHPDC), and tetrahydro-phenazine-1-carboxylate (THPCA) to phenazine-1,6-dicarboxylic acid (PDC) and phenazine-1-carboxylic acid (PCA). PDC and PCA then act as "core" phenazines that strain-specific enzymes convert to the over 150 phenazine derivatives that have been isolated from natural sources until today.

1.1 The Discovery of Phenazines and Phenazine-Producing Bacteria

The unexpected occurrence of light or color in or on apparently inanimate objects has fascinated mankind since its early days and is the basis for fairy tales and even religious beliefs and traditions. For example, the appearance of a red liquid on bread mistakenly interpreted as blood was foreseen as a good omen that led

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Alexander the Great to attack and conquer Tyros in 332 B.C. The same phenomenon provided the transubstantiation "miracle" that convinced the priest Peter of Prague of the trueness of the conversion of bread into the body of Christ while celebrating Eucharist in Bolsena (Italy) in 1263, which gave rise to the still celebrated "Feast of Corpus Christi" by the Catholic Church. "Milky seas" accounted for tales among seafarers for hundreds of years and are also featured in Jules Verne's "Twenty Thousand Leagues Under the Sea" from 1870. References to "blue milk" originating in the sixteenth century appear in Jakob and Wilhelm Grimm's German Dictionary from 1854 and also in books on veterinary medicine from the early days of book printing. All of these phenomena result from the presence of microorganisms: the blue color in dairy products is associated with *Pseudomonas* strains (Martin et al. 2011; Seiler 2006), although ancient texts may also mean skimmed milk, milk diluted with water, or water itself when they refer to "blue milk." "Milky seas" are a consequence of bioluminescent *Vibrio* spp. (Lapota et al. 1988), and the red color in "blood" miracles comes from prodigiosin alkaloids produced by *Serratia marcescens* (Fürstner 2003).

Perhaps of lesser historical consequence is another spontaneous coloration that physicians of the nineteenth century and of earlier times were acquainted with: the occurrence of "blue pus". The basis for this observation was first investigated by Joseph-Maturin Fordos, a French pharmacist who also made important contributions to the development of photography. In 1859, he reported a procedure using chloroform extraction to isolate a compound that he called "pyocyanine" [French spelling; from the Greek words *πύο* (pus) and *κυανό* (cyan)] from purulent wound dressings which had turned blue (Fordos 1859), and he continued to characterize this pigment in subsequent years (Fordos 1860, 1863). It took, however, until 1882, when Carle Gessard identified the source of pyocyanin (English spelling) as being a microorganism (Gessard 1882). The same bacterium already had been described a decade earlier by Schroeter (1872), who called it "*Bacterium aeruginosum*" albeit without referring to the work of Fordos. Later, in his "System der Bakterien" Walter Migula (1900) gave it the name "*Pseudomonas aeruginosa*" which is still in use today and again pays tribute to the fact that this strain can be distinguished by its capacity to synthesize pigments: *aerugo* is the Latin word for verdigris, the blue-greenish copper rust.

It took nearly 100 years before the chemical structure of pyocyanin was established (Fig. 1.1). Ledderhose (1888) was the first to derive a chemical formula, but his results were corrected by others (McCombie and Scarborough 1923; Wrede and Strack 1924a). The phenazine moiety of pyocyanin was first identified by Wrede and Strack (1924b), who subsequently arrived at a dimeric structure of pyocyanin (Wrede and Strack 1929) that contradicted studies performed by others (Elema 1931; Kuhn and Schön 1935), among them Leonor Michaelis (Friedheim and Michaelis 1931), the famous pioneer of quantitative enzyme kinetics. Hilleman (1938) finally determined the correct structure in the late 1930s, albeit it was later shown with dipole measurements that the zwitterionic mesomer 5-N-methyl-1-hydroxyphenazinium betaine is present in considerable amounts [approx. 20 % in dioxane, Fig. 1.1 (Jensen and Holten 1949)].

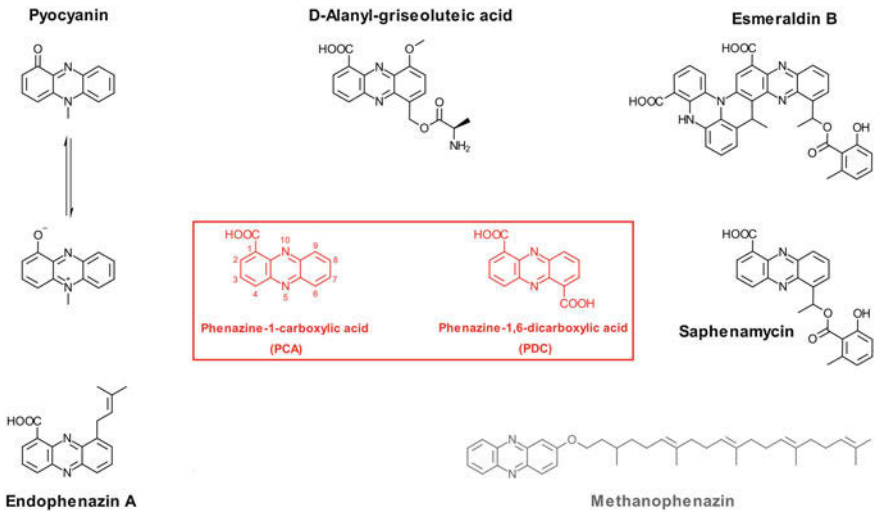

Fig. 1.1 A collection of naturally occurring phenazine derivatives. With the exception of methanophenazin from *Methanosarcina mazei* Gö1, for which the biosynthetic route is unclear, these molecules are synthesized from phenazine-1-carboxylic acid (PCA) or phenazine-1,6-dicarboxylic acid (PDC). *Pseudomonas aeruginosa* produces pyocyanin, which exists in two tautomeric structures. D-Alanyl-griseoluteic acid is generated by *Enterobacter agglomerans* Eh1087 and endophenazin A by *Streptomyces cinnamonensis* DSM1042. Esmeraldin B and saphenamycin are two phenazines biosynthesized by *Streptomyces antibioticus* Tü 2706

In the course of these studies it became clear that pyocyanin, like other phenazines, is a redox-active compound that changes its color depending on pH and oxidation state (Friedheim and Michaelis 1931). This redox activity is also the basis for most of the phenazines' physiological actions, including their function as virulence and survival factors in infections by the opportunistic pathogen *P. aeruginosa* (Lau et al. 2004; Price-Whelan et al. 2006, 2007; Dietrich et al. 2008). Whereas these aspects are covered in other sections of this book, the present chapter summarizes our current understanding of common principles in the biosynthesis of the over 150 phenazine derivatives (http://dnp.chemnetbase.com) that have been isolated from natural sources until today.

1.2 Investigations of Phenazine Biosynthesis in the Pre-genome Era

Before the genes required for phenazine biosynthesis were discovered, research focused on the identification of metabolites that are incorporated into phenazines or have an influence on their generation. This work has been reviewed in detail by Turner and Messenger (1986) and will only be reiterated shortly here.

After Fordos' discovery of pyocyanin and Gessard's isolation of the microorganism responsible for its synthesis, scientists investigated the influence of different media on pyocyanin production. These studies were hampered by the fact that rich media of poorly defined composition together with different bacterial isolates were employed, but nevertheless showed that culture conditions have an impact on pyocyanin biosynthesis. More systematic work with synthetic media was first performed by Jordan (1899) in the late nineteenth century and later led to the development of media that can be used to identify *P. aeruginosa* in the clinic: King's A medium, for example, has a low phosphate content and induces the production of pyocyanin, owing to the fact that phosphate inhibits phenazine biosynthesis in *P. aeruginosa* but not in other strains (King et al. 1954).

The availability of defined synthetic media opened the door for experiments with radioactively labeled carbon sources and, together with the development of techniques for the generation of growth-arrested mutants of microorganisms in the 1940s (Beadle and Tatum 1941), set the scene for research leading to the identification of direct precursors of the phenazine moiety. Millican (1962) and later Ingledew and Campbell (1969) showed that significant amounts of radioactivity from ^{14}C-labeled shikimic acid but not from anthranilate were incorporated into pyocyanin, indicating that phenazine biosynthesis is an offshoot of the shikimate pathway that branches off before shikimate is converted to anthranilate. This was also confirmed for other phenazine natural products (Levitch and Stadtman 1964; Levitch and Rietz 1966; Podojil and Gerber 1967; Chang and Blackwood 1968), suggesting that these compounds share a common biosynthetic route. However, five downstream chemical steps connect shikimic acid with anthranilate, and therefore five metabolites needed to be considered as branching points for phenazine biosynthesis, namely shikimic acid itself, shikimate-5-phosphate, 3-enolpyruvyl-shikimate-5-phosphate, chorismic acid, and 2-amino-2-desoxy-isochorismic acid. By introducing new growth-arrested mutants, two groups independently identified chorismic acid as the common precursor for phenazines in the early 1970s (Calhoun et al. 1972; Longley et al. 1972). The interpretation of these experiments was, however, difficult because chorismic acid is not transported across the cell wall very efficiently and the experiments could not be conducted by observing incorporation of radioactively labeled chorismic acid directly.

The symmetry of the phenazine moiety suggests that two identical or similar precursor molecules are incorporated in phenazine biosynthesis. This attractive hypothesis had already been corroborated by the work of Ingledew and Campbell (1969) mentioned in the previous paragraph, but the question of the relative orientation of these two precursors in phenazines remained. The problem was tackled by several research groups, who employed incorporation of position-specifically labeled shikimic acid followed by chemical degradation of the resulting phenazines, a method developed by Podojil and Gerber (1970) (Fig. 1.2). These experiments finally showed that the phenazine moiety arises from a diagonal symmetrical pairing of the two precursor molecules (Hollstein and McCamey

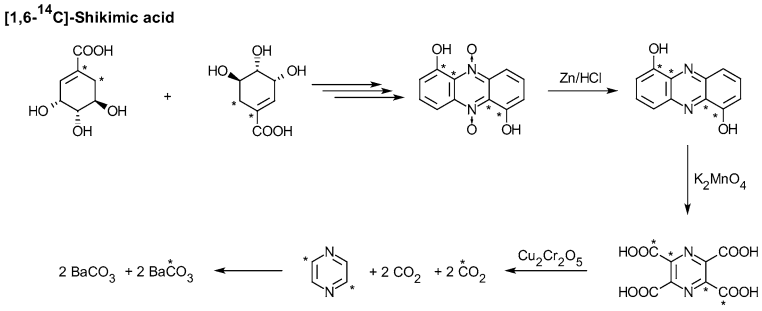

Fig. 1.2 Incorporation of radioactive label from [1,6-^{14}C]-shikimic acid into iodinin, a phenazine derivative that can be degraded into pyrazine and CO_2 by the indicated method (Podojil and Gerber 1970)

1973). However, this model required further refinement because several relative orientations could not be distinguished by this technique. Ambiguities were finally resolved with deuterium exchange (Herbert et al. 1976) and NMR studies (Hollstein et al. 1978), which revealed the orientation shown in Fig. 1.2.

1.3 Discovery and Characterization of Phenazine Biosynthesis Genes

In 1990, Essar et al. reported the discovery of a second heterodimeric anthranilate synthase in the genome of *P. aeruginosa*, which they named *phnA/phnB* because its deletion reduced phenazine levels significantly. The authors concluded that anthranilate must be an intermediate in phenazine biosynthesis, and they hypothesized that this contradiction to the work of others cited in the previous section indicated that PhnAB form a multienzyme complex with other phenazine biosynthesis enzymes into which chorismic acid can enter but anthranilate cannot (Essar et al. 1990). It was shown later, however, that the anthranilate synthase PhnAB is not directly involved in phenazine biosynthesis but rather produces anthranilate for the generation of the *Pseudomonas* Quinolone Signal (PQS) (Farrow and Pesci 2007), which is a quorum sensing regulator required for phenazine biosynthesis in *P. aeruginosa* (Pesci et al. 1999). Nevertheless, anthranilate is still occasionally mistaken as a phenazine precursor in the literature even today.

Genes directly involved in phenazine biosynthesis were first reported by Pierson et al., who isolated and subsequently sequenced five genes in a cluster from *P. aureofaciens* 30-84 that led to production of phenazines when introduced into *E. coli* (Pierson and Thomashow 1992; Pierson et al. 1995). This cluster was, however, lacking a promoter region, suggesting that additional genes in the 5′-region may have been missed. Indeed, a follow-up study conducted with the

phenazine producer *P. fluorescens* 2-79 revealed the presence of two additional upstream genes that subsequently were also discovered in *P. aureofaciens* 30-84 (Mavrodi et al. 1998) and in all other phenazine-producing pseudomonads. *P. aeruginosa* was found to contain two copies of this seven-gene *phz*-operon (Mavrodi et al. 2001), which recently were shown to be under different regulation and play different roles in pathogenesis inflicted by this strain (Recinos et al. 2012). Variations of the *phz*-operon have in the meantime been found in all phenazine-producing Gram-positive and Gram-negative bacteria, and recent analyses have provided evidence that the gene cluster has spread by horizontal gene transfer (Fitzpatrick 2009; Mavrodi et al. 2010). This shows that the general pathway that converts chorismic acid into phenazines is conserved and that the large number of phenazines found in nature derives from a small number of common phenazine precursors.

In the simplest form found in *P. fluorescens* 2-79, the *phz*-operon provides only the genes required for the biosynthesis of phenazine-1-carboxylic acid from chorismate, but other versions of the operon are twice as large and contain phenazine-modifying genes, phenazine resistance and transport factors or regulatory proteins (Fig. 1.3). An exception to encoding all genes essential for phenazine biosynthesis in a single operon is observed in *Streptomyces cinnamonensis* DSM1042, where the required genes are located in two different loci (Seeger et al. 2011). Recently, a plasmid-borne *phz* gene cluster containing 25 genes has been discovered in *Streptomyces antibioticus* Tü 2706. While the directionality of these open reading frames indicates that this cluster is not transcribed into a single mRNA, functional analysis showed that the proteins originating from the different transcripts cooperate to produce strain-specific phenazines in *S. antibioticus* Tü 2706 (Rui et al. 2012). Interestingly, no *phz*-operon can be found in the genome of the archaeal phenazine producer *Methanosarcina mazei* Gö1 (Deppenmeier et al. 2002), even though this strain has been shown to utilize a membrane-anchored phenazine as an electron carrier in membrane-bound electron transport (Abken et al. 1998). This suggests that phenazine biosynthesis follows a different route in archaea.

A comparison of *phz*-operons from different species reveals that five enzymes, PhzB, PhzD, PhzE, PhzF, and PhzG, are conserved among all phenazine-producing bacteria. These enzymes convert chorismate into phenazine-1,6-dicarboxylic acid (PDC) and/or phenazine-1-carboxylic acid (PCA), which act as "core" phenazines that other enzymes encoded in or outside the respective *phz*-operon derivatize to strain-specific phenazines. Importantly, no principal differences in the composition of *phz*-operons from PCA- and PDC-utilizing bacteria have been discovered, making it impossible to distinguish these species by comparing gene sequences alone. An explanation for this finding will be given below.

The first systematic analysis of core phenazine biosynthesis enzymes was presented by Floss and coworkers (McDonald et al. 2001). Their sequence analysis of the *phz*-operon from *P. fluorescens* 2-79 showed that *phzC*, which is not conserved in all phenazine producers, encodes a 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase, an enzyme that catalyzes the first step of the

shikimate pathway in chorismate biosynthesis. DAHP synthases are often feedback-inhibited, but more thorough sequence analysis of PhzC indicated that the enzyme lacks a loop region required for allosteric control (Webby et al. 2005). Because phenazine biosynthesis is normally under quorum sensing control and therefore only initiated at later stages of habitat colonization and at high cell densities, PhzC apparently ensures sufficient precursor generation when other DAHP synthases are inhibited.

Genes for precursor biosynthesis are also found in the *phz* clusters other phenazine producers, e.g., in that of *S. cinnamonensis* DSM1042, which contains genes of the mevalonate pathway to synthesize dimethylallyl diphosphate for the prenyl group of endophenazines that this strain produces (Fig. 1.3).

The crystal structures of the enzymes required for core phenazine biosynthesis have been determined in recent years (Fig. 1.4). This has led to a detailed picture of the pathway's underlying chemistry (Fig. 1.5).

By incubating different potential phenazine precursors with extracts from *E. coli* expressing all or parts of the *phz*-operon from *P. fluorescens* 2-79, McDonald et al. 2001) provided evidence that phenazine biosynthesis begins with the conversion of chorismic acid into 2-amino-2-desoxyisochorismate (ADIC), which had already been postulated as a phenazine precursor by Roemer and Herbert (1982). The reaction is catalyzed by PhzE, an enzyme related to anthranilate synthases but without the capacity to catalyze pyruvate elimination from ADIC to generate anthranilate. Sequence analysis shows that PhzE contains two domains with different enzymatic function concatenated into one chain. The N-terminal part belongs to the menaquinone, siderophore, tryptophan (MST) family, which are proteins that convert chorismic acid into specific precursors for several biosynthetic pathways. The C-terminal domain is a glutamine amidotransferase I (GATase-I) that hydrolizes glutamine to provide the ammonia for ADIC formation in the MST domain. The crystal structure of PhzE from *Burkholderia lata* 383 shows an intertwined homodimer in which the GATase-I domain of one chain generates NH_3 for the MST domain of the other. In the ligand-bound closed form, both active centers are connected by an intramolecular tunnel of approximately 25 Å that channels NH_3 from the GATase-I to the MST domain, thereby avoiding loss of NH_3 to the solvent (Fig. 1.4). Other than the related anthranilate synthases, PhzE is not allosterically regulated, which is a consequence of blocking mutations within the binding site that anthranilate synthases use for feedback inhibition (Li et al. 2011). Kinetic analysis suggests that the MST domain of PhzE catalyzes ADIC formation with a random sequential mechanism (Culbertson and Toney 2012).

The next step in phenazine biosynthesis is catalyzed by PhzD, which hydrolyzes ADIC to pyruvate and *trans*-2,3-dihydro-3-hydroxyanthranlic acid (DHHA) (McDonald et al. 2001), a molecule that Herbert et al. (1979) already had considered as an intermediate of the pathway. Crystal structure analysis revealed that PhzD is an α/β-hydrolase that utilizes an aspartic acid (D38) to protonate the vinyl group of ADIC, which is at variance with related enzymes that require a cysteine in catalysis (Parsons et al. 2003).

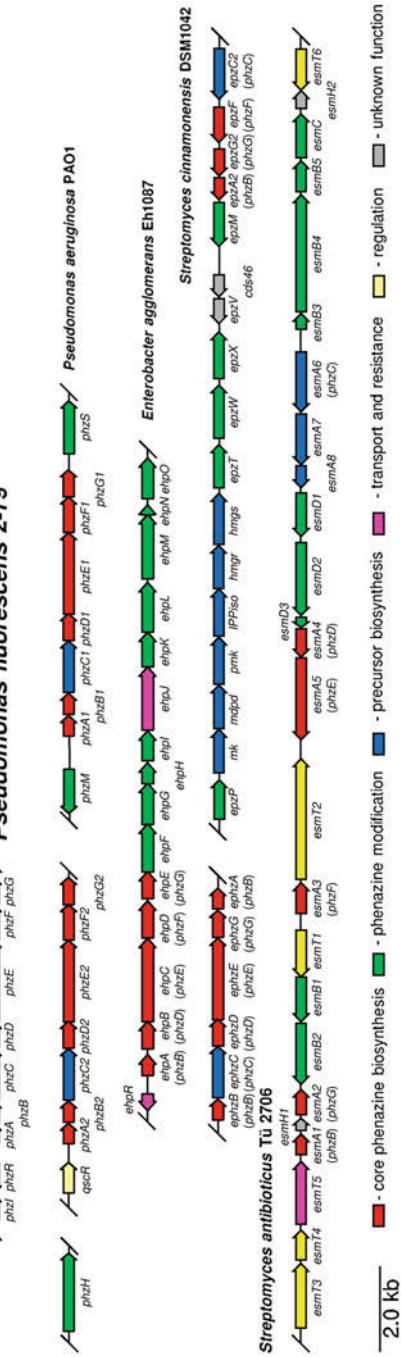

Fig. 1.3 A collection of phenazine biosynthesis operons and gene clusters from phenazine-producing bacteria

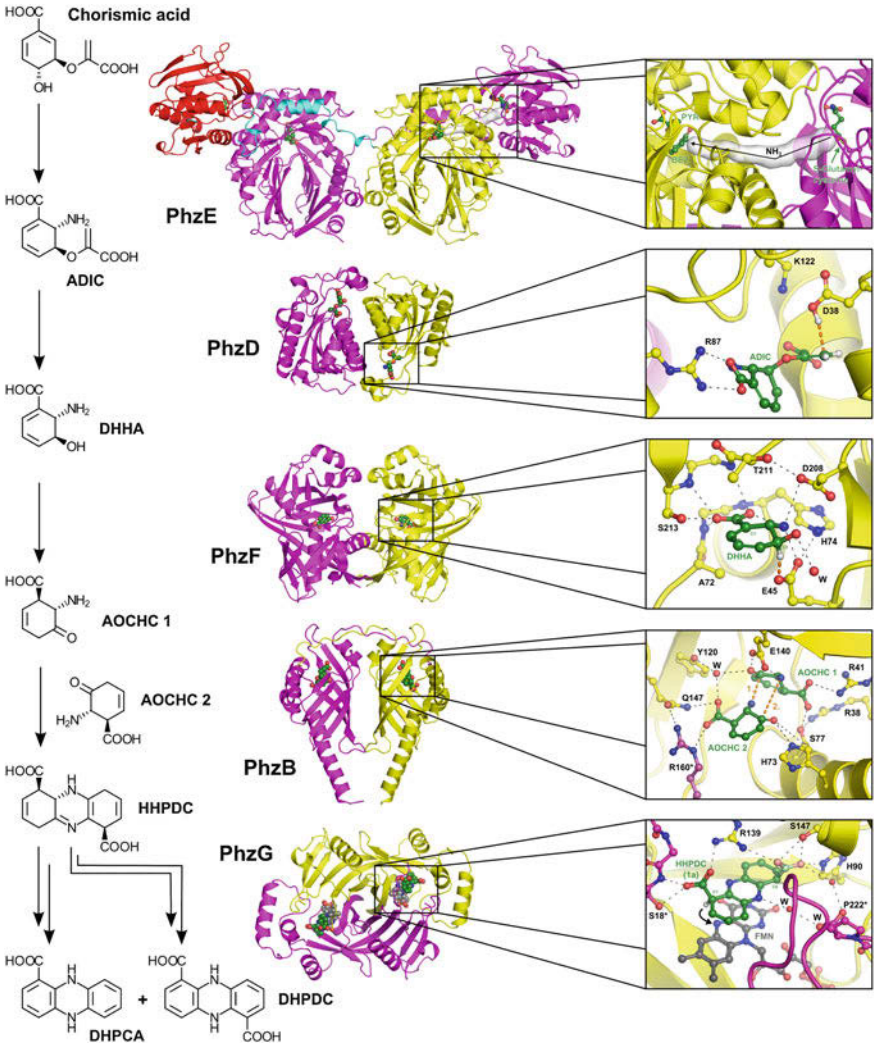

Fig. 1.4 A structural view of core phenazine biosynthesis. The following entries from the Protein Data Bank (Berman et al. 2000) have been used: PhzE, 3R75 (Li et al. 2011); PhzD, 1NF8, and 3R77 (Li et al. 2011); PhzF, 1U1 W (Blankenfeldt et al. 2004); PhzB, 3DZL (Ahuja et al. 2008), PhzG, and 4HMT (Xu and Blankenfeldt 2013)

While Floss and coworkers speculated that DHHA is subsequently oxidized by the FMN-dependent enzyme PhzG (McDonald et al. 2001), it was later shown that DHHA is the substrate of PhzF instead, which isomerizes it to 6-amino-5-oxo-cyclohex-2-ene-1-carboxylic acid [AOCHC (Blankenfeldt et al. 2004; Parsons et al. 2004b)]. PhzF was the second representative of a protein family whose only other characterized member at the time was diaminopimelate epimerase, DapF (Cirilli et al. 1998), an enzyme involved in lysine biosynthesis. The monomers of

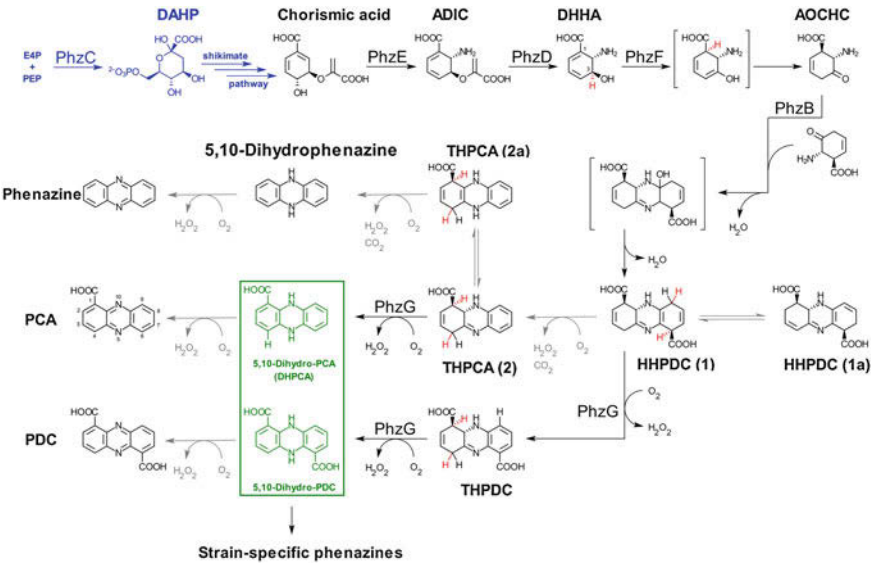

Fig. 1.5 Current understanding of core phenazine biosynthesis. Abstracted or shifted hydrogens are shown in *red*; *grey arrows* indicate uncatalyzed steps. 5,10-Dihydro-PCA and 5,10-dihydro-PDC are the final products of the pathway (*green*). Reactions not directly involved in phenazine biosynthesis are shown in *blue*

PhzF and DapF consist of two structurally similar domains, indicating that this family arose through gene duplication. PhzF is a homodimer in which the two active sites face each other as shown in Fig. 1.4. Unlike DapF, PhzF does not contain two cysteine residues involved in the catalytic mechanism of this PLP-independent racemase. Instead, PhzF employs a glutamic acid to abstract a proton from C3 of DHHA, whose acidity is probably increased by the tight hydrogen bonding network established between protein and substrate. The emerging anion is then reprotonated at C1, generating an enol that tautomerizes to the corresponding ketone AOCHC. NMR spectroscopy revealed that the abstracted and transferred proton are identical, i.e., the reaction catalyzed by PhzF is a [1,5]-proton shift and may thus have sigmatropic character.

AOCHC is highly reactive and cannot be isolated. It was shown by HPLC-coupled mass spectrometry that one of its spontaneous reactions is a 2-fold condensation with a second AOCHC molecule, resulting in an isomer of the double imine hexahydrophenazine-1,6-dicarboxylic acid (HHPDC) (Blankenfeldt et al. 2004). In the light of Ingledew's and Campbell's work cited above, who found that phenazines derive from two shikimate-derived molecules (Ingledew and Campbell 1969), this formation of HHPDC is a logical next step in phenazine biosynthesis. The reaction is bimolecular and accelerates exponentially with increasing AOCHC concentration, but even if it proceeds spontaneously in vitro, the reactive and therefore probably also toxic AOCHC is not expected to accumulate to allow

efficient phenazine production in the cell. Instead, it was shown that PhzB catalyzes the condensation and is hence likely required for phenazine biosynthesis in vivo (Ahuja et al. 2008).

The dimeric PhzB belongs to the ketosteroid isomerase/nuclear transport factor 2 family, and crystal structure analysis of a complex with two AOCHC analogs showed that the two substrate molecules indeed arrange in a diagonal symmetrical fashion as had been derived by experiments with labeled compounds conducted in the 1970s (Fig. 1.2) (Hollstein and Marshall 1972; Hollstein and McCamey 1973; Herbert et al. 1976; Hollstein et al. 1978). The crystal structure further revealed that PhzB accelerates HHPDC formation in two acid/base-catalyzed steps, using a glutamic acid for the first and probably a histidine/serine pair for the second condensation (Fig. 1.4).

Because the product HHPDC is not stable, HPLC-coupled NMR spectroscopy was required to elucidate its structure as (1R,5aS,6R)-1,2,5,5a,6,7-hexahydrophenazine-1,6-dicarboxylic acid **1a**. This molecule probably arises through spontaneous isomerization of isomer **1** shown in Fig. 1.5, gaining stability through conjugation of its four double bonds.

Interestingly, phenazine-producing pseudomonads contain a second copy of the *phzB* gene named *phzA* in their phenazine biosynthesis operons. PhzA is approximately 70 % identical to PhzB but lacks the histidine/serine diad for the second condensation step. Recombinant PhzA homodimers were indeed found not to catalyze HHPDC formation, despite the fact that Floss and coworkers found that both *phzA* and *phzB* were required for complete conversion of ADIC into phenazine (McDonald et al. 2001). The molecular basis for this observation and hence the function of PhzA is unclear at present.

It should be mentioned that PhzB may be an attractive target for pharmaceutical intervention: in an effort to repurpose already approved drugs, it recently was demonstrated that the enzyme can be inhibited with the cancer drug raloxifene, resulting in attenuated pyocyanin production and virulence of *P. aeruginosa* (Ho Sui et al. 2012). It is possible that inhibition of PhzB not only reduces phenazine biosynthesis but is also harmful to phenazine-producing bacteria because of the accumulation of the reactive AOCHC.

HHPDC needs to lose six electrons to gain aromaticity. HPLC-coupled mass spectrometry and work with oxygen- and carbon dioxide-selective electrodes showed that HHPDC undergoes a rapid spontaneous oxidative decarboxylation to an isomer of tetrahydrophenazine-1-carboxylic acid (THPCA) (Ahuja et al. 2008; Blankenfeldt et al. 2004). This accounts for two of the six electrons and provides an explanation why the Phz enzymes from pseudomonads do not convert PDC into PCA (McDonald et al. 2001). The second two-electron oxidation is carried out by PhzG (Xu and Blankenfeldt 2013), an FMN-dependent enzyme that is structurally closely related to pyridoxine-5′-phosphate oxidase PdxH (Parsons et al. 2004a). The product of this reaction is 5,10-dihydrophenazine-1-carboxylic acid (DHPCA), which is identical to the reduced electron shuttle form of PCA. DHPCA is easily oxidized by air, making further enzymatic oxidations unnecessary and explaining why the *phz*-operon contains only one oxidase while three two-

electron-oxidations and hence three enzymes seem necessary to complete phenazine biosynthesis. This is also corroborated in work with the phenazine-modifying prenyltransferase from *Streptomyces anulatus*, which was found to convert DHPCA, but not PCA into strain-specific phenazine derivatives (Saleh et al. 2009). From a chemical viewpoint, DHPCA will be more reactive than PCA because it does not possess the larger aromatic electron system of the latter. In the cell, on the other hand, phenazine modification will be coupled to core phenazine biosynthesis such that freshly synthesized DHPCA can be converted to strain-specific phenazine derivatives before it is oxidized to PCA.

If PCA biosynthesis relies on a non-catalyzed oxidative decarboxylation, how is this reaction avoided in phenazine producers that use PDC as a precursor for strain-specific phenazine derivatives? Initial insight into this question has recently been provided by Rui et al., who expressed combinations of Phz enzymes from *P. fluorescens* 2-79 and from *S. antibioticus* Tü2706 in *E. coli*. The authors then quantified PDC, PCA, and unsubstituted phenazine, which arises from THPCA as a "shunt" product after a second uncatalyzed oxidative decarboxylation. Their experiments showed that the relative amounts of the three phenazines are determined by the specific combination and provenience of the PhzB and PhzG enzymes: whereas the *P. fluorescens* 2-79 enzymes synthesized approximately 300 times more PCA than PDC, *S. antibioticus* Tü2706 enzymes produced nearly equal levels, while total quantities were around six times lower. This was interpreted as an indication for "stringent catalytic cooperation" of PhzB and PhzG toward either PCA or PDC production (Rui et al. 2012). Data from our own laboratory refines this hypothesis further: by soaking crystals of PhzG from *P. fluorescens* 2-79 with mixtures of PhzB and PhzF turning over high concentrations of DHHA, it was possible to trap HHPDC bound to the active center if soaking solutions were freshly prepared (Fig. 1.4). Soaking with reaction mixtures older than 1 day led to the trapping of THPCA (Xu and Blankenfeldt, unpublished). This suggests that in the biosynthesis of PDC, PhzG performs two rounds of oxidation on HHPDC by first generating tetrahydro-PDC and then rebinding this intermediate after reoxidation of the FMN-cofactor. Further, the complex structure with HHPDC indicates that even the PCA-producer *P. fluorescens* 2-79 can synthesize PDC, as was indeed observed by Rui et al. (2012). In the biosynthesis of PCA, on the other hand, PhzG oxidizes THPCA to DHPCA, which was confirmed in HPLC–MS experiments (data not shown). Notably, the active center of PhzG from different species is completely conserved, arguing that it is not the specificity of PhzG that distinguishes PCA- from PDC-producers, but rather the relative activities of PhzB and PhzG from the respective strains: if the activity of PhzB is high, larger amounts of HHPDC will accumulate such that the oxidation by PhzG cannot compete with the uncatalyzed oxidative decarboxylation and PCA is produced; if, on the other hand, the activity of PhzB is low, emerging HHPDC will immediately be oxidized and PDC, together with smaller amounts of PCA, will be generated. This is further substantiated by the fact that Rui et al. measured significantly increased amounts of unsubstituted phenazine when PhzG was omitted in their experiments (Rui et al. 2012). Here, oxidative decarboxylations are the main

means to gain aromaticity. Apparently, the competition between FMN-dependent oxidations by PhzG and uncatalyzed oxidative decarboxylations determines the outcome of core phenazine biosynthesis. It can at present not be ruled out that the Phz enzymes form a multienzyme complex, as has been suggested by McDonald et al. (2001). This could have further influence on the PCA/PDC ratio, but evidence for such interactions is lacking.

Compared to the many phenazine derivatives that have been isolated from natural sources, knowledge about phenazine-modifying enzymes is still scarce. Of the more complicated *phz* gene clusters, only the ones from *Enterobacter agglomerans* Eh1087 producing D-alanyl-griseoluteic acid (Giddens et al. 2002), from *S. cinnamonensis* DSM 1042 (Haagen et al. 2006) and *Streptomyces anulatus* 9663 (Saleh et al. 2012) producing endophenazines, and from *S. antibioticus* Tü 2706 leading to saphenamycins and esmeraldins (Rui et al. 2012) have been analyzed experimentally. Crystal structures of only four phenazine-modifying enzymes are available at present, namely of the SAM-dependent N-methyltransferase PhzM (Parsons et al. 2007) and the FAD-dependent hydroxylase PhzS (Greenhagen et al. 2008), which together convert PCA into pyocyanin in *P. aeruginosa*, as well as of a prenyltransferase from *S. cinnamonensis* DSM 1042 involved in endophenazine biosynthesis (Zocher et al. 2012) and of EhpF, a member of the ANL (acyl-CoA synthetases, NRPS adenylation domains, luciferase enzymes), catalyzing in the first step of converting PDC into D-alanyl-griseoluteic acid in *E. agglomerans* Eh1087 (Bera et al. 2010). Of these, only EhpF has been crystallized in complex with a phenazine ligand, which may have to do with the low solubility of phenazines and with the fact that it has not generally been recognized that phenazine-modifying enzymes probably have higher affinity toward reduced phenazines, as laid out above.

Another area related to phenazine modification that currently is gaining attention is phenazine degradation. Yang et al. recently isolated a *Sphingomonas* strain that can use PCA as the sole source of carbon and nitrogen (Yang et al. 2007), degrading the phenazine moiety via interesting azacyclobutyl metabolites (Chen et al. 2008). The genome of this organism has been sequenced (Ma et al. 2012), but the responsible catabolic enzymes await identification. Clearly, this field is expected to expand in the future.

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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.

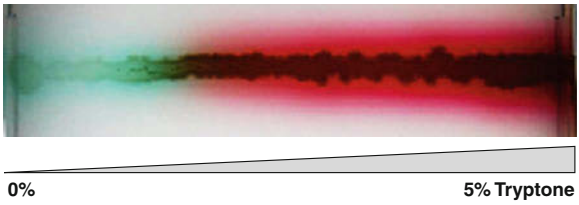


Fig. 2.1 *P. aeruginosa* PA14 grown on an agar plate containing a gradient of tryptone

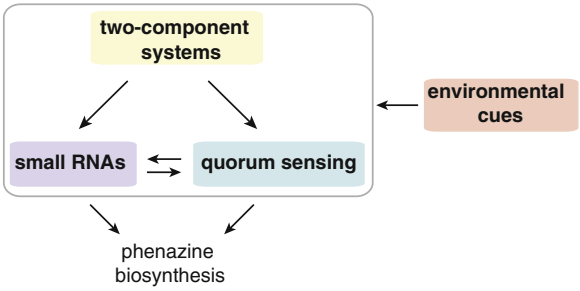


Fig. 2.2 Conceptual hierarchy of the phenazine regulation network

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

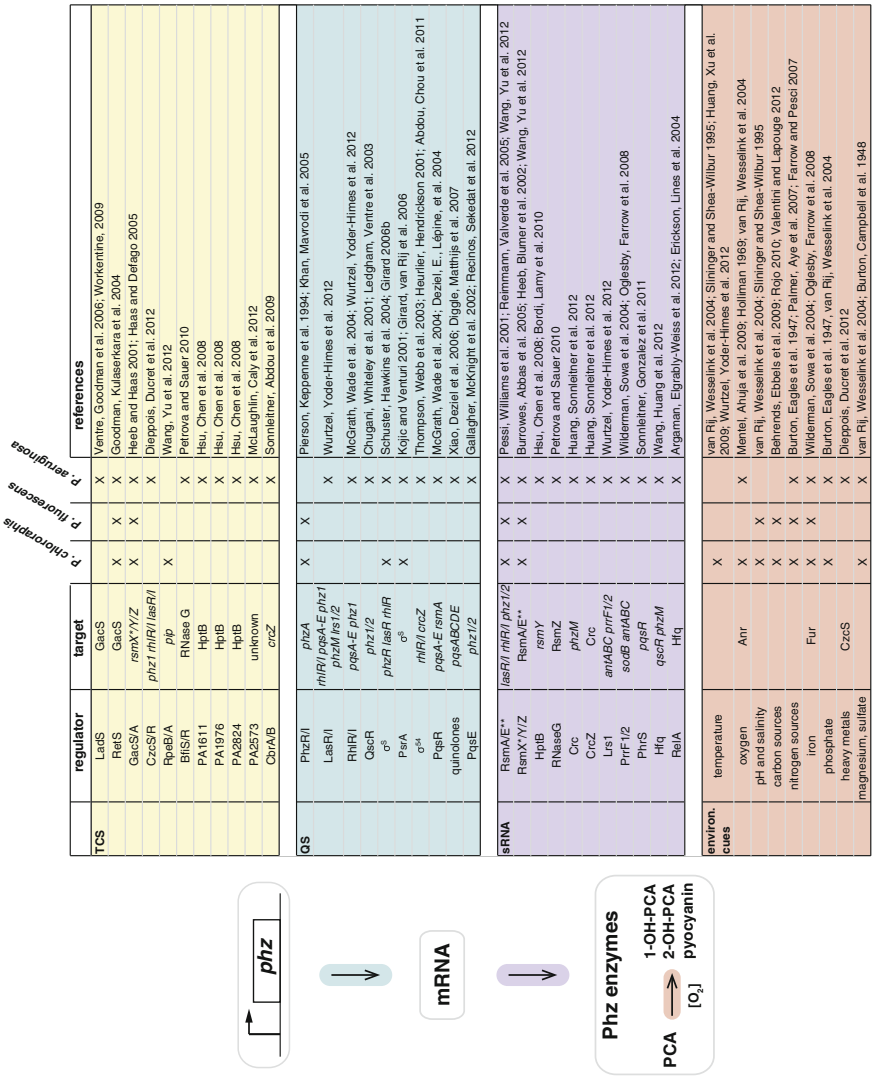
Table 2.1 Phenazine derivatives produced by *Pseudomonas* spp. and other bacteria

Phenazine derivative	References		actinobacteria	beta-proteo-bacteria	gamma-proteobacteria
1-phenazine-carboxamide	Lasseur (1911); Birkofer (1947)	R1: $CONH_2$			*P. aeruginosa* *P.chlororaphis*
5-methyl-phenazine-1-carboxylic acid	Byng, Eustice et al. (1979) Hansford and Hollman et al. (1972)	R1: COOH; R5: CH_3			*P.aeruginosa*
Aeruginosin A	Hollman (1969)	R1: COOH; R5: CH_3; R7: NH_2			*P. aeruginosa*
Aeruginosin B	Herbert and Hollman (1969)	R1: COOH; R3: SO_3; R5: CH_3; R7: NH_2			*P. aeruginosa*
4,9-dihydroxyphenazine-1,6-dicarboxylic acid dimethyl ester	Ballard, Palleroni et al. (1970)	R1: $COOCH_3$; R4: OH; R6: $COOCH_3$; R8: OH		*Burkholderia cepacia*	
D-alanylgriseoluteic acid	Giddens, Feng et al. (2002)	R1: COOH; R6: $C_3O_2NH_6$; R9: OCH_3			*Erwinia herbicola* *Vibrio spp.*
1-hydroxyphenazine	Gerber (1967); Schoental (1941)	R1: OH			*P. aeruginosa*
Pyocyanin	Fordos (1859); Von Saltza, Last et al. (1969)	R1: O; R5: CH_3			*P. aeruginosa*
1-phenazine-carboxylic acid	Turner and Messenger (1986); Laursen and Nielsen (2004); Mentel, Ahuja et al. (2009)	R1: COOH	All phenazine-producing bacteria		
Iodinin	Clemo and Daglish (1948); Gerber and Lechevalier (1964); Gerber and Lechevalier (1965); Lechevalier and Lechevalier (1965); Tanabe and Obayashi (1971); Gerber (1967)	R1: OH; R5: O; R6: OH; R10: O	*Brevibacterium iodinum* *Micrococcus paraffinolyticus* *Actinomadura dassonvillei* *Amycolata hydrocarbonoxydans* *Microbispora spp.* *Corynebacterium stationis* *Corynebac. hydrocarboclastum* *Arthrobacter paraffineus* *Nocardia hydrocarbonoxydans* *Streptomyces tholuteus*		
Iomofungin	Johnson and Dietz (1969); Tipton et al. (1970)	R1: COH; R2: OH; R4: O; R6: $COOCH_3$; R9: OH	*Streptomyces lomodensis*		
Solphenazine A	Rusman, Eppegard et al. (2013)	R4, R9: $C_7H_{11}O_6$	*Streptomyces sp.*		
Endophenazine A	Gebhardt et al. (2002); Haagen, Gluck et al. (2006)	R4: C_4H_9; R6: COOH	*Streptomyces anulatus* *Streptomyces cinammonensis*		
1,6-phenazinedimethanol	Choi, Kwon et al. (2009)	R1, R2: CH_2OH	*Brevibacterium 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



	regulator	target	*P. chlororaphis*	*P. fluorescens*	*P. aeruginosa*	references
TCS	LadS	GacS			X	Ventre, Goodman et al. 2006; Workentine, 2009
	RetS	GacS	X	X	X	Goodman, Kulasekara et al. 2004
	GacS/A	*rsmX'Y'Z*	X	X	X	Heeb and Haas 2001; Haas and Defago 2005
	CzcS/R	*phz1 rhlR/I lasR/I*			X	Dieppois, Ducret et al. 2012
	RpeB/A	*pip*	X			Wang, Yu et al. 2012
	BfiS/R	RNase G			X	Petrova and Sauer 2010
	PA1611	HptB			X	Hsu, Chen et al. 2008
	PA1976	HptB			X	Hsu, Chen et al. 2008
	PA2824	HptB			X	Hsu, Chen et al. 2008
	PA2573	unknown			X	McLaughlin, Caly et al. 2012
	CbrA/B	*crcZ*			X	Sonnleitner, Abdou et al. 2009
QS	PhzR/I	*phzA*	X	X		Pierson, Keppenne et al. 1994; Khan, Mavrodi et al. 2005
	LasR/I	*rhlR/I pqsA-E phz1 phzM lrs1/2*			X	Wurtzel, Yoder-Himes et al. 2012
	RhlR/I	*pqsA-E phz1*			X	McGrath, Wade et al. 2004; Wurtzel, Yoder-Himes et al. 2012
	QscR	*phz1/2*			X	Chugani, Whiteley et al. 2001; Ledgham, Ventre et al. 2003
	σ^S	*phzR lasR rhlR*	X		X	Schuster, Hawkins et al. 2004; Girard 2006b
	PsrA	σ^S	X		X	Kojic and Venturi 2001; Girard, van Rij et al. 2006
	σ^{54}	*rhlR/I crcZ*			X	Thompson, Webb et al. 2003; Heurlier, Hendrickson 2001; Abdou, Chou et al. 2011
	PqsR	*pqsA-E rsmA*			X	McGrath, Wade et al. 2004; Deziel, E., Lépine, et al. 2004
	quinolones	*pqsABCDE*			X	Xiao, Deziel et al. 2006; Diggle, Matthijs et al. 2007
	PqsE	*phz1/2*			X	Gallagher, McKnight et al. 2002; Recinos, Sekedat et al. 2012
sRNA	RsmA/E**	*lasR/I rhlR/I phz1/2*	X	X	X	Pessi, Williams et al. 2001; Reimmann, Valverde et al. 2005; Wang, Yu et al. 2012
	RsmX'Y'Z	RsmA/E**	X	X	X	Burrowes, Abbas et al. 2005; Heeb, Blumer et al. 2002; Wang, Yu et al. 2012
	HptB	*rsmY*			X	Hsu, Chen et al. 2008; Bordi, Lamy et al. 2010
	RNaseG	RsmZ			X	Petrova and Sauer 2010
	Crc	*phzM*			X	Huang, Sonnleitner et al. 2012
	CrcZ	Crc			X	Huang, Sonnleitner et al. 2012
	Lrs1	*antABC prrF1/2*			X	Wurtzel, Yoder-Himes et al. 2012
	PrrF1/2	*sodB antABC*			X	Wilderman, Sowa et al. 2004; Ogilesby, Farrow et al. 2008
	PhrS	*pqsR*			X	Sonnleitner, Gonzalez et al. 2011
	Hfq	*qscR phzM*			X	Wang, Huang et al. 2012
	RelA	Hfq			X	Argaman, Elgrably-Weiss et al. 2012; Erickson, Lines et al. 2004
environ. cues	temperature			X		van Rij, Wesselink et al. 2004; Slininger and Shea-Wilbur 1995; Huang, Xu et al. 2009; Wurtzel, Yoder-Himes et al. 2012
	oxygen	Anr	X		X	Mentel, Ahuja et al. 2009; Hollman 1969; van Rij, Wesselink et al. 2004
	pH and salinity		X	X		van Rij, Wesselink et al. 2004; Slininger and Shea-Wilbur 1995
	carbon sources		X	X		Behrends, Ebbels et al. 1947; Palmer, Aye et al. 2007; Farrow and Pesci 2007
	nitrogen sources		X	X	X	Burton, Eagles et al. 1947; Palmer, Aye et al. 2007; Farrow and Pesci 2007
	iron	Fur	X	X	X	Wilderman, Sowa et al. 2004; Ogilesby, Farrow et al. 2008
	phosphate		X		X	Burton, Eagles et al. 1947, van Rij, Wesselink et al. 2004
	heavy metals	CzcS			X	Dieppois, Ducret et al. 2012
	magnesium, sulfate		X		X	van Rij, Wesselink et al. 2004; Burton, Campbell et al. 1948

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 (Reimmann 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

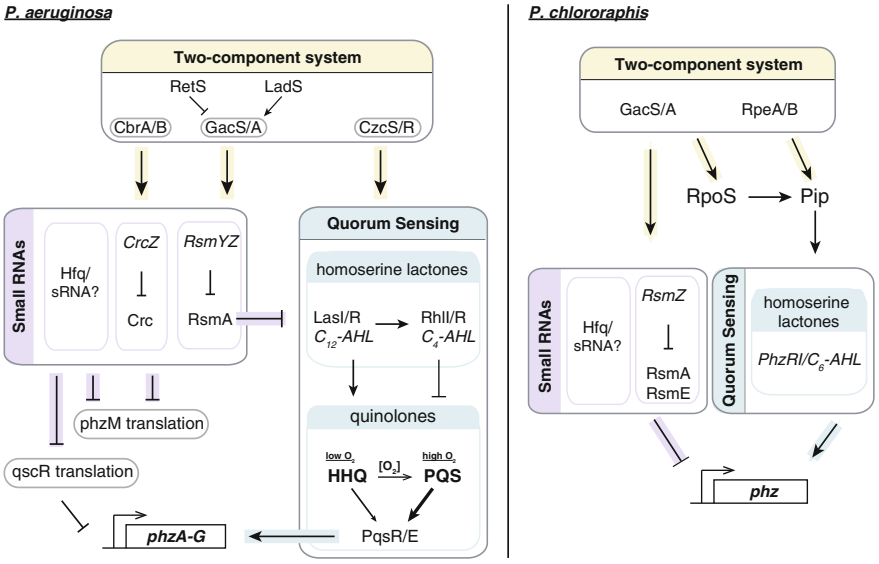


Fig. 2.4 Summary of two component, quorum sensing, and sRNA systems that control phenazine production in *P. aeruginosa* and *P. chlororaphis*. The *P. fluorescens* regulatory system is likely similar to that of *P. chlororaphis*

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 *phz1* promoter when the system is activated by zinc. In this way, CzcR could inhibit *phz1* 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–C_6–HSL). Although additional LuxR/I type systems in some of these strains produce and respond to other AHLs, 3–OH–C_6–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–C_{12}–HSL) and *N*-butanoyl-*L*-homoserine lactone (C_4–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–C_{12}–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 PsrA 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

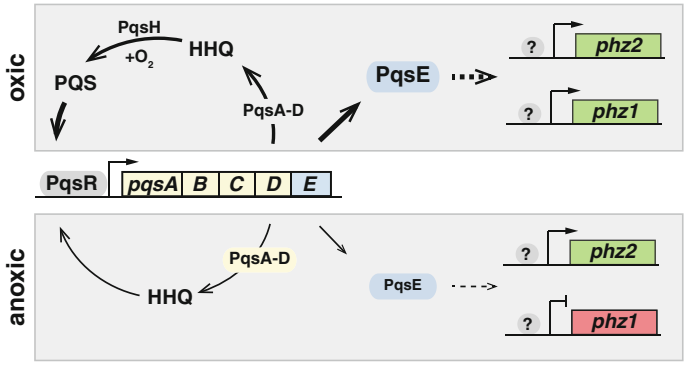


Fig. 2.5 Quinolone-mediated expression of the *pqs* operon and its influence on transcription of the two *phz* operons in the presence and absence of oxygen

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

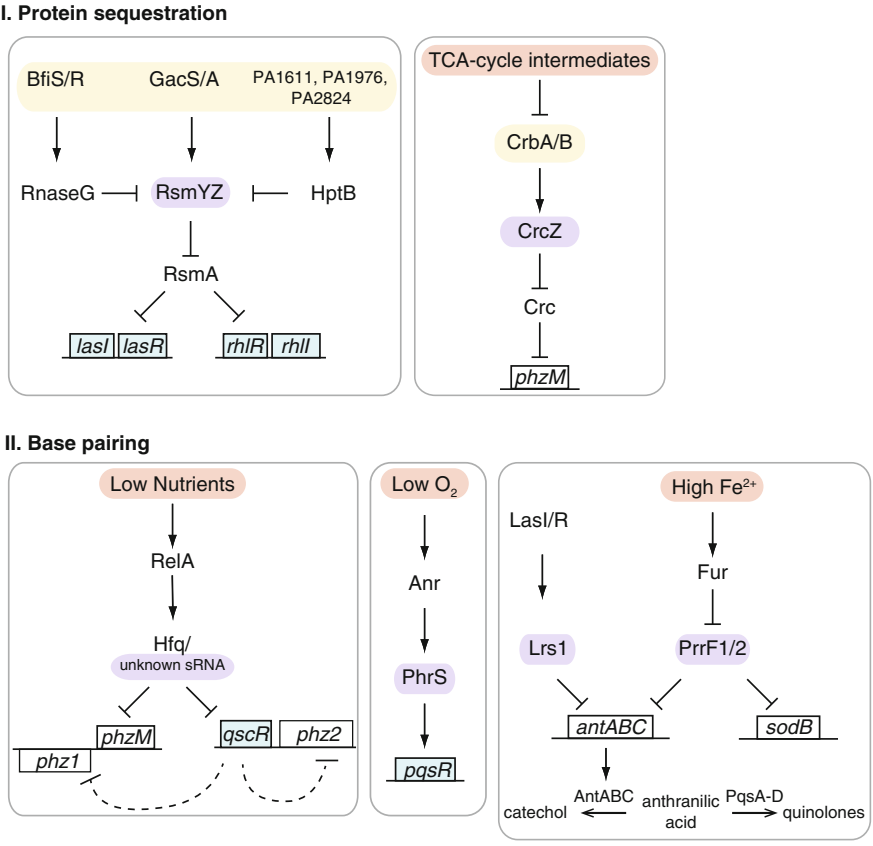


Fig. 2.6 sRNAs and proteins that regulate phenazine production post-transcriptionally in *P. aeruginosa*. Some sRNAs regulate target genes by base pairing with target mRNAs, while others sequester proteins that interact with mRNAs. Known environmental cues that regulate these elements are indicated

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 ANGGA motifs near the ribosomal binding site; by stabilizing a stem loop that contains the ANGGA 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 (Reimmann 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 ANGGA 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 *prrF1/prrF2* mutant shows increased expression of the *antABC* operon, an effect that seems to contradict the simultaneous upregulation of *antABC* and *prrF1/prrF2* 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.

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Chapter 3
The Yin and Yang of Phenazine Physiology

Nora Grahl, Suzanne E. Kern, Dianne K. Newman and Deborah A. Hogan

Abstract Microorganisms are seldom solitary. They are surrounded by both clonal cells and other members of the local microbial community, and they often exist in, on, or in close proximity to multi-cellular host organisms like plants and humans. Whether in vivo during infection or in situ in the nutrient rich rhizosphere, microorganisms affect each other and the host. Phenazines, a class of secondary metabolites secreted by diverse bacteria, are best known for their antibiotic properties and have been shown to affect a broad spectrum of organisms ranging from bacteria over fungi, plants, nematodes, parasites, and humans. However, phenazines are also involved in numerous aspects of bacterial physiology like survival, iron acquisition, signaling, and biofilm formation in ways that have the potential to increase the fitness of both the phenazine-producing strain and non-producers alike. The overarching theme of this chapter is that phenazines can be beneficial or detrimental to organisms, depending on the milieu and one's perspective. In this chapter, we will highlight specific examples to discuss the yin and yang of phenazine physiology.

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3.1 Introduction

> What's in a name? That which we call a rose by any other name would smell as sweet.
>
> *Romeo and Juliet, William Shakespeare, Act II Scene II*

Names shape the way we think about things. Phenazines are best known for their toxic effects (reviewed in Laursen and Nielsen 2004) and are conventionally called "antibiotics," "virulence factors" or "secondary metabolites." Yet, studies of the direct and indirect effects of phenazines on diverse biological systems have shown that these compounds have versatile functions extending well beyond their toxicity (Fig. 3.1). In order to gain a complete understanding of the roles of phenazines in different environments, we must embrace the fact that for these molecules, as for so many others in biology, context matters. Phenazine activity can be positive or negative, depending on one's point of view. Furthermore, the consequences of phenazines can vary with dose, and the chemical context. Fundamental advances in our understanding of microbial metabolism and microbial community dynamics have now given us the opportunity to better appreciate the physiological functions of phenazines in both nature and disease. Although most of what is known about the physiological roles of phenazines comes from the study of pseudomonads, much of what we discuss likely applies to the roles of phenazines produced by other microbes and may also be relevant for other redox-active pigments. In this chapter, we highlight the beneficial and antagonistic roles of phenazines in microbial interactions within single-species populations, mixed-species communities, and during microbe-host interactions, to convey the complexity and importance of phenazine physiology.

3.2 Properties of Phenazines

In 1899, Emmerich and Löw (1899) discovered that subcutaneous injection of *Pseudomonas aeruginosa* supernatants could cure rabbits of anthrax and diphtheria. The heat-resistant factors responsible for this phenomenon were likely phenazines, and these important secreted factors were subsequently described as potent antibiotics decades later (Schoental 1941; Young 1947; Knight et al. 1979; Hassan and Fridovich 1980). Even in these early days of phenazine research, it was proposed that the underlying chemical properties that make them potent antibiotics can also be harnessed by bacteria to their benefit. In the 1930s, Barron, Friedheim, and others postulated that redox-cycling compounds such as phenazines are "accessory respiratory pigments" that can sustain bacterial "respiration" based on their ability to stimulate oxygen consumption in suspensions of many different types of cells (Harrop and Barron 1928; Barron and Hoffman 1930; Friedheim 1931, 1934). Since this early work, there has been great growth in our knowledge of the types of phenazines produced by different species, the regulation of their production, and the factors that influence their activity.

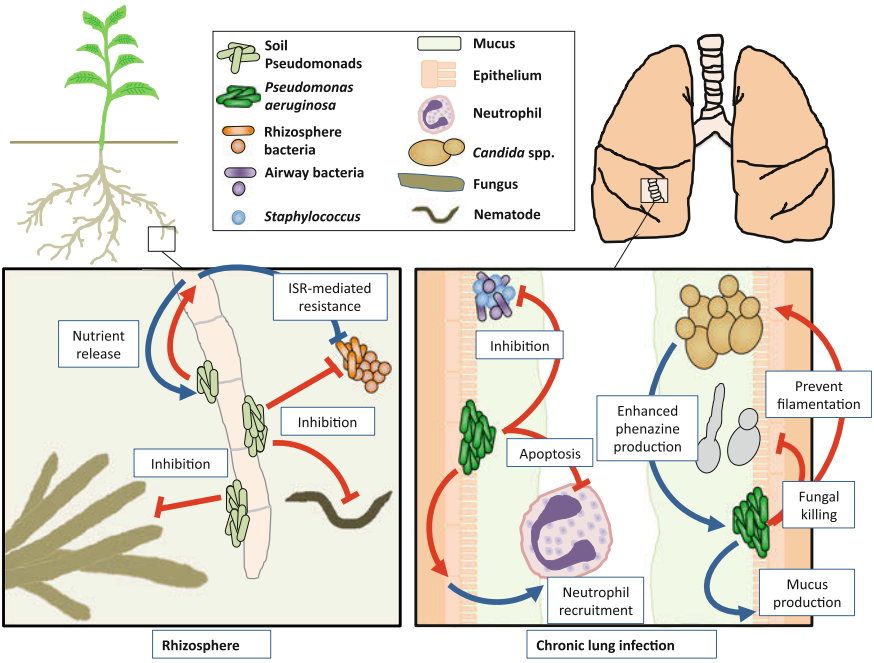

Fig. 3.1 The effects of phenazines in the rhizosphere and in multi-species chronic infections in the cystic fibrosis lung. (*Left panel*) In the soil a number of species of *Pseudomonas* form biofilms on plant roots. Phenazines produced by these pseudomonads protect plants from other phytopathogenic soil bacteria, fungi, and nematodes. Furthermore, *Pseudomonas* biofilms and phenazine secretion cause nutrient release by the plants into the rhizosphere, which may enhance pseudomonal growth. Phenazines also activate the plant's induced systemic resistance (*ISR*) which may result in increased protection against attacks by phytopathogens. (*Right panel*) In chronic lung infections associated with the genetic disease cystic fibrosis, phenazines are secreted by *Pseudomonas aeruginosa* and the levels of these phenazines have been shown to negatively correlate with the complexity of the microbial community in the lung. In vitro studies showed that *Candida albicans*, a fungal pathogen that frequently co-infects with *P. aeruginosa*, is efficiently killed by *P. aeruginosa* when phenazine levels are high (*grey colored fungal cells*). However, when phenazine levels are low they prevent *C. albicans* filamentation in vitro. This likely also occurs in vivo and might be the reason why *C. albicans* is only found in its yeast form in the lungs of cystic fibrosis patients. Furthermore, phenazines may alter the chemistry of the local environment in such a way as to repress or favor different microbial species under these conditions. For example phenazines cause mucus overproduction by lung epithelial cells resulting in low-oxygen environments. Furthermore, increased neutrophil recruitment in response to phenazines might result in increased clearance of some microorganisms. However, phenazines also increase apoptosis of neutrophils and decrease ciliary activity which might actually allow survival and growth of other bacterial species. *Red arrow*—direct effects of phenazines; *blue arrow*—reaction of a recipient to phenazines

3.2.1 Phenazine Chemistry

Phenazines accept and donate two electrons, stepwise, according to the redox properties of nearby compounds, and they are soluble in aqueous solution at micro- to millimolar levels (Price-Whelan et al. 2006). Phenazines can accept electrons from reduced nicotinamide cofactors (NAD(P)H, $E^{\circ\prime} = -320$ mV) and donate electrons to electron acceptors with more positive reduction potentials. Major electron acceptors for phenazines are oxygen ($E^{\circ\prime} = +816$ mV) and ferric iron ($E^{\circ\prime} = +770$ mV for conversion to Fe^{2+}, although this varies considerably with pH or complexation to organic ligands) (Hernandez et al. 2004; Cox 1986). Some of these redox reactions can proceed spontaneously in vitro. Phenazine oxidation by denitrifying *P. aeruginosa* has also been observed, but only in the presence of live cells, suggesting that this particular reaction may be cell-mediated (Price-Whelan 2009). Furthermore, specific redox properties vary according to the phenazine structure. For example, reduced 5-methyl-1(5H)-phenazinone (pyocyanin, PYO) reacts quickly with oxygen and slowly with ferric iron, while phenazine-1-carboxylic acid (PCA) shows the opposite tendency (Wang and Newman 2008). One of the interesting and important aspects of phenazine physiology is that these redox reactions can occur both intracellularly and in the extracellular milieu.

3.2.2 Regulation of Phenazine Production

In the pseudomonads, phenazine production is often greatest when populations reach high cell densities (Mavrodi et al. 2010, 2012). In dense populations, secreted autoinducers stimulate "quorum sensing" regulatory pathways that lead to striking changes in gene expression. In *P. aeruginosa* and *Pseudomonas chlororaphis*, it has been shown that phenazine biosynthesis genes are controlled by quorum sensing. In addition, various chemical, physical, and biological determinants can affect the identity and quantity of phenazines produced (Kanner et al. 1978; Slininger and Jackson 1992; Slininger and Shea-Wilbur 1995; Recinos et al. 2012; Wurtzel et al. 2012). For example, in a strain of *P. chlororaphis* (PCL1391), levels of phenazine carboxamide vary by more than 20-fold in response to different combinations of carbon and nitrogen sources, levels of oxygen, phosphate, magnesium, and iron, as well as differences in temperature or pH (van Rij et al. 2004).

The "fine-tuning" of phenazine production may increase the fitness of *P. aeruginosa* strains in particular environments. For example, Recinos et al. (2012) recently dissected the differential regulation of the two core phenazine biosynthesis operons (*phzABCDEFG*) in *P. aeruginosa* PA14, showing that *phzA1-G1* is more expressed in liquid cultures whereas *phzA2-G2* dominates in colony

biofilms and is involved in lung colonization in a murine infection model. Huang et al. (2009) noted that the soil isolate *P. aeruginosa* strain M18, which exhibits strong biocontrol properties, shares remarkably similar sequence identity with *P. aeruginosa* strain PAO1, a known animal pathogen. While both strains encode two copies of the core phenazine biosynthesis operon, as well as *phzM* and *phzS*—the genes encoding proteins required for converting PCA to PYO—they produce dramatically different amounts of the two phenazines. Huang et al. (2009) observed environment-specific regulation of phenazine production, with levels of plant-protective PCA produced in the highest amounts by the rhizosphere isolate at soil temperatures, and the virulence factor PYO more highly produced by the pathogenic strain PAO1 at the more elevated temperature of an animal host. Both strains produce more PYO at 37 °C than at 28 °C, but the pathogen PAO1 produced over twice as much as M18. Levels of PCA were highest in cultures of M18 grown at 28 °C, over four times higher than at 37 °C. PAO1 produced much lower levels of PCA at either temperature (Huang et al. 2009). A recent study by Wurtzel et al. (2012) similarly found enhanced transcription of phenazine biosynthetic genes from the pathogenic strain PA14 at 37 °C, the body temperature of mammalian hosts. It is worth noting that, apart from the PhzS enzyme that requires molecular oxygen for its activity, all other enzymes involved in phenazine biosynthesis can operate under anoxic conditions (Gohain et al. 2006).

Phenazine production by *P. aeruginosa* can also be affected by products secreted by other species. For example, farnesol, a quorum sensing molecule secreted by the fungus *Candida albicans*, increases phenazine levels in *P. aeruginosa* when the two species are cultured together (Cugini et al. 2010). *P. aeruginosa* chronically infects the lungs of individuals with the genetic disease cystic fibrosis (CF), and during these chronic infections, approximately 30 % of *P. aeruginosa* CF clinical isolates lose quorum sensing regulation and, consequently, their ability to produce phenazines in laboratory culture conditions (Hoffman et al. 2010). Even in these mutants, *C. albicans* or farnesol strongly induces phenazine production by activating RhlR and the expression of *pqsH* and thus the *Pseudomonas* quinolone signal (PQS) biosynthesis pathway (Cugini et al. 2010). This stimulation of toxic phenazine production may also occur in vivo during mixed-species infections (Fig. 3.1, right panel). Because phenazines can be damaging to the host, as discussed below, this interaction might explain why co-infections of *P. aeruginosa* and fungal pathogens like *C. albicans* are correlated with a worse disease status of CF patients (Navarro et al. 2001; Chotirmall et al. 2010). However, when *C. albicans* cells outnumber *P. aeruginosa*, farnesol can inhibit PqsR-mediated induction of the PQS biosynthesis genes with resulting inhibition of PYO production. Farnesol has a lower affinity for the transcriptional regulator PqsR than the endogenous ligand PQS, and thus is most influential in low-density situations where PQS production has not yet been induced (Cugini et al. 2007, 2010). For a full account of phenazine regulation, see Chap. 2.

3.3 Beneficial Effects of Phenazines on Producing Organisms

While phenazines have long been recognized for their importance in virulence and microbial killing, their positive physiological roles for the bacteria that produce them are becoming more appreciated (Fig. 3.2). The pseudomonads, particularly *P. aeruginosa*, *P. chlororaphis*, and *Pseudomonas fluorescens*, are well-studied examples of phenazine producers, and they share a common operon (*phzABC-DEFG*) encoding the enzymes needed for synthesizing PCA, a precursor to the other phenazines (Mentel et al. 2009). The *phzS* and *phzM* genes (which encode the enzymes necessary for the last two steps in PYO biosynthesis) are only found in *P. aeruginosa* but not other pseudomonads. Preservation of the *phzA-G* operon throughout the evolution of this genus may point to a role for phenazines in core physiological functions (Mavrodi et al. 2010). In comparison, among many non-pseudomonads, such as species of *Burkholderia*, *Pectobacterium*, and members of the *Actinomycetales*, this core phenazine biosynthesis operon appears to be present due to horizontal gene transfer, and is encoded on plasmids or in transposons (Fitzpatrick 2009; Mavrodi et al. 2010, 2012). In such instances, phenazines might primarily serve as antimicrobial agents against competing organisms, maintained

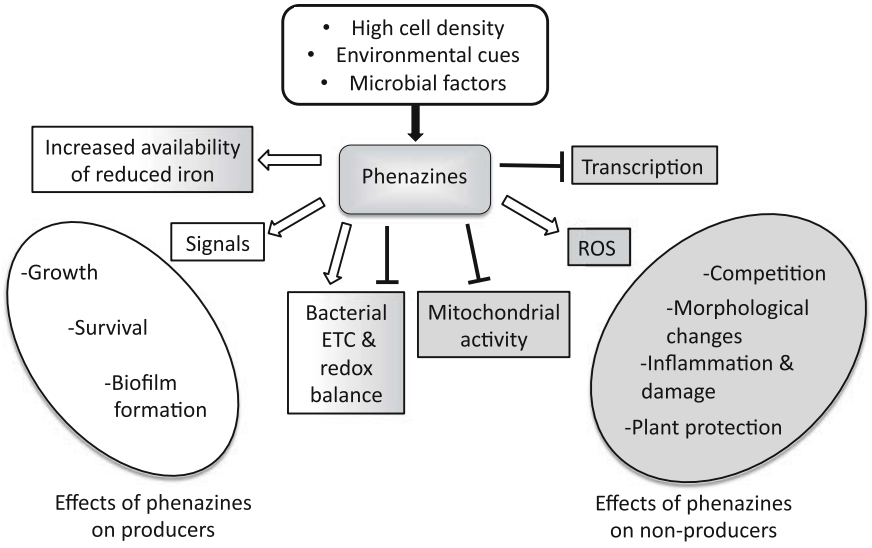

Fig. 3.2 Summary of factors that influence phenazine production, the effects of phenazines and the consequences of these effects. Phenazines can affect cells in many ways that impact phenazine producers (*white rectangles*), phenazine non-producers (*grey rectangles*), or may have general effects that impact many species in a community (*rectangles with a grey-white gradient*). For phenazine-producing microbes, these effects are beneficial (*white oval*). In non-phenazine-producing microbes and hosts, phenazines can have either beneficial or detrimental roles (*gray oval*)

only in specific ecological contexts (Fitzpatrick 2009; Mavrodi et al. 2010; Parejko et al. 2012). It is interesting to note that many strains produce a spectrum of different phenazines, and this may reflect the fact that different phenazines have distinct functions that manifest under particular environmental conditions.

3.3.1 Intracellular Reactivity: Redox Balance and Anaerobic Survival

Phenazines can alter the redox state of key cellular metabolites, and thus, phenazine-producing cells are subject to phenazines' potential benefits and harms from the moment they are synthesized. Early work in *P. aeruginosa* revealed that PYO is chemically reduced through radical intermediates within the cell (Hassett et al. 1992). It was suggested that *P. aeruginosa's* ability to withstand this potential source of redox stress could be attributed to limited redox-cycling of PYO and increased activity of catalase and superoxide dismutase (SOD). While an enhanced response to reactive oxygen species (ROS) may indeed be helpful, phenazines can also react with other things in the cell, such as iron-sulfur (Fe-S) clusters within proteins. For example, recent in vitro studies of the transcription factor SoxR from *Escherichia coli* have shown that the SoxR protein directly senses redox-cycling compounds such as phenazine methosulfate (PMS) in addition to superoxide (Gu and Imlay 2011). The same is likely to hold true for SoxR from *P. aeruginosa*, as in vivo transcriptional studies have shown that PYO can regulate SoxR-dependent gene expression under anoxic conditions (Dietrich et al. 2006). Very little attention has been paid to how producing cells prevent phenazines from wreaking havoc by indiscriminately reacting with intracellular redox-sensitive proteins or cofactors. It is possible that phenazine "chaperones" exist and/or that phenazine production and export are so well coordinated that phenazines are removed before they can do harm. Understanding the mechanisms of phenazine tolerance by producing cells is an important area for future research.

Regardless of how cells circumvent phenazine toxicity, a number of studies have shown that phenazine producers can benefit from their production. For example, late-exponential and stationary phase planktonic cultures of *P. aeruginosa* grown with glucose as the main carbon source have a more oxidized pool of nicotinamide adenine dinucleotide (NAD(P)(H)) when phenazines are synthesized (Price-Whelan et al. 2007) and this is correlated with survival (Wang et al. 2010; Glasser et al. submitted). The same is true for *P. aeruginosa* colony biofilms grown on agar plates (Dietrich et al. 2013). Multiphoton microscopy measurements clearly indicate that the reduction of phenazines over time is accompanied by the oxidation of the NAD(P) pool in planktonic *P. aeruginosa* (Sullivan et al. 2011). However, this technique cannot determine whether this redox coupling is direct or indirect—for example, through a component of the respiratory chain. Efforts are underway to identify the catalysts of phenazine reduction and oxidation in *P. aeruginosa* (Glasser et al., submitted; Fig. 3.3a).

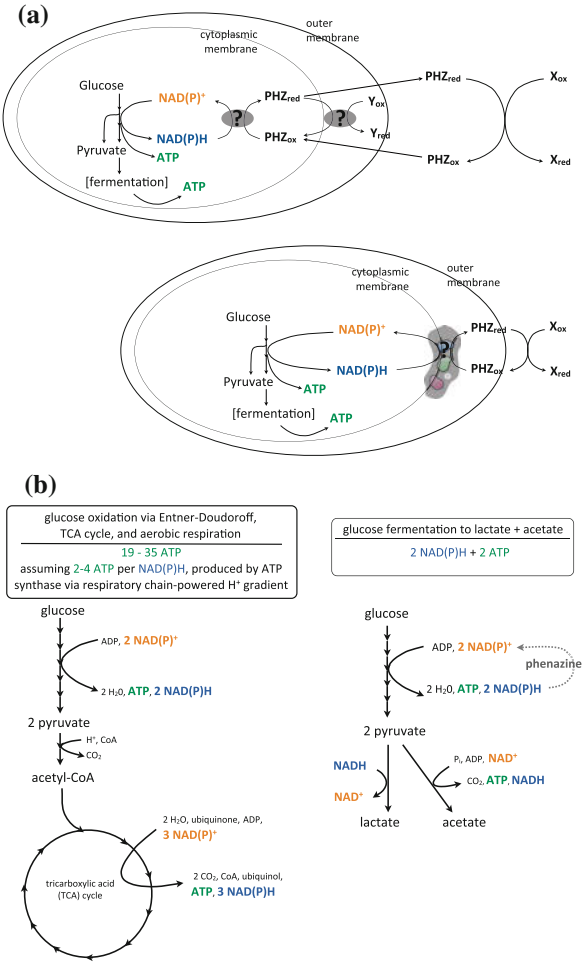

Fig. 3.3 Phenazine reduction and glucose metabolism in *P. aeruginosa*. **a** Possible locations for phenazine reduction and oxidation within or outside the cell. *Top diagram* assumes phenazines are reduced in the cytosol without being coupled to the membrane electron transport chain, with oxidation either in the periplasm (by oxidant Y) and/or extracellularly (by oxidant X). *Bottom diagram* assumes phenazines are reduced by a constituent of the membrane electron transport chain. Although the figure, for simplicity, shows reduction on the periplasmic face, it is possible that this occurs on the cytoplasmic side. **b** Simplified pathways for glucose metabolism: *left panel* shows complete oxidation of glucose to CO_2 via the Entner-Doudoroff and TCA cycle; *right panel* shows partial oxidation of glucose to the predominant fermentation products in *P. aeruginosa,* lactate and acetate. The theoretical amount of ATP and NAD(P)H generated from these alternative routes for glucose oxidation are indicated on the diagram

Oxidation of the NAD(P)H pool can be key for microbial fitness in oxygen-limiting environments. Emde et al. (1989) and Emde and Schink (1990) have demonstrated that anaerobically fermenting cultures of *Propionibacterium freudenreichii* and *E. coli* produce more oxidized end products when redox-active small-molecule mediators and an oxidizing electrode are present. Phenazines can also act as soluble electron shuttles. Dense *P. aeruginosa* cultures grown aerobically with glucose excrete and subsequently consume pyruvate in late stationary phase, while a phenazine-null mutant does not (Price-Whelan et al. 2007). Pyruvate fermentation in *P. aeruginosa* enables anaerobic survival, but not growth, via production of acetate and lactate with ATP generation and NAD(H) balance as byproducts (Eschbach et al. 2004); therefore, the consumption of pyruvate in late stationary phase would seem to provide a useful and timely energy source. The transient accumulation of pyruvate in wild-type, but not phenazine-null mutant, cultures may be due to PYO-mediated inhibition of the pyruvate dehydrogenase complex, thereby blocking the downstream utilization of pyruvate in the TCA cycle, resulting in its buildup and excretion (Price-Whelan et al. 2007). Subsequently, pyruvate may be consumed through its fermentation to lactate and acetate promoted by phenazine oxidization of NAD(P)H, facilitating ATP production (Glasser et al., submitted) (Fig. 3.3b). In this manner, phenazines may contribute to survival by balancing the cellular redox state under oxidant-limited conditions by transferring electrons to an extracellular oxidant at a distance from the cell (Fig. 3.3a).

When bacteria grow in aggregates or in association with a surface, collectively referred to as biofilms, hypoxic or even anoxic zones develop (Stewart and Franklin 2008). As discussed above, phenazine production occurs under conditions of high cell density and hypoxia, which are two important aspects of the biofilm environment. Maddula et al. (2008) reported that phenazines have distinct roles in the development of *P. chlororaphis* 30-84 biofilms: 2-hydoxy phenazine (2-OH-PHZ) enhanced biofilm attachment and growth and diminished dispersal rates, while PCA only impacted growth and dispersal. Biofilms of the PCA-overproducer, like the wild type, formed mushroom-shaped flow-cell biofilms, while cells overproducing 2-OH-PHZ created a thick, but flat biofilm structure (Maddula et al. 2008). Other recent work has shown that phenazines influence the morphology of *P. aeruginosa* colony biofilms (Dietrich et al. 2008; Ramos et al. 2010) in ways that promote intracellular redox balance (Dietrich et al. 2013). Colony biofilms produced by the phenazine-null mutant of *P. aeruginosa* PA14 increase their surface-to-volume ratio, becoming wrinkled earlier in biofilm development than the parent strain. Similar morphological consequences in colony biofilms have been reported for the Gram-positive *Streptomyces coelicolor* in relation to the secondary metabolites actinorhodin and undecylprodigiosin (Dietrich et al. 2008). Measurements of the ratio of reduced to oxidized NAD ($NADH/NAD^+$) in the *P. aeruginosa* studies indicate similar ratios between the smooth wild type colonies and the wrinkled mutant colonies. Consistent with the hypothesis that phenazines counteract a buildup of NADH under conditions with limited terminal oxidants, a high $NADH/NAD^+$ ratio immediately precedes the initiation of wrinkling in the

phenazine-null mutant, and the ensuing increase in the ratio of surface area to volume correlates with a return of the NADH/NAD^+ levels to earlier, wild-type levels (Dietrich et al. 2013).

3.3.2 Extracellular Reactivity: Electrodes and Iron

In addition to their intracellular reactivity, phenazines can profoundly affect the extracellular environment. As redox shuttles, any given phenazine molecule is transported into and out of individual producing cells multiple times (Wang et al. 2010). Once outside, phenazines can react with many different oxidants with higher reduction potential. For example, electrodes can be poised at the appropriate potential to oxidize phenazines, which can promote survival in anoxic conditions (Wang et al. 2010; Glasser et al. submitted) and may impact the function of microbial fuel cells (see below). Another important extracellular oxidant for phenazines is ferric iron [Fe(III)]. Both in vitro and in vivo studies have shown that phenazines can liberate iron from minerals and chelators in the form of ferrous iron [Fe(II)] (Cox 1986; Hernandez et al. 2004; Wang and Newman 2008; Wang et al. 2011). During planktonic growth, the fraction of iron present as Fe(II) in cultures of *P. aeruginosa* PA14 increases as the cell density and phenazine concentrations increase (Kreamer et al. 2012). Certain phenazines react more readily with iron than others: for example, PCA is a better reductant of Fe(III) minerals than PYO, which preferentially reduces molecular oxygen (Wang and Newman 2008). Importantly, while phenazines can stimulate Fe(III)-reduction, *Pseudomonas* can also catalyze iron reduction via other mechanisms under certain conditions (Cox 1980).

Phenazine-mediated reduction of Fe(III) to Fe(II) increases its bioavailability, which can have important consequences both for producers and others in their vicinity. For example, phenazines can promote biofilm development in part due to Fe(II) generation (Wang et al. 2011). Iron is an important signal for *P. aeruginosa*, affecting twitching motility and thereby biofilm attachment (Singh et al. 2002; Harmsen et al. 2010). We hasten to note, however, that phenazines can promote biofilm formation through mechanisms separate from those involving iron (Ramos et al. 2010; Dietrich et al. 2013). Moreover, not all iron concentrations promote biofilm formation: above a critical threshold, the buildup of Fe(II) in fact has the opposite effect (Musk et al. 2005). Interestingly, the recently-discovered Fe(II)-specific two-component system BqsR/S was first described in a screen for mutants defective in biofilm dissolution (Dong et al. 2008; Kreamer et al. 2012), suggesting that as the Fe(II) concentration crosses a critical threshold, *Pseudomonas* senses this and returns to a planktonic state. It will be exciting to explore the relevance of these laboratory findings to the more complex environments *Pseudomonas* inhabits. For example, a systematic study of phenazine and iron concentrations within sputum samples from a cross-section of CF patients at different disease states found that PCA abundance positively correlates with higher proportions of

Fe(II) (Hunter et al. 2013). This finding underscores the importance of understanding phenazine reactivity in situ, as antimicrobial therapies involving Fe-chelation appear to hold promise as a novel therapeutic strategy (Banin et al. 2008; Moreau-Marquis et al. 2009). The effect of phenazines on iron acquisition is particularly striking in light of the well characterized suite of iron scavenging compounds known to be produced by *P. aeruginosa* (Cornelis and Matthijs 2002). These studies highlight the need to consider environmental conditions in assessing the importance of different microbial molecules.

3.4 The Roles of Phenazines in Interspecies Interactions

Since the initial discovery of the antibiotic properties of phenazines, their ill effects on many microbial species and multicellular hosts have been described (reviewed in Kerr 2000; Laursen and Nielsen 2004). Many phenazines are active against numerous species, suggesting that one of their roles is to broadly decrease competition for resources. At the same time, however, phenazines should also be recognized for their growth-promoting roles even in species that do not produce phenazines themselves. Below, we describe the context-dependent roles of phenazines in the interactions between phenazine-producing bacteria and their neighbors, whether they be other microbes (bacteria or fungi) or plant and animal host species.

3.4.1 Phenazines' Effects on Bacteria

P. aeruginosa-produced PYO is toxic to many diverse bacterial species that are found in very different environments, including mycobacteria, Gram-positive species, such as *Staphylococcus aureusaureus*, and other Gram-negative organisms, such as *E. coli* and *Vibrio cholerae* (Schoental 1941; Young 1947; Knight et al. 1979; Baron and Rowe 1981). PYO has a number of properties that contribute to its biological activity including its redox activity and its zwitterionic character that enables easy penetration of biological membranes. PYO can directly accept electrons from intracellular reductants, such as NADPH and NADH, and studies suggest that reduced PYO then transfers electrons to oxygen, resulting in the generation of toxic ROS such as superoxide and hydrogen peroxide (Hassett et al. 1992). Oxygen radicals have been implicated in PYO toxicity towards *E. coli* (Hassan and Fridovich 1980) and, in *P. aeruginosa* and *Bacillus subtilis,* SOD levels positively correlate with resistance to PYO (Hassan and Fridovich 1980; Hassett et al. 1995). However, across species, SOD activity and susceptibility to phenazines do not necessarily correlate with one another (Baron et al. 1989).

Several lines of evidence suggest that the other effects of phenazines on "target" cells may be strongly influenced by the organism's mode of metabolism at the time of phenazine exposure. First, PYO seems to short-circuit respiration as

evidenced by a restriction in oxygen consumption in target cells (Baron et al. 1989). Interestingly, inhibition of respiration is not limited to aerobically growing cells. Bacteria that are growing by nitrate respiration in the absence of oxygen are also inhibited by PYO. In contrast, bacteria growing via a fermentative metabolism are more resistant to phenazines (Baron and Rowe 1981). Second, the inhibition of growth by phenazines in conditions that support respiration of oxygen or nitrate may be due to the formation not only of damaging ROS, but also of reactive nitrogen species with toxic effects (Baron and Rowe 1981; Baron et al. 1989). It is not known if electrons within the ETC of target species can be transferred to phenazines, thereby facilitating or hastening the formation of ROS. Third, recent work shows that phenazines oxidize Fe-S clusters that are essential for the catalytic activity and/or conformation of numerous proteins (Gu and Imlay 2011). Phenazine-mediated oxidation of Fe-S clusters could impair critical protein functions in the cell. It is interesting to consider how some of the same electron transfer reactions that promote growth within oxygen-limited *P. aeruginosa* cultures can have very toxic effects on other bacterial species.

Phenazines can also have positive roles for neighboring bacterial species. In microbial fuel cells, a potential source of "green" energy (Rabaey et al. 2004), phenazines produced by *P. aeruginosa* act as soluble redox mediators, shuttling electrons and significantly increasing power output generated through bacterial metabolism. Phenazines may contribute to this by maintaining electrical conductivity throughout multilayered biofilms of heterologous bacterial species growing on the anode (Rabaey et al. 2005). This indicates that when microbes are in close proximity, phenazines may mediate interspecies metabolic interactions. In support of this hypothesis, *Enterobacter aerogenes* was found to use PYO produced by *P. aeruginosa* as an electron acceptor for respiration resulting in enhanced growth in a bioelectrochemical system setting. Secretion of the fermentation product 2,3-butanediol by *E. aerogenes* in turn enhances PYO production by *P. aeruginosa* (Venkataraman et al. 2011). In addition, phenazines stimulate iron reduction by phenazine non-producers (Hernandez et al. 2004). The ecological implications of this are intriguing: it would not be surprising if phenazines served as a "public good," as is known to be the case for siderophores (Harrison et al. 2008). While higher phenazine concentrations were found to correlate with a less diverse microbial community in a study of different CF patients, it is noteworthy that many of the organisms that persisted in the presence of high phenazine concentrations were anaerobes (Hunter et al. 2012). It would not be farfetched to speculate that while some members of the microbial community are harmed by phenazines, other organisms might benefit from phenazines over the progress of infection as lung chemistry co-evolves. It will be interesting to see whether this prediction withstands future experimental tests.

Phenazines may also have nucleic acid targets due to their propensity for intercalation. PYO, PCA, phenazine-1-carboxamide, and other phenazine derivatives bind non-specifically to double-stranded DNA (Hollstein and Van Gemert 1971). Phenazines can inhibit transcription to varying extents, and preliminary data indicate that this inhibition is due in part to DNA intercalation. Studies with

phenazine-1-carboxamide suggest that the effect is not due to inhibition of RNA polymerase (Hollstein and Van Gemert 1971). While this activity may have biological importance, it is important to note that phenazine affinity for DNA is rather weak compared to other DNA-complexing antibiotics (Hollstein and Van Gemert 1971).

In general, Gram-positive bacteria, such as staphylococci, appear to be more susceptible to phenazine toxicity than Gram-negative organisms. In an invertebrate model for *P. aeruginosa* infection, the presence of Gram-positive microbiome members, peptidoglycan fragments or GlcNAc activates pyocyanin production (Korgaonkar et al. 2013). Furthermore, the increased production of phenazines leads to reduction in the Gram-positive flora at the infection site, as well as increased virulence (Korgaonkar et al. 2013). The susceptibility of Gram-positive species suggests that some resistance might be conferred by the Gram-negative outer membrane, a structure known to play a role in antibiotic resistance (Baron and Rowe 1981). Interestingly, some non-pathogenic *Staphylococcus* species like *S. carnosus*, *S. pisciferm*entans, and *S. gallinarum* are resistant to PYO due to structural alterations in CydB, a component of the cyanide-insensitive cytochrome bd quinol oxidase (Voggu et al. 2006). The biological relevance of this resistance is not yet clear as these *Staphylococcus* spp. are not typically found in environments particularly known for high levels of *P. aeruginosa* or other phenazine-producing organisms, but these strains indicate the potential for organisms to have this type of resistance mechanism. Interestingly, *S. aureus* and other pathogenic *Staphylococci* that form co-infections with *P. aeruginosa* do not have this unusual form of PYO-resistant cytochrome system (Voggu et al. 2006). *S. aureus* does, however, have another mechanism for survival in the presence of PYO. In *P. aeruginosa*-*S. aureus* co-infections associated with CF, PYO-resistant *S. aureus* strains are recovered, and their resistance is due to defects in the electron transport chain (Kahl et al. 2003; Proctor et al. 2006; McNamara and Proctor 2006). It is not known whether the phenazines themselves provide the selection pressure for the appearance of respiration-defective staphyloccal mutants in the CF lung, or if other conditions such as the low oxygen content in the airway mucus drives their appearance (Worlitzsch et al. 2002). Whatever the cause, these adaptations appear to allow the coexistence of *S. aureus* with *P. aeruginosa* (Fig. 3.2, right panel), and the interactions between these two pathogens have been proposed to negatively affect disease outcome; CF patients co-infected with *P. aeruginosa* and *S. aureus* have more rapid progression of their disease, and clearance of *S. aureus* improves disease status (Marks 1990; Avital et al. 1995; Kahl et al. 2003).

3.4.2 Phenazines in Bacterial–Fungal Interactions

Due to rising concerns about the use of chemical pesticides in the 1980s, researchers became very interested in the antifungal properties of the phenazines produced by soil pseudomonads, and their potential uses in the control of fungal

diseases of plants. *P. fluorescens* 2–79 was one of the first strains whose purified phenazines were shown to have antifungal activity (Gurusiddaiah et al. 1986). This strain was isolated from the roots of wheat growing in a field where the wheat root disease "take-all" caused by *Gaeumannomyces graminis* var. *tritici* had spontaneously declined. It was found that *P. fluorescens* 2–79 colonizes the plant roots and suppresses growth of the pathogen (Shipton 1975; Weller and Cook 1983). In situ studies with phenazine-non-producing *P. fluorescens* or PCA-resistant isolates of *G. graminis* var. *tritici*, as well as inverse correlations between soil PCA levels with disease rates, have further established phenazines as important players in preventing fungal infections of plant roots (Fig. 3.1, left panel) (Gurusiddaiah et al. 1986; Thomashow and Weller 1988; Thomashow et al. 1990; Bull et al. 1991; Mazzola et al. 1992). Interestingly, studies with *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 2000) found that phenazine production alone is not sufficient for biocontrol. Bacterial mutants incapable of root colonization are also impaired in their ability to control tomato foot and root rot, despite the fact that they produce wild-type levels of phenazines. This study suggests that the colonization of the root enables the production of the right levels of these antifungal compounds in the right place; phenazines may only reach high enough local concentrations to cause pathogen killing if a biofilm is formed. Phenazines produced by organisms such as *P. fluorescens*, *P. chlororaphis*, and *P. aeruginosa* are able to inhibit a wide variety of phytopathogens (discussed further in the chapters on phenazines and biocontrol) and to shape rhizosphere communities. Because of this, researchers are also examining how phenazine-producing bacteria influence the beneficial mycorrhizal fungi. A recent study suggests that phenazine production is not necessarily harmful to the arbuscular mycorrhizal fungus-plant association (Dwivedi et al. 2009). Phenazine effects on a subset of fungi may protect niches for symbiotic fungi that confer a clear growth advantage to the plant.

Beside inhibiting growth of a variety of phytopathogenic fungi, *P. aeruginosa* and members of the *Burkholderia cepacia* complex inhibit in vitro growth of human fungal pathogens like *C. albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida lusitaniae*, *Candida parapsilosis*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, and others (Kerr 1994a, b; Rella et al. 2012). *C. albicans* and *A. fumigatus* are fungi commonly detected in association with humans (Amin et al. 2010; Leclair and Hogan 2010), and co-infections with *P. aeruginosa* and these fungi are frequently found in clinical infections–for example in mixed-species biofilms on catheters, ventilator tubing, and other devices, in wounds and burns, or in chronic lung infections associated with CF (Hughes and Kim 1973; Bauernfeind et al. 1987; Hermann et al. 1999; Gupta et al. 2005; de Macedo and Santos 2005; Chotirmall et al. 2010). The work of several groups shows or strongly suggests that *P. aeruginosa* limits *C. albicans* growth in vivo and may suppress *Candida* infections (Fig. 3.1, right panel) (Hughes and Kim 1973; Kerr 1994a; Burns et al. 1999; Leclair and Hogan 2010). Kerr et al. (1999) showed that PYO and 1-hydroxyphenazine produced by *P. aeruginosa* display antifungal activity against *Candida* spp., *Saccharomyces cerevisiae*, and *A. fumigatus*. While phenazine concentrations in CF respiratory sputum are below the reported minimal inhibitory

concentrations (MICs) for *C. albicans* and *A. fumigatus* (Wilson et al. 1988; Kerr et al. 1999; Hunter et al. 2012), local concentrations of phenazines may be substantially higher and thus may suffice to severely inhibit fungal growth or cause killing. Specific non-lethal effects of low concentrations of phenazines on fungi are discussed below.

Recently, new *P. aeruginosa* phenazines as well as phenazine modifications have been identified in the presence of fungi. Physical interactions between *C. albicans* and *P. aeruginosa* increase the release of PYO and cause the release of 5-methyl-phenazinium-1-carboxylate (5MPCA), a newly described secreted phenazine (Gibson et al. 2009; Morales et al. 2010). 5MPCA was the proposed intermediate in the conversion of PCA to PYO, and has not been found to accumulate in the supernatants of *P. aeruginosa* monocultures (Byng et al. 1963). 5MPCA is more toxic to *C. albicans* than PCA or PYO (Gibson et al. 2009). In the cytoplasm, 5MPCA covalently links to protein-associated cellular amines, thereby promoting its accumulation in the fungal cell. The redox activity of 5MPCA is maintained after covalent modification, and ROS generated by modified 5MPCA appear to play an important role in its toxicity (Morales et al. 2010). In contrast, PYO is not intracellularly modified, does not accumulate in fungal cells, and was less toxic than 5MPCA (Gibson et al. 2009; Morales et al. 2010). Recent studies showed that 5MPCA is also released in the presence of *A. fumigatus* and *S. cerevisiae* (Gibson et al. 2009; Morales et al. 2010; Moree et al. 2012). Using a combination of MALDI-TOF, MALDI-IMS, and MS–MS networking, Moree et al. (2012) were able to identify and visualize the complex molecular interplay between *P. aeruginosa* and *A. fumigatus*. The authors found not only 5MPCA in and around the fungal colony, but also showed that *A. fumigatus* biotransforms *P. aeruginosa* PCA into 1-hydroxyphenazine, 1-methoxyphenazine, and phenazine-1-sulfate. These compounds have distinct chemical properties, including enhanced toxicities and the ability to induce fungal siderophores (Moree et al. 2012).

In the same way that biofilm formation on plant roots is critical for phenazine-mediated protection against fungi, the development of mixed bacterial-fungal biofilms shapes phenazine production and transformation. For example, due to its instability in its unmodified form, 5MPCA is not freely detected in culture supernatants, but it represents a powerful antifungal agent in mixed-species communities where producer and target cells are in close proximity. 5MPCA, like other phenazines, enters *C. albicans* cells much more readily in its reduced form. In fact, environmental conditions created within fungal biofilms cause the reduction of an exogenously supplied 5MPCA-derivative PMS through unknown mechanisms (Morales et al. 2010).

The effects of phenazines on fungi are not limited to overt killing. In 1999, Kerr et al. reported that PYO repressed the transition of *C. albicans* to its invasive and adherent filamentous morphology, a transition that is important for virulence and biofilm formation (Lo et al. 1997; Mitchell 1988; Kerr et al. 1999). Recently it was shown that sub-lethal (low micromolar) concentrations of PCA, PMS, and PYO inhibit the fungal yeast-to-filament transition, intercellular adherence, and biofilm development (Morales et al. 2013). These *P. aeruginosa* phenazines inhibit *C.*

albicans respiration (Morales et al. 2013), and this observation is consistent with published data that phenazines can impact mitochondrial activity (French et al. 1973; O'Malley et al. 2003a, b). Subsequent analysis indicated that the decreased ability of *C. albicans* to develop wrinkled colonies (consisting of filaments and yeast forms) or robust biofilms on plastic was due to alterations in electron transport chain activity (Morales et al. 2013). Early studies with mammalian mitochondria showed that phenazines uncouple oxidative phosphorylation by shunting electrons from endogenous pathways (Armstrong and Stewart-Tull 1971; Armstrong et al. 1971; Stewart-Tull and Armstrong 1971), and this is most likely how respiration is inhibited in *C. albicans*. Furthermore, *S. cerevisiae* mutants with defects in proton gradient maintenance and respiration were among those more susceptible to PYO (Ran et al. 2003). Phenazine-mediated repression of *C. albicans* filamentation may occur through effects on ATP levels, as ATP is the precursor to cAMP, a second messenger that is a key positive regulator of hyphal growth (reviewed in Hogan and Sundstrom 2009). PYO reduces levels of both cAMP and its precursor ATP in human epithelial cells (Kanthakumar et al. 1993) due to PYO's effects on respiration and oxidative phosphorylation. Other signals generated by phenazine exposure, such as changes in intracellular redox status, generation of ROS, and binding to specific ligands also have the potential to impact key signaling pathways. Future experiments will determine if increases in cAMP are sufficient to restore hyphal growth in phenazine-treated cells.

In sputum samples collected from CF patients, *C. albicans* and *P. aeruginosa* are frequently found together (Chotirmall et al. 2010; Leclair and Hogan 2010), and it is interesting to consider how phenazines may shape their interactions. *P. aeruginosa* forms dense biofilms only on the surface of fungal hyphae, and not on the yeast forms, and biofilm formation allows for very high local concentrations of phenazines (including 5MPCA) and other factors that result in fungal death (Hogan and Kolter 2002; Bolwerk et al. 2003; Gibson et al. 2009). In bulk sputum, phenazine levels within CF sputum range between 5 and 80 μM (Hunter et al. 2012). These concentrations repress *C. albicans* filamentation and biofilm formation, but do not kill the fungus (Morales et al. 2013). In addition, some evidence suggests that phenazines might have a greater toxicity towards hyphal forms as compared to the yeast form of *C. albicans* possibly due to differences in the mitochondrial electron transport system. In 1982, Aoki and Ito-Kuwa showed that *C. albicans* yeast cells develop a cyanide-resistant respiration after aging while hyphal forms always only exhibit the usual form of cyanide-sensitive respiration (Aoki and Ito-Kuwa 1982) As the cyanide-resistant respiration of the phenazine-producer *P. aeruginosa* is also resistant to PYO, one could speculate that *C. albicans* yeast forms are more resistant to PYO than hyphal forms because of their ability to switch to a resistant electron transport system. Therefore, the change in *C. albicans* morphology in response to phenazines may, in part, explain how these two species co-exist. Indeed, a study by Bhargava et al. (1989) that assessed fungal morphology in the CF lung suggests that *C. albicans* exists only as yeast forms in CF airways (Fig. 3.1, right panel). In future clinical studies it will be interesting to see whether

some *C. albicans* filamentation can be observed in the lungs of CF patients when *P. aeruginosa* levels are decreased by antibiotic treatment.

Overall, phenazines are able to inhibit and kill a wide variety of pathogenic fungi, and secretion of phenazines by *Pseudomonas* spp. biofilms on roots has been shown to represent a critically important line of defense for plants against fungal pathogens. While antifungal properties against fungi have been rather well studied, their activities towards parasites are much less well characterized. PYO possesses antimalarial activity (Makgatho et al. 2000; de Andrade-Neto et al. 2004; Kasozi et al. 2011), and micromolar concentrations of PMS, a synthetic analogue of 5MPCA, are able to selectively kill *Trypanosoma cruzi*, *Toxoplasma gondii*, and *Leishmania mexicana amazonensis* while these parasites are inside of macrophages (Rabinovitch et al. 1982; Alves and Rabinovitch 1983; Chang and Pechere 1989). Future research is needed to determine if the mechanisms of action are similar to what has been described in fungi and bacteria. Indeed, one study by Nabi and Rabinovitch (1984) suggests that ROS are important for PMS-mediated killing of *L. m. amazonensis*.

3.5 Effects of Phenazines on Multicellular Eukaryotes

While phenazines are best known for their effects on other microorganisms, they can also impact multicellular organisms in both positive and negative ways. Phenazines can alter the dynamics between the phenazine producer and its host. In addition, phenazine effects on plants and animals may impact how these hosts interact with other, non-phenazine-producing microbial species.

3.5.1 Effects of Phenazines on Plants

Few negative effects of phenazines on plants have been reported. PCA and 2-hydroxyphenazine-1-carboxylic acid can be directly toxic to algae and higher plants (Toohey et al. 1965), and toxicity varies based on species. For example, plants are more sensitive to PCA, while its hydroxyl derivative is more potent against algae (Toohey et al. 1965). However, most plants greatly benefit from phenazine production by bacteria as phenazines can inhibit fungal and bacterial pathogens as discussed above, and in Chaps. 7–10 (Fig. 3.1, left panel). In addition to the previous examples, *Pantoea agglomerans* secretes the phenazine D-alanylgriseoluteic acid on apple flowers contributing to suppression of *Erwinia amylovora*, which causes fire blight disease (Giddens et al. 2003).

The benefits of phenazines for plants do not end with the suppression of harmful microbes. For example, phenazines produced by plant-associated *Pseudomonas* spp. may increase bacterial and plant access to iron and other nutrients associated

with soil mineral phases (Hernandez et al. 2004). Through mechanisms that have not yet been described, PYO produced by *P. aeruginosa* TO3 enhances the symbiosis between *Arachis hypogaea* L. (groundnut) and a rhizobial bacterial strain isolated from groundnut root nodules resulting in improved growth and productivity of groundnut (Khare and Arora 2011). PYO is also involved in the stimulation of the induced systematic resistance (ISR) in tomato and bean against the fungus *Botrytis cinerea* (Leeman et al. 1995; Audenaert et al. 2002). ISR is a plant defense mechanism induced by nonpathogenic biocontrol bacteria resulting in long-lasting resistance and protection against a broad range of pathogens (Fig. 3.2, left panel) (van Loon et al. 1998; Pieterse et al. 2001). In contrast to the wild type, a PYO-deficient mutant of *P. aeruginosa* is unable to induce systemic resistance against *B. cinerea* (Audenaert et al. 2002). A more recent study by De Vleesschauwer et al. (2006) shows that elevated H_2O_2 levels caused by PYO secreted by *P. aeruginosa* trigger ISR which results in the protection of rice roots or distal leaves against *Magnaporthe grisea*. However, increased ROS levels caused by PYO also resulted in enhanced susceptibility of rice to another fungal pathogen, *Rhizoctonia solani*, suggesting that ROS produced by the redox activity of PYO are a double-edged sword (de Vleeswschauwer et al. 2006). Phenazines can also affect the release of plant metabolites. For example, treatment of alfalfa roots with 200 μM PCA results in an increased total net efflux of amino acids by over 200 % in 3 h (Phillips et al. 2004), and those amino acids probably then serve as nutrients for the bacterial populations surrounding the roots (Fig. 3.2, left panel).

3.5.2 Phenazine-Mediated Killing of Nematodes

Nematodes that reside in the rhizosphere, like the root-knot nematodes of the genus *Meloidogyne,* infect plants causing decreased yield and plant death. Interestingly, *Pseudmonas oryzihabitans* colonization of the plant roots was shown to decrease plant damage by these nematodes (Vagelas et al. 2007). This inhibition is probably at least in part due to phenazine production as another study showed that phenazines produced by *P. chlororaphis* completely suppressed the hatching of *M. incognita* juveniles (Kavitha et al. 2005). Furthermore, the soil nematodes *R. similis* and *C. elegans* are also sensitive to phenazine-mediated killing by *P. aeruginosa* (Fig. 3.1, left panel). PYO, 1-hydroxyphenazine, and PCA are capable of killing nematodes in a matter of hours and seem to exert their toxic effect through the generation of ROS (Mahajan-Miklos et al. 1999; Cezairliyan et al. 2013). Interestingly, Cezairliyan et al. (2013) showed that 1-hydroxyphenazine is toxic over a wide pH range, whereas PCA is active at low pH and PYO at high pH. Similar condition-dependent toxic activities of phenazines have been shown for PCA (active at low pH) and phenazine-1-carboxamide (active at high pH) produced by *P. fluorescens* 2–79, *P. aureofaciens* 30–84, and *P. chlororaphis* (Brisbane et al. 1987; Chin-A-Woeng 1998).

3.5.3 Phenazines in Human Disease

Many studies have characterized the toxic effects of phenazines on the mammalian lung and a few excellent recent publications review this data in great detail (Lau et al. 2004; Liu and Nizet 2009; Rada and Leto 2013). Here, we restrict our discussion to how phenazines may impact the course of mixed-species infections.

Above, we described how phenazines may shape chronic infections associated with the genetic disease CF focusing on bacterial–bacterial and bacterial–fungal interactions. In addition to a direct role for phenazines in microbe–microbe interactions, phenazines may also alter the growth and survival of co-infecting microbes through effects on the host in a number of ways (Fig. 3.1, right panel). First, phenazine-generated ROS and inhibition of host catalase activity by PYO (O'Malley et al. 2003a, b) may lead to the suppression of ROS-sensitive species and provide a competitive edge to those microbes that tolerate ROS well. Second, PYO exposure of the epithelium results in mucin hypersecretion (Rada et al. 2011), which likely changes the local lung environment in a number of ways including the creation of low oxygen niches, protecting bacteria from immune clearance, and altering the nutrient availability profile. Increased mucus secretion can also promote the growth of microbial species (Rada et al. 2011). Third, PYO and PCA have been shown to modulate the host immune response by upregulating the expression of the proinflammatory cytokine, interleukin-8 (IL-8) (Denning et al. 1998a, b; Leidal et al. 2001). IL-8 is a very potent chemokine capable of recruiting neutrophils and, together with a number of other proinflammatory mediators induced by PYO (Rada et al. 2011), results in uncontrolled neutrophil infiltration in CF lungs (Lauredo et al. 1998; Sadik et al. 2011; Ratner and Mueller 2012). At the same time, however, PYO inhibits superoxide production by neutrophils (Miller et al. 1987) and induces their apoptosis. PYO-induced apoptosis is triggered by increased ROS and subsequent decrease in intracellular cAMP (Usher et al. 2002). These effects seem to be neutrophil-specific since it was not observed in monocyte-derived macrophages and airway epithelial cells (Usher et al. 2002). In addition, PYO inhibits the release of IL-2, thus limiting proliferation of T-lymphocytes and secretion of immunoglobulin by B-lymphocytes (Muhlradt et al. 1986). These complex effects on the immune response broadly affect levels of microbial killing or may impact the clearance of particular species (Usher et al. 2002; Allen et al. 2005). Fourth, PYO negatively affects levels of CFTR, the CF transmembrane conductance regulator, and decreased CFTR activity may lead to disruption of normal microbial clearance pathways (Kong et al. 2006). The effects on CFTR may also be due to decreased ATP/cAMP levels (Stewart-Tull and Armstrong 1971; Kanthakumar et al. 1993; Usher et al. 2002; Schwarzer et al. 2008) which regulate the expression and trafficking of CFTR. Fifth, decreased levels of ATP and cAMP have also been shown to affect calcium homeostasis (Denning et al. 1998a, b) and ciliary motility (Wilson et al. 1988; Jackowski et al. 1991; Kanthakumar et al. 1993, 1996). Sixth, phenazines inhibit the dual oxidase-based antimicrobial system involved in microbial killing (Schwarzer et al. 2008;

Rada et al. 2008). Lastly, it is possible that phenazine-linked changes to the redox potential and other chemical attributes of mucus, such as the iron oxidation state, may influence which microbial species thrive in this environment. The net impact of these myriad effects of phenazines on acute and chronic infections is not yet understood, but how the environment and the cells within it co-evolve is an important area for future research.

3.6 Conclusions and Future Directions

In summary, phenazines are versatile compounds that impact both single-species populations and interspecies interactions in both positive and negative ways (Fig. 3.1). In some cases, the same chemical role for phenazines (such as ROS generation or NAD(P)H oxidation) can have different consequences in different species, between strains within a species (Cugini et al. 2010), or within a strain under different conditions (Wang et al. 2010). The complexities increase as one considers that the spectrum of phenazines produced is altered by environmental conditions and by the presence of other species, as well as by the host, and that a given phenazine has different activities under different conditions (such as pH or oxygen availability). Furthermore, the effects of phenazines vary based on concentration and thus distance from phenazine-producing species (Gibson et al. 2009; Morales et al. 2013). Together, these findings highlight the need to assess the roles of phenazines in situ or in vivo in order to truly understand their importance in different biological processes. Existing biochemical and genetic approaches are useful for deciphering how phenazines may affect diverse cell types, and the development of new technologies, such as those at the interface between imaging and chemistry, hold promise in helping us determine the extent to which these powerful (and colorful) chemicals shape their environment—from the roots of plants to infected tissues, and the myriad microbes in between.

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Chapter 4
Phenazines and Bacterial Biofilms

Dmitri V. Mavrodi and James A. Parejko

Abstract Most bacteria in the environment exist in biofilms—structured, surface-attached multicellular communities that are enmeshed in a self-produced polysaccharide matrix. Biofilms allow bacteria to participate is social interactions, survive under harsh conditions and successfully resist antimicrobials, invasion by competitors, predation, and destruction by components of the immune system. Fluorescent *Pseudomonas* spp. are prolific biofilm formers and some members of the genus have become model organisms for the study of biofilm biology. Several economically important groups of pseudomonads produce phenazines, pigmented, redox-active metabolites that have long been recognized for their broad-spectrum antibiotic activity. The current chapter focuses on the emerging close link between phenazine production and biofilm formation in *Pseudomonas* spp., and on the important role of phenazines in biofilms associated with human infectious diseases and highly competitive environmental niches such as soil and the plant rhizosphere.

4.1 Introduction

Bacteria in the environment rarely lead a planktonic lifestyle and more often form "biofilms"—structured, surface-attached multicellular communities that are enmeshed in a self-produced polysaccharide matrix (Hall-Stoodley et al. 2004). Recent research in the area of biofilm biology has revealed that biofilm formation is an ancient and integral component of the prokaryotic lifestyle and has even led

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to the hypothesis that the planktonic cell phenotype has evolved as a dispersion mechanism in biofilm communities (Stoodley et al. 2002). Recent studies have demonstrated that mature biofilms are highly resilient to environmental stresses and play an important role in the prolonged survival of bacteria under harsh conditions (Flemming and Wingender 2010). Biofilms allow bacteria to successfully resist antimicrobials (Mah and O'Toole 2001), invasion by competitors (Burmolle et al. 2006), predation (Matz et al. 2008), and destruction by components of the immune system (Bordi and de Bentzmann 2011). Finally, biofilms also facilitate co-metabolic interactions and the exchange of genetic material via horizontal gene transfer, thus giving bacteria the flexibility to rapidly adapt to a changing environment. These findings have led to an enhanced appreciation of the importance of biofilms in both environmental and clinical settings.

The application of modern imaging and molecular techniques over the past three decades has revealed the structural and developmental complexity of bacterial biofilms. It is now well accepted that the establishment of biofilms involves sophisticated cell-to-cell signaling and regulatory networks that govern the process of biofilm development in response to environmental stimuli (Stewart and Franklin 2008). Generally, biofilm formation follows a defined pathway that starts with reversible attachment of planktonic cells to a surface due to simple surface attraction forces and the action of outer membrane assemblages (i.e., pili or fimbriae). In the second stage, the biofilm develops into an irreversibly bound bacterial community as a result of the production of exopolysaccharides and other secreted biopolymers (Flemming and Wingender 2010). Later stages involve biofilm maturation and are characterized by structural and architectural changes in the biofilm matrix. The process of biofilm maturation is accompanied by an increase in nutrient, metabolite, and gaseous gradients, which creates increasingly stressful microenvironments. The bacteria respond to changes in local environmental conditions by altering gene expression patterns and physiological activity (Stewart and Franklin 2008). Stressful conditions in mature biofilms also generate genetic diversity and lead to the enrichment of new subpopulations that can better survive in a given microenvironment (McDougald et al. 2008; Bordi and de Bentzmann 2011). The final stage is characterized by a transient return to motility and the release of planktonic cells into the environment, leading to biofilm dispersion.

Pseudomonas spp. are prolific biofilm formers and some phenazine-producing (Phz^+) members of the genus have become model organisms for the study of biofilm biology. In particular, *Pseudomonas aeruginosa* is currently used as an important model system for understanding the molecular basis of sessile growth and processes involved in bacterial biofilm development, physiology, and adaptation in human opportunistic pathogens. Among saprophytic Phz^+ species, *Pseudomonas chlororaphis* has increasingly been explored as a model to study the establishment of surface-attached communities in non-clinical environments. The current review focuses on the emerging tight link between phenazine production and biofilm formation in *Pseudomonas* spp. and the important role of phenazines in biofilms associated with human infectious diseases and highly competitive environmental niches such as soil and the plant rhizosphere.

4.2 Biofilms and Phenazine Production in Fluorescent *Pseudomonas* spp.

The tight connection between phenazine production and the development of biofilms was first revealed in *P. chlororaphis,* where mutations in regulatory and structural phenazine (*phz*) genes caused deficiency in biofilm formation (Maddula et al. 2006; Selin et al. 2010). Conversely, the constitutive expression of the phenazine biosynthesis pathway positively affected sessile growth and correlated with premature formation of biofilms. The identity and ratio of phenazine compounds also influenced biofilm architecture and dispersal rates in flow cells, suggesting that individual phenazines produced by *P. chlororaphis* might play different functional roles in surface adhesion and the development of mature biofilms (Maddula et al. 2008) (see more below). Similar studies with phenazine-deficient mutants of *P. aeruginosa* and species of the *Pseudomonas fluorescens* complex provided further support of the role of phenazines in biofilm formation. In *P. aeruginosa*, the loss of phenazine production did not affect the ability to adhere to surfaces (Ramos et al. 2010). However, the Phz^- mutant had higher swarming motility and in flow cell experiments formed biofilms with altered architecture (thinner and higher surface-to-volume ratio), leading the authors to suggest that phenazines in *P. aeruginosa* are important primarily for the process of biofilm maturation. The Phz^- mutant also formed highly rugose colonies, but readily reverted to the wild-type smooth phenotype on media supplemented with phenazines. As in *P. chlororaphis*, the identity and amounts of amended phenazines affected the morphology of *P. aeruginosa* in colony biofilms (Ramos et al. 2010).

We recently conducted experiments with phenazine-nonproducing mutants of several species of the *P. fluorescens* complex (Parejko et al. 2013) and observed that the ability to produce phenazine-1-carboxylic acid (PCA) influenced biofilm formation by some, but not all, Phz^+ strains. The effect of PCA also varied with the degree of matric or osmotic stress (Parejko, Mavrodi, Thomashow, unpublished data). For example, we found that in the presence of upwards of 100 μg ml^{-1} of exogenous PCA, biofilm formation by Phz^- mutants was restored to wild-type levels in *Pseudomonas orientalis*, but not in *Pseudomonas cerealis*. Ultrastructural characteristics of colony biofilms grown under control and stress conditions also differed among members of the *P. fluorescens* complex, and in some cases, between wild-type Phz^+ strains and their isogenic Phz^- mutants.

To summarize, the production of phenazines is universally linked with the process of biofilm formation in all Phz^+ *Pseudomonas* spp. studied to date. However, the exact mechanisms by which phenazines influence the dynamics of surface-attached growth is often affected by environmental conditions and appears to be species-specific. Recent findings, discussed in more detail below, strongly suggest that the observed link between biofilms and phenazines is due to the unique redox-cycling properties of these metabolites and their capacity to act as endogenous electron shuttles in biofilms formed by *Pseudomonas* spp.

4.3 Regulation of Biofilm Formation in *P. aeruginosa* and Role of Phenazines as Molecular Signals

Biofilm formation is an important defense response employed by many bacteria to survive abiotic and biotic stresses (Lopez et al. 2010). The transition from the planktonic lifestyle to surface attachment and the buildup of multicellular communities encased in an extracellular polymeric matrix consisting of exopolysaccharides, lipopolysaccharides, DNA, and proteinaceous attachment fibers is tightly regulated. Phenazine-producing *Pseudomonas* spp., and in particular the opportunistic human pathogen *P. aeruginosa*, are among the best understood model systems in terms of molecular signals and pathways regulating the switch from the planktonic to the attached form of growth. In *P. aeruginosa*, regulatory mechanisms governing the transition from planktonic to sessile modes of growth are complex and include sensing and integration of environmental signals via two-component systems, extracytoplasmic function (ECF) pathways, and regulatory pathways that rely on secondary messengers and quorum sensing (QS) signals.

The two-component GacS/A system forms part of the Gac/Rsm pathway, which plays a fundamental role in controlling the production of virulence factors, exoproducts and the formation of biofilms in *P. aeruginosa*. It is thought that the membrane-bound histidine kinase GacS senses an unidentified environmental signal and activates the cognate response regulator protein GacA. Once activated, the GacS/A two-component system activates the transcription of two small untranslated RNAs (sRNAs), RsmY, and RsmZ, which sequester the mRNA-binding protein RsmA (Kay et al. 2006; Brencic et al. 2009). RsmA is a translational regulatory protein that, in the absence of RsmY and RsmZ, represses expression of multiple genes by binding to the untranslated 5′ ends of target mRNAs and occluding ribosome binding sites (Lapouge et al. 2008). The function of the GacS/A system is further modulated by several accessory regulators that exert both positive and negative effects on the expression of the *rsm* genes. The sequestration of RsmA by sRNAs represses flagellar motility and the production of acute virulence factors, and facilitates the synthesis of Pel and Psl exopolysaccharides, which are important components of the *P. aeruginosa* biofilm matrix (Burrowes et al. 2006; Brencic and Lory 2009).

Signaling pathways that rely on the secondary messengers bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) and adenosine 3′-5′-cyclic monophosphate (cAMP) coordinately regulate virulence and the transition between planktonic and surface-attached lifestyles in *P. aeruginosa* in response to adaptation to novel habitats (McDougald et al. 2008; Romling 2012). Cyclic di-GMP signaling controls fundamental physiological processes such as transcription, translation, post-translational events, and proteolysis, which in turn affect a wide range of highly complex phenotypes including pathogenesis, secretion, motility, production of secondary metabolites, stress adaptation, and biofilm formation. The cellular levels of c-di-GMP are regulated by diguanylate cyclases and phosphodiesterases that, correspondingly, produce and break the secondary messenger, and

the genome of *P. aeruginosa* encodes multiple proteins with predicted diguanylate cyclase and phosphodiesterase activity (Kulasakara et al. 2006). Cyclic di-GMP plays a critical role in biofilm formation, and its accumulation positively regulates production of the matrix expolysaccharides (Hickman et al. 2005; Lee et al. 2007; Merighi et al. 2007; Mikkelsen et al. 2011), surface adhesins (Borlee et al. 2010), and surface appendages that are important for different stages of biofilm formation such as the Cup fimbriae (Meissner et al. 2007) and type IV pili (Jain et al. 2012).

Cyclic AMP is another secondary messenger in *P. aeruginosa* and is produced by the enzymatic activity of two adenylate cyclases. Through allosteric activation of the transcriptional factor Vfr, cAMP influences expression of a large group of genes involved in the production of virulence factors including type II and III protein secretion systems, secreted toxins, and degradative enzymes (Wolfgang et al. 2003; Smith et al. 2004). Although cAMP is generally recognized in *P. aeruginosa* as a key mediator of the acute virulence phenotype and planktonic lifestyle, some lines of evidence suggest the importance of cAMP/Vfr regulation for biofilm formation, particularly for initial surface attachment during the transition to the sessile lifestyle. The effect is probably mediated by the capacity of the cAMP/Vfr system to activate the production of type IV pili and components of QS systems (Beatson et al. 2002; Wolfgang et al. 2003; Croda-Garcia et al. 2011; Coggan and Wolfgang 2012).

Quorum sensing is a form of bacterial communication that uses diffusible molecules known as autoinducers to sense population density and coordinate population-wide gene expression. QS regulation in *P. aeruginosa* involves two *N*-acylhomoserine lactone (HSL)-based systems, Las and Rhl, and a third system based on the *Pseudomonas* quinolone signal 2-heptyl-3-hydroxy-4-quinolone (PQS) (Dubern and Diggle 2008). Although the relationship between QS and biofilm formation has not been fully elucidated, there is evidence that QS plays an important role in the process. It has been demonstrated that Las-deficient mutants form abnormal flat biofilms that are more susceptible to antibiotic treatment (Davies et al. 1998). Similarly, biofilm architecture was affected in mutants deficient in the production of rhamnolipids, a process controlled in *P. aeruginosa* by the Rhl quorum system (Davey et al. 2003). Finally, both Las and Rhl QSs have been shown to influence the expression of *pel* genes that are involved in biosynthesis of the biofilm matrix (Sakuragi and Kolter 2007).

It has become increasingly clear that in *P. aeruginosa,* the production of phenazines is connected with the formation of biofilms and adaptation to diverse environments. *P. aeruginosa* has two almost identical seven-gene phenazine biosynthesis operons, *phz*1 and *phz*2, that have very distinct promoter regions (Mavrodi et al. 2001). The expression of the phenazine operons is differentially controlled by HSL- and quinolone-based QSs in relation to the transition between the planktonic and sessile modes of growth. The *phz*1 operon is associated with genes (*phzM* and *phzS*) that are involved in the conversion of PCA to pyocyanin. The expression of the *phz*1 operon is regulated by a temporal cascade formed by

the Las, Rhl, and quinolone QSs and is mediated by the PQS-binding transcriptional regulator PqsR (Whiteley et al. 1999; Deziel et al. 2005; Xiao et al. 2006). A recent study by Recinos et al. (2012) revealed that the *phz*1 operon is expressed during planktonic growth, whereas the second phenazine operon, *phz*2, is expressed primarily in biofilms. The authors hypothesized that the differential regulation of *phz*1 and *phz*2 during the planktonic versus the biofilm mode of growth is driven by the response of *P. aeruginosa* to oxygen. The expression of *phz*2 was dependent on 2-heptyl-4-quinolone (HHQ), an immediate precursor of PQS, and contributed to host colonization by *P. aeruginosa* in a murine model of infection. Interestingly, phenazines themselves also act as molecular signals downstream of the PQS system and affect the expression of a small subset of genes in *P. aeruginosa* (Dietrich et al. 2006). Phenazine-mediated signaling is linked with biofilm formation by a yet unknown mechanism, since PCA represses swarming and stimulates biofilm formation in *P. aeruginosa* (Ha et al. 2011).

A recent review by Coggan and Wolfgang (2012) argued that the planktonic and sessile environmental lifestyles of *P. aeruginosa* parallel the acute and chronic infection phenotypes observed in humans infected by this bacterium. During the environmental planktonic phase, free-living *P. aeruginosa* cells rely on virulence factors that are controlled by cAMP/Vfr signaling to defend themselves against eukaryotic predators (Hilbi et al. 2007). In this growth phase, *P. aeruginosa* expresses surface appendages that help to evade predation, but also promote surface attachment and the establishment of sessile communities that quorum sense and produce toxins and degradative enzymes for additional protection against predators and competitors. Many of these same virulence factors are produced and contribute to tissue damage, sepsis, and bacterial dissemination observed during acute *P. aeruginosa* infections. A review of published literature suggests that phenazines are actively produced by *P. aeruginosa* in saturated planktonic cultures, in immature biofilms, or during acute infections. The production of phenazines clearly requires QS, and pyocyanin and PCA were identified as important pathogenicity factors contributing to the ability of *P. aeruginosa* to infect and kill different protozoan and non-mammalian organisms (Rahme et al. 1997, 2000; Mahajan-Miklos et al. 1999; Lau et al. 2003; Cezairliyan et al. 2013).

What happens to phenazine production in mature biofilms is less clear. As biofilms mature and bacteria further adapt to the sessile lifestyle, the virulence pathways ultimately become repressed, and c-di-GMP and Gac/Rsm signaling activate the synthesis of the biofilm matrix. This stage resembles the non-cytotoxic, mucoid, and antibiotic-resistant phenotypes often associated with chronic human infections caused by *P. aeruginosa*. There is ample experimental evidence that phenazines are produced by *P. aeruginosa* in patients with cystic fibrosis (CF) and other chronic pulmonary diseases, and that pyocyanin contributes to the persistent inflammation and pathological changes observed in the infected airways (Wilson et al. 1988; Denning et al. 1998; Lau et al. 2004a, b; Caldwell et al. 2009). There is also a report documenting the abundance of isolates that overproduce pyocyanin in

sputa of CF patients with chronic infection (Fothergill et al. 2007). On the other hand, small colony variant (SCV) isolates commonly recovered from patients with chronic pulmonary infection do not quorum sense by PQS or produce pyocyanin (Wei et al. 2011). This is in agreement with results of a recent study by Hunter et al. (2012), who measured phenazines in the sputum of CF patients and demonstrated that patients with the highest loads of phenazine harbored isolates of *P. aeruginosa* that produced pyocyanin poorly. However, poor pyocyanin production was compensated by the high cell density of *P. aeruginosa* in the infected airways.

4.4 Phenazines and the Physiology of Biofilm Growth in *P. aeruginosa*

A number of exciting recent studies have revealed that phenazines play an important role in the biology of surface-attached growth in fluorescent *Pseudomonas* spp. This phenomenon is directly linked to the redox-cycling properties of phenazine compounds. High levels of phenazines are toxic to most organisms, as they upset intracellular redox homeostasis by inducing the accumulation of reactive oxygen species, depleting-reduced glutathione, and triggering apoptosis (Jacob et al. 2011). In phenazine-nonproducing microorganisms such as *Escherichia coli*, the defense response to redox stress is mediated by the transcriptional regulators SoxR and SoxS, which control the expression of more than 100 genes whose primary function is to protect the cell against the toxicity of exogenous redox-cycling agents (Gu and Imlay 2011). As discussed above, exposure to phenazines also affects gene expression in Phz^+ *Pseudomonas* spp., and the mechanism of this cellular response also involves a SoxR homolog (Dietrich et al. 2006). However, in contrast to *E. coli*, the SoxR regulon of *P. aeruginosa* includes genes encoding an RND type efflux pump, an MFS transporter, and a monooxygenase. Similar types of genes were upregulated by SoxR in a strain of *Streptomyces coelicolor* that produces the redox-active polyketide actinorhodin (Shin et al. 2011). These studies suggest that the primary function of the SoxR response in *Pseudomonas* and *Streptomyces* is not defense from redox stress, but rather the proper shuttling of phenazines and other redox-active secondary metabolites (Dietrich and Kiley 2011).

Phenazines can act as electron carriers between the bacterium and an external substrate, and can react extracellularly with higher potential oxidants such as ferric iron and oxygen (Wang and Newman 2008). Pseudomonads are strict aerobes, and it has long been postulated that under certain conditions, redox-active phenazines function in these organisms as accessory "respiratory pigments" (Friedheim 1931; Trutko et al. 1988). Recent studies in *P. aeruginosa* have confirmed this hypothesis by revealing the capacity of phenazines to act as electron acceptors for the reoxidation of NADH that accumulates under conditions of oxygen limitation during the biofilm mode of growth. A recent study by Koley et al. (2011) inves-

tigated chemical gradients and the redox status of metabolites in submerged biofilms of *P. aeruginosa* using a noninvasive technique called scanning electrochemical microscopy. The authors demonstrated that *P. aeruginosa* biofilms are surrounded by a 400-micron-thick layer of reduced pyocyanin called the "pyocyanin electrocline." Under electron acceptor-limiting conditions, the electrocline formed in wild-type biofilms but not in biofilms of a cytochrome bc1 mutant that lacked the ability to reduce pyocyanin. The electrocline also rapidly collapsed when the biofilm was flooded with an alternative electron acceptor such as nitrate. The pyocyanin electrocline was not affected by nitrate in a nitrate reductase mutant that does not reduce NO_3^- under aerobic conditions (Koley et al. 2011). Finally, the importance of phenazines for the maintenance of redox homeostasis in colony biofilms formed by *P. aeruginosa* was demonstrated by Dietrich et al. (2013). The authors revealed that colony biofilms experience steep gradients in oxygen availability and that phenazines promote survival of cells in deeper anoxic layers of the biofilm. Furthermore, redox perturbations associated with shifts in the availability, production, and utilization of electron acceptors (including phenazines) acted as a major mechanism that drives the morphogenesis of *P. aeruginosa* colonies.

It is known that elevated levels of iron are among the factors that trigger the transition from planktonic to biofilm growth (Banin et al. 2005; Berlutti et al. 2005; Patriquin et al. 2008). A recent study by Wang et al. (2011) explained a molecular mechanism linking iron acquisition with biofilm formation and phenazine production in *P. aeruginosa*. That study revealed that a combination of ferric iron and PCA restored biofilm formation in siderophore mutants of *P. aeruginosa* that normally cannot form biofilms. The effect was mediated by the Fe^{2+} uptake protein FeoB, thus suggesting that phenazines promote the reduction of ferric iron and sequestration of the resultant Fe^{2+} by the bacteria. The results are in agreement with the findings of Koley et al. (2011), who reported the accumulation of soluble iron in the medium surrounding the biofilm upon formation of the pyocyanin electrocline. Taken collectively, these results further support earlier observations that, under aerobic conditions, phenazines can reduce Fe^{3+} to Fe^{2+} and provide an important additional source of iron for *P. aeruginosa* (Wang and Newman 2008; Wang et al. 2011).

4.5 Role of Phenazines in Biofilms Formed by Saprophytic *Pseudomonas* spp.

The plant root, or rhizosphere, microecosystem serves as a carbon and energy source for soil-dwelling fluorescent *Pseudomonas* spp. Motile cells move toward the root by root exudate-based chemotaxis and/or come into contact with the root as it grows through the soil profile. Depending on the relative competitiveness of the microbes, successful root colonization may occur (Lugtenberg et al. 2001;

Buchan et al. 2010). The exact determinants of colonization likely depend in part upon soil type, plant root structure, and exudate composition, but the production of surface adhesins like pili, fimbriae, flagella, and O-antigen lipopolysaccharides contribute to the process of bacterial colonization by promoting reversible cellular attachment to the root (Vesper 1987; Lugtenberg et al. 2001). When successful colonization occurs, the competency of microbes in the rhizosphere is highly dependent on the utilization of root exudates (Lugtenberg and Dekkers 1999). Once cell-to-root attachment has occurred, polysaccharide biosynthesis initiates a phase of irreversible cellular attachment that structurally resembles a biofilm (Danhorn and Fuqua 2007). Throughout both phases of attachment, social coordination by QS allows cells to aggregate into microcolonies and regulate specific population-wide processes (Danhorn and Fuqua 2007). Many of the phenotypes involved in rhizosphere colonization and competition are also regulated by the global two-component GacA/GacS system although its influence on biofilm formation and rhizosphere competency is still unclear. As a result of biofilm-like microcolony establishment on plant roots, populations of Phz$^+$ *Pseudomonas* spp. are likely more competitive for limited available resources in the rhizosphere niche and are more resilient to abiotic disturbances like limited water availability.

In Phz$^+$ *Pseudomonas* spp. associated with plant hosts, colonization (Mazzola et al. 1992), and biofilm formation are profoundly affected by phenazines *in vitro* and *in planta*. Presently, much of what is known about biofilm formation in plant-associated and Phz$^+$ *Pseudomonas* spp. has been studied in strains of *P. chlororaphis*. The impact of phenazines on biofilm establishment and development on abiotic surfaces in addition to plant seeds and roots was first described in *P. chlororaphis* subsp. *aureofaciens* strain 30-84 (Maddula et al. 2006), a strain that was isolated from wheat roots and has biocontrol activity toward soilborne plant pathogens (Pierson and Pierson 2010). Strain 30-84 produces three phenazines, PCA, 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA), and 2-hydroxyphenazine (2-OH-PHZ), which play distinct roles in biofilm dynamics and architecture (Maddula et al. 2008). For example, a mutant of 30-84 (30-84PCA) that produced only PCA formed thicker and more structured biofilms compared to those formed by wild-type 30-84, which produced PCA and 2-OH-PCA (Maddula et al. 2008). Mutant derivatives of wild-type strain 30-84 producing only PCA produced cell-dense biofilms with more heterogeneous structure that were less prone to dispersion forces than biofilms composed of cells producing wild-type levels of PCA, 2-OH-PCA, and 2-OH-PHZ (Maddula et al. 2008). Furthermore, a 2-OH-PCA overproducing derivative of wild-type 30-84 (30-84O*) reached the irreversible attachment stage earlier than the wild-type or the strain producing only PCA, suggesting a distinct role for 2-OH-PCA in attachment and biofilm structural progression (Maddula et al. 2008).

Biofilm formation by other plant-associated *P. chlororaphis* strains is also highly dependent upon phenazine biosynthesis. The phyllosphere-colonizing *P. chlororaphis* strain PA23, which shows biocontrol activity toward Sclerotinia stem

rot caused by *Sclerotinia sclerotiorum* (Savchuk and Fernando 1994), forms a biofilm *in vitro* with influence from endogenous PCA and 2-OH-PHZ (Selin et al. 2010). Cell physiological state as a function of intracellular levels of guanosine phosphates, or (p)ppGpp, is intimately linked to the expression of specific stationary phase genes aided by the sigma factor RpoS due to nutrient deprivation. This physiological change is known as the stringent response and it plays a significant role in strain PA23 *phz* gene expression and phenazine production through RpoS (Selin et al. 2010; Manuel et al. 2012), but not other stringent response genes. The importance of RpoS to phenazine production was also established in phenazine-1-carboxamide (PCN)-producing *P. chlororaphis* strain PCL1391 (Girard et al. 2006), however specific regulatory networks may be strain- and niche-specific. In colonizing the nutrient-limited phyllosphere, phenazine stringent response regulation would provide tight control over factors necessary for resource competition and biofilm development. In *Pseudomonas* spp. colonizing plant surfaces in nature, the relative impact of phenazines on colonization and survival as a function of biofilm development varies with strain and ecological source. However, the evolutionary and physiological role of phenazines in establishing and maintaining microbial communities appears largely conserved.

P. aeruginosa is an important opportunistic pathogen of humans and animals and its population biology has been the subject of multiple studies (Wiehlmann et al. 2007; Pirnay et al. 2009; Selezska et al. 2012). In contrast, the large-scale ecology of saprophytic Phz$^+$ *Pseudomonas* spp. until recently remained poorly understood. The topic has recently been examined in detail in a series of studies focused on indigenous antibiotic-producing communities from the rhizosphere of wheat grown in the inland Pacific Northwest, USA. The low-precipitation zone of the Pacific Northwest encompasses 1.56 million cropland hectares in central Washington State and northern Oregon that are farmed almost exclusively to wheat (Schillinger and Papendick 2008). The area receives only 150–350 mm of precipitation annually and represents an ideal agroecosystem to test on a large scale how management practices and climate impact the populations of biocontrol agents. Over a period of 4 years, the region was extensively surveyed for the presence of indigenous antibiotic-producing rhizobacteria using a combination of traditional and culture-independent approaches. Results of the survey revealed the widespread abundance of indigenous phenazine-producing *Pseudomonas* spp. in the rhizosphere of field-grown cereals (Mavrodi et al. 2010; Parejko et al. 2012). The indigenous Phz$^+$ *Pseudomonas* community exhibited extensive genetic diversity, with at least four distinct genetic species (Parejko et al. 2013). Two of these species were provisionally described as novel and named *P. cerealis* and *Pseudomonas aridus.* Phenazine-nonproducing mutants of these species, like those of *P. aeruginosa* and *P. chlororaphis*, gave rise to colony biofilms altered in morphology (Fig. 4.1). Most interestingly, Phz$^+$ pseudomonads were predominantly associated with dryland cereals and their population levels and plant col-

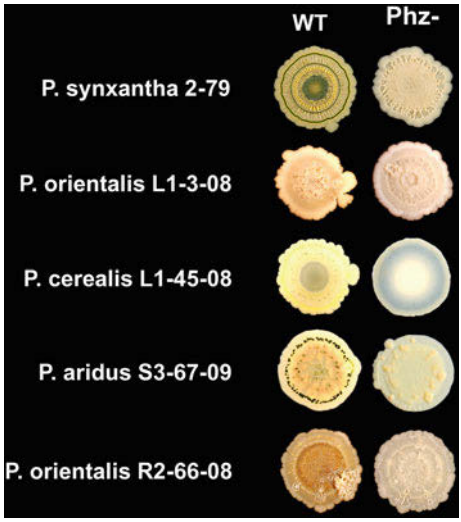

Fig. 4.1 The effect of phenazine production on colony morphology in PCA-producing Pseudomonas strains from the rhizosphere of wheat

onization frequency were markedly reduced under higher annual precipitation and in irrigated fields (Mavrodi et al. 2012a, b). Roots of dryland wheat also contained microgram amounts of PCA, suggesting that Phz^+ rhizobacteria were highly active and thriving in the arid soils of the Inland Pacific Northwest.

The amount of water available for cells in the environment is a function of the water potential (Ψ), which is expressed as a negative value, where $\Psi = 0$ is a fully hydrated environment. Water potential is the sum of six components that include a reference correction (Ψ_0), the solute potential (Ψ_π), the pressure potential (Ψ_p), the gravimetric potential (Ψ_s), the potential due to humidity (Ψ_v), and the matric potential (Ψ_m) (Or et al. 2007). On plant roots growing in most non-saline soils, the overwhelming component contributing to the water potential is Ψ_m, with some positive influence from Ψ_π in saline soils and a negative influence from Ψ_p in the root zone due to the hydraulic pull of water into the plant roots. Furthermore, in unsaturated soil, as water content decreases, the influence of Ψ_m and Ψ_π on bacterial cells increases drastically (Or et al. 2007). Some strains of *Pseudomonas putida* are able to survive a decrease in Ψ_m to -1.5 MPa by forming thick biofilms *in vitro* (Chang and Halverson 2003; Gulez et al. 2012). Biofilms are resistant to abiotic stress imposed by low water availability and are rich in extracellular polymers like exopolysaccharides and cellulose that help cells survive water-limited conditions (Chang and Halverson 2003; Chang et al. 2007, 2009). These biofilm components each have a distinct function in resistance to water limitation, biofilm formation, and rhizosphere colonization by *P. putida* mt-2 (Nielsen et al. 2011), and homologous compounds likely are produced by other *Pseudomonas* spp. found in the environment (Mann and Wozniak 2012). One mechanism for coping with reactive oxygen species in water-limited biofilms is mediated by the

production of the exopolysaccharide alginate (Chang et al. 2007), which is produced by most pseudomonads. It is plausible that the production of similar exopolysaccharides, surface adhesins, and phenazines contributes to the capacity of Phz$^+$ *Pseudomonas* to form robust biofilms and thus adapt to the rhizosphere of dryland wheat (Mavrodi et al. 2012a, b).

4.6 Concluding Remarks

Recent advances in the area of biofilm biology have revealed that biofilm formation is an ancient and integral component of the prokaryotic life cycle and suggest that most bacteria in Nature exist in biofilms. Biofilms play an important role in the response of bacteria to environmental stimuli and adaptation to stressful environments in both clinical and environmental settings. It is now well accepted that the establishment of biofilms is a complex and tightly regulated process. The shift from the planktonic to the sessile lifestyle involves major changes in cell-to-cell signaling, gene expression patterns, and bacterial physiology. Among the sophisticated regulatory and physiological adaptations that govern the process of biofilm development in fluorescent *Pseudomonas* spp. is the production of redox-active phenazine compounds. A number of exciting recent studies have revealed that the production of phenazines is universally linked with sessile growth in all Phz$^+$ *Pseudomonas* spp. studied to date, and that phenazines strongly affect colony morphology and the process of biofilm establishment and maturation. This phenomenon is directly linked to the electrochemical properties of phenazines and their capacity to act as alternative electron shuttles in microaerobic conditions similar to those found in mature biofilms. These findings help to explain the high degree of conservation of phenazine genes and the importance of phenazines for ecological fitness in Phz$^+$ *Pseudomonas* spp.

Most of our knowledge regarding the contribution of phenazines to the biofilm lifestyle was gained from the ubiquitous opportunistic human pathogen *P. aeruginosa*. However, recent studies have also revealed a widespread abundance of indigenous saprophytic and symbiotic phenazine-producing species. Very little is known about the relevance of phenazines in the microbial habitats where these bacteria reside, and elucidation of the role of phenazines in biofilms formed in diverse natural and managed ecosystems represents and important an exciting avenue of future research.

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Chapter 5
Fermentative Production of Bacterial Phenazines

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Abstract Phenazines, a nitrogen containing heterocyclic antibiotic biosynthesized by a diverse range of bacteria. Owing its enormous importance as (1) electron shuttles to alternate terminal acceptors in bacteria, (2) modify cellular redox states to modify host response, (3) contributing to biofilm formation and cell signaling, as well as (4) biotechnological applications such as environmental sensor, microbial fuel cell, antitumor, and biocontrol activity attracted attention of scientific community to target phenazine as lead molecule. Similarly, emerging application of phenazines insisted high productivity fermentative process. Current chapter focuses on sources of natural phenazines and impact of nutritional as well as environmental dynamics on fermentative production of phenazine in different bacteria.

5.1 Introduction

Phenazines cover nitrogen containing colored redox active heterocyclomers of biological and chemical origin. More than 6,000 phenazine derivatives have been described with one or other bioactivity (Mavrodi et al. 2006; Pierson and Pierson 2010). However, nearly 100 natural phenazines were reported exclusively from bacteria of diverse vicinity. Based on the types and position of functional groups present on structure, phenazines were known for a long time as pigments and antifungal/antibacterial compounds (Schoental 1941; Haynes et al. 1956; Mann 1970).

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Chemical synthesis of phenazines renders toxic chemicals including, aniline, azobenzoate, lead oxide, o-phenylenediamine which are potentially toxic as well as this process, (1) shows relatively less productivity, (2) needs harsh reaction process, and (3) produce toxic by-product. Whereas, selective phenazine synthesis can be possible using bacterial fermentation with added advantage of noncytotoxicity and growth limiting potential for eukaryotes, hence can be used as effective therapeutic agent for eukaryotic organisms (Laursen and Nielsen 2004). Also natural phenazine derivative have proven to be the more effective biocontrol agent than synthetic one (Nansathit et al. 2009). Thus, natural phenazine can always be a choice of selection for wide applications.

Phenazine production has been studied in different bacterial strains including florescent *Pseudomonas* (Mavrodi et al. 2006; Maddula et al. 2008; Li et al. 2008; Shanmugaiah et al. 2010), *Streptomyces* sp. (Gebhardt et al. 2002; Clinton et al. 1993; Zendah et al. 2012; Ohlendorf et al. 2012; Fotso et al. 2010; Kondratyuk et al. 2012), *Bacillus* sp. B-6 (Kim 2000; Li et al. 2007), *Brevibacterium* (Podojilt and Gerber 1967), *Burkholderia* (Mavrodi et al. 2006), and archae *Methanosarcina mazei Gö*1 (Abken et al. 1998; Beifuss et al. 2000) and few others. Among all phenazine producers *Pseudomonas* sp. and *Streptomyces* sp. have been studied at metabolic and genomic level. It was observed that the structural complexity of phenazines increase from *Pseudomonas* sp. to S*treptomyces* sp. (Saleh et al. 2009) and *Methanosarcina mazei* (Beifuss et al. 2000) as long side chain where it served as final electron acceptor in electron transport chain (Abken et al. 1998; Beifuss et al. 2000). The change in structure related to change in phenazine properties.

The extra ordinary potential of phenazines is due to their physicochemical properties, i.e., oxidation–reduction (redox) and their bright pigmentation, which changes with pH and redox state (Pierson and Pierson 2010). These prosperities have been used for biotechnological applications such as (1) biocontrol agent (Rane et al. 2007a, b), (2) microbial fuel cell (Torres et al. 2010; Sanderson et al. 1987), (3) organic light emitting devices (OLED) like phenanthroline-fused phenazine (Chen and Xiao-Chang 2004), (4) antitumor agent (Laursen and Nielsen 2004; Mavrodi et al. 2006; Kondratyuk et al. 2012), (5) biosensor like glucose sensor (Ohfuji et al. 2004), H_2O_2 (Santos et al. 2005), (6) biocolor for dyeing silk fabrik (Saranya et al. 2012), (7) mineral reduction (Hernandez et al. 2004), (8) oil degradation (Norman et al. 2004), (9) anticandidal (Morales et al. 2010), (10) food colorent (Saha et al. 2008) etc.

5.2 Phenazine Production

Broad range of bioactivity and applicability left impact on researcher to increase the productivity of phenazine from laboratory to fermentation scale production using potential phenazine producer. A wide variety of phenazines are biosynthesized by bacteria are given as follows.

5.2.1 Pseudomonad Phenazines

Pseudomonas is foremost phenazine producing bacteria with almost one-third of all known phenazines. Among all bio-chemo origin phenazines, pyocyanin was the first isolate one by Fordos in the 1850s from isolated *Pseudomonas*. Till date, fluorescent pseudomonads are the best studied phenazine producer which includes *Pseudomonas aeruginosa*, *P. fluorescens*, and *Pseudomonas chlororaphis.* Production profiling of *P. aeruginosa*, include (1) phenazine-1-carboxylic acid (PCA) (Rane et al. 2007a, b; Mavrodi et al. 2006); (2) phenazine-1-carboxamide (PCN) (Shanmugaiah et al. 2010), (3) pyocyanin (PYC) (Ra'oof and Latif 2010; Kaleli et al. 2006); (4) 1-hydroxyphenazine (1-OHPHZ) (Kerr et al. 1999), (5) Aeruginosin A and B (Holliman 1969; Herbert and Holliman 1969), etc. While, *P. chlororaphis* was reported for production of (1) PCA, (2) orange-colored 2-Hydroxyphenazine-1-carboxylic acid, (3) brick red-colored 2-Hydroxyphenazine. More than one phenazine derivative can be produced by *P. aeruginosa* and *P. chlororaphis* depending upon the genetic and environmental makeup (Mavrodi et al. 2006). *P. fluorescens* have found to produce only PCA production (Mavrodi et al. 2006). *P. aeruginosa* is the only known species capable of producing the very distinctive water-soluble pigment pyocyanin (Gohain et al. 2006), however pyocyanin negative *P. aeruginosa* strains are also reported (Mavrodi et al. 2006).

5.2.2 Streptomyces Phenazines

A vast diversity of phenazines was noticed in *Streptomyces* sp. like endophenazines (Gebhardt et al. 2002), diphenazines (Ding et al. 2011), phenazinomycin, D-alanylgriseoluteic acid (Giddens and Bean 2007), Geranylphenazinediol (Ohlendorf et al. 2012), esmeraldin, and saphenamycin (Clinton et al. 1993), also been studied till the date.

Apart from these major groups' phenazines like pelagiomicins A/B/C, myxin, PCA have been reported from *Pelagiobacter variabilis*, *Sorangium* sp. and *Bacillus* sp. respectively.

5.3 Phenazine Regulation and Environmental Factors

Phenazine biosynthesis is based on the phz gene expression, which turn on or off and allowing control of phenazine production (Linares et al. 2006). Different nutritional (carbon and nitrogen), metal (iron and phosphate), and environmental/process (pH, oxygen) parameters were found to regulate phz gene expression (Slininger and Jackson 1992; Slininger and Shea-Wibur 1995; Siddiqui and Shaukat 2004; van Rij et al. 2004).

Many reports had suggested, growth rate and phase-dependent phenazine production in pseudomonads, i.e., maximum phenazine production at late exponential and early stationary growth phase, while comparatively less product accumulation in early and mid-exponential phase (Chin-A-Woeng et al. 2001). With most important concern, quorum sensing, cell density dependant genome regulation are the most influencing factor for PCA and PCN production in *P. aureofaciens* (Pierson et al. 1995) and PCN in *P. chlororafis* (Chin-A-Woeng et al. 2005), respectively. The PCA molecule is thought to be the precursor for all other phenazine derivative (Gohain et al. 2006). Addition of exogenous PCA, as a precursor molecule in fermentation medium showed enhanced phenazine production in *P. chlororaphis* GP72, postulating that exogenous PCA may act as final electron acceptor and autoinducer providing more energy for bacterial growth and metabolite production (Huang et al. 2011).

5.4 Nutritional Requirement for Phenazine Production

Media components of production media specify the productivity of the metabolite during fermentation. Pierson and Pierson (2010) had suggested phenazine production is depending upon the nutritional condition. Hence, qualitative and quantitative effect of nutrients in production medium has to be optimised during process optimization studies. Depending upon the type of phenazine producer nutrient condition was found to be changing. Although phenazine production is aged process, however, the assessments of its nutritional as well as fermentation parameters for its production are still not well documented.

5.4.1 Nutritional Factor for Pseudomonad

Different media were studied earlier for pseudomonad phenazines production like (1) Pigment Production Medium D (Kluyver 1956), (2) Synthetic medium (Chang and Blackwood 1969), (3) Alanine medium (Frank and DeMoss 1959; Meyer and Abdallah 1978), (4) 1 % Casamino Acids-salts medium (Whooley and McLoughlin 1982), etc. Detailed of these media (Table 5.1) suggests requirement of sodium and potassium metal requirement during phenazine fermentation. Similarly, except 1 % Casamino Acids-salts medium other media comprising glycerol nutrition for phenazine production.

Yuan et al. (2008) showed glucose and soytone as influencing factors for PCA production in *Pseudomonas* sp. M-18q using Plackett Burman design (PBD) where, increasing the glucose concentration and decreasing the soytone concentration result in increasing the accumulation and secretion of PCA in growth medium (Yuan et al. 2008). Similarly, effect of carbon and nitrogen source on gacA-deficient *Pseudomonas* sp. M18G mutant suggest the glucose as influencing

Table 5.1 Phenazine production media and producer

Media	Ingredient	Organism	Phenazine	Reference
Pigment production medium D (PPMD)	Peptone, sodium chloride, glycerol, KNO_3	*P. aeruginosa* ID 4365	PCA	Rane et al. (2007a, b)
Synthetic medium	$MgCl_2.6H_2O$, Na_2SO_4, $FeSO_4.7H_2O$, K_2HPO_4, urea, glycerol	*P. aeruginosa* Mac 436	PCA, PYC, oxychlororaphine	Chang and Blackwood (1969)
Alanine medium	$MgCl_2.6H_2O$, Na_2SO_4, ferric citrate, K_2HPO_4, DL-alanine, glycerol	*P. aeruginosa*	PYC	Frank and DeMoss (1959)
1 % Casamino acids-salts medium	Casamino acids, NaCI, KC1, K_2HPO_4, $CaC1_2{\cdot}2H_20$, $MgSO_4{\cdot}7H_20$, tris (hydroxymethyl) methylamine, trace element	*P. aeruginosa* ATCC 10145	PYC	Whooley and McLoughlin (1982)
NA	Glucose, glycerol, starch, corn steep powder, casein peptone, yeast extract, NaCl, $CaCO_3$	*S. anulatus*	Endophenazine A–D PCA	Gebhardt et al. (2002)
TSB 10, modified trypticase soy broth	BD trypticase soy broth, NaCl, $CaCO_3$	*Streptomyces* sp. HB202	Streptophenazin	Mitova et al. (2008)
GYM1	Glucose, yeast extract, malt extract	*Streptomyces* sp. HB202	Streptophenazin	Mitova et al. (2008)
GYM2	Glucose, yeast extract, malt extract	*Streptomyces* sp. HB202	Streptophenazin	Mitova et al. (2008)
M1	Peptone, meat extract	*Streptomyces* sp. HB202	Streptophenazin	Mitova et al. (2008)
LB	Tryptone, yeast extract, NaCl	*Streptomyces* sp. HB202	Streptophenazin	Mitova et al. (2008)
MB	Difco marine broth	*Streptomyces* sp. HB202	Streptophenazin	Mitova et al. (2008)
Soybean–mannitol medium	Soybean flour, mannitol	*S. diastaticus* YIM DT26	Phenazinolins A–E	Ding et al. (2011)

(continued)

Table 5.1 (continued)

Media	Ingredient	Organism	Phenazine	Reference
GOT medium	Glycerol, oatmeal, tomato paste, $CaCO_3$	*Streptomyces* sp. ICBB8198	Griseolutein A Griseoluteic acid Compound 2 Compound 3	Fotso et al. (2010)
M2 medium	Malt extract, yeast extract, glucose	*Streptomyces* sp. Ank 315	Chromophenazines A–F	Zendah et al. (2012)
SPD medium	Starch, peptone from soymeal, artificial sea salt	*Streptomyces* sp. strain LB17	Geranylphenazinediol	Ohlendorf et al. (2012)
NA	KH_2PO_4, $(NH_4)2SO_4$, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, biotin, octanoic acid	*Bacillus* sp. B-6	PCA	Kim (2000)
Marine broth	Peptone, yeast extract, $FeSO_4$	*Bacillus* strain 39	Compound 1	Li et al. (2007)
NA	K_2HPO_4, KH2PO4, NH_4Cl, $MgSO_4{\cdot}7H_20$, $CaCl_2{\cdot}2H_20$, NaCl, $FeSO_4{\cdot}7H_20$, resazurin, vitamin solution, trace elements, yeast extract (Difco), casitone (Difco), $NaHCO_3$, methanol, cysteine hydrochloride, $Na_2S{\cdot}9H_20$	*Methanoscina mazei Göl*	Methanophenazine	Abken et al. (1998), Beifuss et al. (2000)

NA not assign

carbon source; likewise yeast extract (0.28 %) was found most influencing factor for growth and soy peptone for maximum PCA production (He et al. 2008). The PCA biosynthesis at optimised conditions in *Pseudomonas flurescence* 2–79 have found to be accelerated by glucose as carbon source with unnoticeable influence of nitrogen source (Slininger and Shea-Wilbur 1995). Similarly, zinc sulfate, ammonium molybdate, and cytosine as micronutrition had shown increased PCA biosynthesis in *P. flurescence* 2–79 (Slininger and Jackson 1992).

Effect of individual amino acids on PCN production studied in *P. chlororaphis* PCL1391 illustrated the influence of aromatic amino acids and casamino acids (van Rij et al. 2004). In another study, Fusaric acid, a self defensive molecule of *Fusarium oxysporum* was shown to suppress the PCN production which can be overcome by presence of phenylalanine in *P. chlororaphis* PCL1391 (van Rij et al. 2005).

Labeyrie and Neuzil (1981) showed enhanced growth rate as well as enhanced pyocyanin secretion of *P. aeruginosa* A237 in amino acids (tyrosine and phenylalanine) supplemented media. The concentration of glycerol and paraffins was found to stimulate the production of pyocyanin and phenazine derivatives in *P. aeruginosa* (MacDonald 1967). The selective and increased production of PCA and PCN was achieved by Byng et al. (1979) using *m* or *p*-aminobenzoic acid as selective inhibitor of PYC specifically inhibit of phenazine methylation.

5.4.2 Nutritional Factors for Streptomyces

As like pseudomonad phenazines various media were exploited for *Streptomyces* phenazine production which mainly includes, (1) soybean–mannitol medium (Ding et al. 2011), (2) GOT medium (Fotso et al. 2010), (3) M2 medium (Zendah et al. 2012), (4) SPD medium (Ohlendorf et al. 2012), (5) TSB 10, modified Trypticase Soy broth (Mitova et al. 2008), (6) GYM1 (Mitova et al. 2008), (7) GYM2 (Mitova et al. 2008), (8) M1 (Mitova et al. 2008), (9) LB (Mitova et al. 2008), (10) MB (Mitova et al. 2008). In all studied media (Table 5.1) a complex protein source was used like casein peptone, soy protein digest, yeast extract, and oatmeal. The change in streptophenazine biosynthesis in presence of antibiotics like, i.e., tetracycline and bacitracin in fermentation medium suggest two- to threefold increase in synthesis (Mitova et al. 2008). In marine *Streptomyces* sp., subinhibitory concentrations of antibiotics were found to enhance and modulate the production of new phenazines, i.e., streptophenazines A–H (Mitova et al. 2008).

The phenazine production media for *Bacillus* sp. as mentioned in Table 5.1 by Kim (2000) and Marine broth (Li et al. 2007) stipulate iron (Ferrous) requirement for phenazine secretion apart from other nutrition factors. Phenazine production using *Bacillus* strain 39 deep sea sediment isolate, has also been studied in marine broth by Li et al. (2007).

Very few reports have been seen in case of methanophenazine (Abken et al. 1998; Beifuss et al. 2000) where a complex media (Table 5.1) was tried for phenazine production from *Methanoscina mazei Gö1*.

5.5 Fermentative Conditions of Phenazine Production

Environmental and process parameters, i.e., temperature, pH, and dissolved oxygen, respectively affect growth and hence secondary metabolites secretion. Change in pH condition, i.e., from neutral to slight acidic or alkaline minimizes the phenazine production while phenazine production at pH 7 has given considerable phenazine yield, while optimum pH-control during late phase of fermentation has been studied with effective PCA production (Li et al. 2010). pH and temperature sensitive PCA production was detected in *P. flurescence* 2–79, where optimum productivity was recorded at pH 7 and 25–27 °C with greater cell density (Slininger and Jackson 1992).

Influenced of abiotic environmental factors, i.e. pH, O_2 exchange and temperature on PCN production in *P. chlororaphis* PCL1391, showed PCN productivity was increased at 1 % oxygen and at low magnesium concentrations, while noticeable decrease observed at pH 6 and temperature 16 °C. Also, production of autoinducer during cell growth in pseudomonas is influenced by cell density, which directly affects the PCN production positively by increasing the PCN yield during fermentation process (Rij et al. 2004; Mavrodi et al. 2006).

In current scenario insufficient known knowledge is available about DO requirement for phenazine production during fermentation. However, reports claimed that the higher productivity of PCA fermentation yielded at 20 % of DO with optimized agitation and aeration condition. However, increased in DO (50 %) drastically decrease the PCA yield during fermentation, which might be due to cell lysis caused by increased agitation condition and unsuitable pH (Li et al. 2010).

5.6 Phenazine Productivity

PCA production in *Pseudomonas* sp. M18G gacA mutant showed 30-fold increase productivity from 0.02 to 0.6 gL^{-1} compared to the wild-type strain (Ge et al. 2004). With optimum glucose and yeast extract in fermentation media increased PCA production from 673.3 up to 966.7 μgmL^{-1} in *Pseudomonas* sp. M18G gacA mutant (He et al. 2008). The same strain at optimized fermentation conditions after 60 h of incubation showed 1.89 gL^{-1} of PCA production (Li et al. 2008). Rane et al. (2007a, b) had recovered 18 gm of crystalline PCA at large-scale fermentation (125 L working volume in synthetic medium) from *P. aeruginosa* ID 4365. Phenazine production in *Bacillus* sp. B-6 yielded 400 μgmL^{-1} PCA in chemically defined medium (Kim 2000).

5.7 Conclusion

Characteristics of phenazine and its derivative opened its applicability in different biotechnological segments. Current research in evaluation of new properties and its exploitation of same warrants economic fermentative production of phenazines. In the same regard, this chapter rationalizes the factors influencing phenazine fermentation. To a great extent, nutritional and environmental factors supporting growth as well as phenazine production have been discussed in different Pseudomonads and *Streptomyces* strains. The future debate on alone phenazine gene expression will certainly lead to techno economic production of selective phenazines.

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Chapter 6
Methods for Purification and Characterization of Microbial Phenazines

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Abstract Phenazines are nitro aromatic redox-active antibiotics produced under the control of quorum sensing mechanism by a diverse range of bacterial genera with various color intensities varies from blue, green, purple, yellow, red to even brown. Production of phenazine showed (1) taxonomic value when related to few genera, (2) functional role in environment and (3) bioactive potential with respect to structure; however due to poor structural understanding its correlation is still incomplete with diversity and bioactivity. Thus isolation and identification of phenazine antibiotic is highly desirable. Current chapter resolves the methodologies for various phenazines isolation as well as its identification through different spectroscopic and electrophoretic methods in different bacterial systems.

Abbreviations

MS	Mass spectrometry
EI-MS	Electron impact mass spectroscopy
HRMS	High-resolution mass spectrometry
FI MS	Field ionization mass spectrometry
HRESIMS	High resolution electron spray ionization mass spectroscopy
LRCIMS	Low resolution chemical ionization mass spectrometry
HRCIMS	High resolution chemical ionization mass spectrometry
HR-FAB-MS	High resolution fast atom bombardment mass spectrum
DCI-MS	Desorption-chemical ionization mass spectrometry
LC/MS	Liquid chromatography/mass spectrometry
GC-MS	Gas chromatography–mass spectrometry

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6.1 Introduction

Bioactive secondary metabolites of bacterial origin may serve as lead compound in a number of industrial applications (Burja et al. 2001); where due to technoeconomic value and environmental acceptance naturally produced metabolites are always choice of consideration (Ozdemir et al. 2004). Newman and Cragg (2007) have reported different natural products which were approved as drug molecules. Function of such metabolites was found to relied on (1) environmental conditions of production, (2) release mechanism of metabolite from bacteria, (3) self protection mechanism of bacteria against metabolite and (4) extraction or isolation process (Smith and Doan 1999; Volk 2007), where the metabolite synthesis was found to be associated with growth phase i.e. at exponential and stationary phase (Namikoshi and Rinehart 1996; LeFlaive and Ten-Hage, 2007).

To ascertain new compounds, which has potential in biotechnological appliance and understanding their interactions at in vivo conditions, isolation and chemical analysis of compound i.e. chemical structure analysis in relation to biosynthetic pathways are highly intended (Schlegel et al. 1999). The methodologies for screening of potential drug molecules are time consuming and hence new strategies are highly obligatory. For the last decade's efficient extraction methodologies were utilized for successful isolation of secondary metabolite and number of techniques was continually attempted to improve efficacy of extraction by optimizing (1) polarity of solvent, (2) ionic condition (pH) of crude broth, (3) number of extraction steps and (4) combination of solvents (aqueous and inorganic). Following compound extraction, analytical technique including HPLC, UV–Visible, FTIR, NMR and MS were used to resolve (1) functional group present in structure, (2) structure and (3) molecular formula.

Despite the function, these metabolites remained undetermined for their structural and functional relationship based research. Amongst different metabolites phenazines, a nitro aromatic antibiotic mainly biosynthesized by *Pseudomonas* sp. and *Streptomyces* sp. have been studied so far. However structure based function and taxonomic correlation of phenazines are still lagging due to poor isolation and characterization knowledge. Arbiser and Moschella (1995) stated that, the phenazine can be a good candidature as a drug molecule. Hence high throughput screening of different phenazines is needed. Genetic approaches for screening of phenazine molecule have been precisely described by Schneemann et al. (2011), but these are the most expensive and time consuming schemes. Thus the analytical technique such as chromatographic and spectroscopic techniques can be an identification tool. Looking forwarded for the need, current chapter enlightens isolation i.e. purification and characterization i.e. structural elucidation methodologies of different phenazines in different bacterial genera.

6.2 Purification

Generally phenazines are associated with specific bacterial genera e.g. phenazine-1-carboxylic acid biosynthesis in *P. aureofaciens*, pyocyanine biosynthesis in *P. aeruginosa* etc. However, Kanner et al. (1978) showed incubation condition dependent multi phenazines production viz. Pyocyanin, Oxychlororaphin (phenazine-1-carboxaminde), Oxychlororaphin precursor and Oxychlororaphin/chlororaphin (reduced Phenazine-1-Carboxamide) in *P. aeruginosa* at 37 °C/shaking, 37 °C/stationary, 28 °C/shaking and 28 °C/stationary, respectively in minimal salt medium. However, to isolate these phenazines different isolation methods were used i.e. (a) Chlororaphin isolated by filtering culture broth through a fine (200-mesh) sieve, the crystal retained washed with 2N NaOH and water, then dried at 25 °C over diphosphorous pentaoxide, (b) oxychlororaphin was extracted from cell free broth with chloroform and (c) Pyocyanine was isolated by passing cell free supernatant through polystyrene Amberlite XAD-2 and eluting with methanol which was partitioned with chloroform and the resultant blue solution was extracted with acid followed by neutralization with alkali and extracted with chloroform (Kanner et al. 1978). Thus isolation of phenazine is state of art and it differ with respective type i.e. structure and properties of phenazine molecule. In view of this, some approaches like bioactivity-guided phenazine (griseoluteic acid) isolation by fractionation using liquid–liquid partitioning followed by chromatographic separation like (1) thin layer, (2) vacuum liquid, (3) column (CC) and (4) preparative high-performance reversed-phase liquid chromatography have been described by Wang et al. (2011). Table 6.1, summarizes different isolation methods in different bacterial system.

6.3 Characterization

A fast and efficient approach to identify phenazine biosynthesizing ability of bacterium needs efficient phenazine characterization methodology. To type the phenazines, number of different methodologies has been used, like for easy and fast detection (1) chromatographic techniques like TLC and HPLC and (2) spectroscopic method like UV–Visible techniques were employed. Similarly, for determining the structural features spectroscopic techniques viz. (1) Fourier transform infrared spectroscopy (FTIR), (2) Nuclear magnetic spectroscopy (NMR—^{1}H and ^{13}C) and Mass spectroscopy were used.

6.3.1 Thin Layer Chromatography

Thin Layer Chromatography (TLC) is an easy and rapid technique and generally used for partial characterization of metabolite i.e. (1) detection, (2) quantification and (3) quality (during purification) on C18 reversed phase plates (Brelles-Mariño

Table 6.1 Purification schemes for different phenazine

Phenazine derivative	Organism	Purification method	Reference
Phenazine-1-carboxylic acid	*P. aeruginosa* ID 4365	Acidified cell free broth extracted with benzene, the benzene layer separated by adding small amount of water and extracted in sodium bicarbonate, washed with benzene. Phenazine re-extracted in benzene after acidification of bicarbonate layer and chromatographed on silica gel (benzene–acetic acid,19:1)	Rane et al. (2007a)
	Pseudomonas fluorescens 2–79	Extraction with equal volume of chloroform	Brisbane et al. (1987)
	P. aeruginosa	Cell free broth passed on an Amberlite XAD-2 column and eluted with methanol, further chromatographed on silica gel eluting with chloroform. Final purification by RP HPLC using 50 % aqueous methanol	Jayatilake et al. (1996)
	Pseudomonas chlororaphis GP72	Acidified supernatant (pH 2) extracted with equal volume of ethyl acetate followed by separation in methanol using preparative HPLC (57 % methanol + 43 % 5 mM NH_4AC, pH 5.0)	Liu et al. (2007)
Phenazine-1-Carboxamide	*Pseudomonas aeruginosa* MML2212	Cell-free culture supernatant extracted with ethyl acetate, concentrated and mixed with silica gel (100–120 mesh) and air-dried. The powder then gradiently eluted with Hexane, hexane/ethanol and ethanol in column. The partially purified phenazine further chromatographed silica-gel (230–400 mesh)	Shanmugaiah et al. (2010)
Phenazine-1,6-dicarboxylic acid	*Streptomyces cinnamonensis* DSM 1042T	Cells were extracted with methanol in an ultrasonic bath, mixed with sodium acetate buffer and extracted with dichloromethane, which further concentrated and redissolved in methanol	Haagen et al. (2006)
	Pseudomonas aeruginosa PUPa3	Cell free supernatant extracted with equal volume of ethyl acetate and concentrated. The crude extract further chromatographed over silica gel eluting with chloroform, chloroform–methanol mixtures (9:1)	Kumar et al. (2005)
Aeruginosin A	*P. aeruginosa*	Acidified (pH 4.5) red culture filtrated added with Celite 535, the supernatant get adsorbed on charcoal-Celite column, further washed with water, 50 % (v/v) aqueous ethanol and gradient elution with 0–12 % aqueous pyridine	Holliman (1969)

(continued)

Table 6.1 (continued)

Phenazine derivative	Organism	Purification method	Reference
Aeruginosin B	*P. aeruginosa*	As per Aeruginosin A except elution with 2 % pyridine	Herbert and Holliman (1969)
Pyocyanin	*P. aeruginosa* ID 4365	Cell free broth extracted in chloroform and fractionated with 0.1 N HCl (2:1 ratio). The fraction subjected for washing with chloroform in least amount of dilute HCl.	Rane et al. (2008)
	P. aeruginosa	Culture filtrate added with potassium chloride (10 %) extracted by chloroform, further extracted with a 0.33 volume of 0.1 N HCI. The acid layer washed with chloroform and titrated with 1.0 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 11.0) till the blue color reappeared.	Baront and Rowe (1981)
	P. aeruginosa	Organisms washed from the surface of King's A agar medium, phenazines extracted into chloroform and crude pyocyanin crystallized with petroleum ether further purified by reverse phase HPLC	Watson et al. 1986
2-hydroxy-phenazine-1-carboxylic acid	*Pseudomonas aurantiaca* Strain PB-St2	Extraction of alkaline broth with chloroform, further acidified followed by chloroform extraction and concentration. Extract further chromatographed on silica gel (chloroform:acetic acid, 49:)	Samina et al. (2009)
2-hydroxy phenazine-l, 6-dicarboxylic acid	*Streptomyces* sp.	Acidified broth (pH 3) extracted with butanol, back-extracted with water and dried. Further, Ion-exchange chromatographed, eluted to purer product with 0.5 M NaCl and desalted on Diaion HP-20 adsorbent afforded two active fractions i.e. (A) eluted with water, further chromatographed on silica gel (n-butanol-ethanol–water, 2:1:1) and subsequently purified by gradient HPLC on a preparative C18 column (100 % water to 50 % methanol) and	Gilpin et al. (1995)
Derivative of 2-hydroxy phenazine-l, 6-dicarboxylic acid.)		(B) eluted with methanol, further chromatographed on silica gel (n-butanol-ethanol–water, 4:1:1) and subsequently purified by HPLC on a preparative Cl8 column (27.5 % methanol in ammoniumacetate buffer)	

(continued)

Table 6.1 (continued)

Phenazine derivative	Organism	Purification method	Reference
1-hydroxy phenazine	*P. aeruginosa*	Crude crystallized phenazine hydrolysed in alkaline condition (0.2 M NaOH, 18 h, 20 °C) and purified by reverse phase HPLC	Watson et al. (1986)
	P. aeruginosa SD12	Cell free extract loaded on TLC grade silica prepared in hexane and gradiently eluted with mixtures of hexane, chloroform, and methanol in increasing order of polarity and further purified by preparative thin layer chromatography (PTLC) developed in chloroform: methanol (99.5:0.5)	Seema et al. (2012)
	P. aeruginosa	The crude cell free supernatant passed on a Nova Pak octadecylsilyl column and eluted with acetonitrile/water/trifluoroacetic acid (100:0:0.04) isocratically for 5 min followed by a 10-min linear gradient to acetonitrile/water/TFA (60:40:0.04)	Wilson et al. (1987)
2-hydroxy phenazine	*P. chlororaphis subsp. ureofaciens* DSM 6698T	Alkaline cell free broth extracted in chloroform which then neutralized and added with sodium phosphate 0.01 M. The extract adsorbed on Whatman cellulose powder made column and eluted with 0.05 M sodium phosphate (pH 7)	Levitch and Rietz (1966)
	P. aurantiaca Strain PB-St2	Cell-free supernatant (pH 9) extracted with chloroform and re-extracted with same in acidic condition (pH 3), where the organic fractions were water washed, dried and redissolved in methanol. The methanolic extract chromatographed on silica gel (chloroform: acetic acid, 49:1)	Samina et al. (2009)
1,6-dihydroxy phenazine 1,6-dihydroxy-2-chlorophenazine	*Streptosporang-ium* sp.	The whole broth extracted with equal volumes of ethyl acetate, concentrated to oil and precipitated with petroleum ether. The vacuum filtered precipitate chromatographed on silica gel in benzene–methanol (9:1) followed by column chromatography on neutral alumina (chloroform–methanol–water, 2:2:1) and active fraction crystallized with methylene chloride	Patel et al. (1984)
Endophenazine A, B, C	*S. cinnamonensis* DSM 1042T	Cells extracted with methanol in an ultrasonic bath, mixed with sodium acetate buffer and extracted with dichloromethane, which further concentrated and redissolved in methanol	Haagen et al. (2006)

(continued)

Table 6.1 (continued)

Phenazine derivative	Organism	Purification method	Reference
Esmeraldin A	*S. antibioticus*	Acidified supernatant (pH 2) extracted with methylene chloride and breaks in dichloromethane/methanol (1:2); dissolved in aqueous sodium bicarbonate (5 %) and washed with *n*-hexane. After acidification (pH 1–2) the aqueous solution extracted with methylene chloride and chromatographed on silica gel (*n*-hexane/acetone, 1:1) eluting yellow and green fractions	McDonald et al. (1999)
Griseolutein A	*Streptomyces* sp. ICBB8198	The brown culture broth mixed with Celite and filtered under vacuum. The filtered medium passed through an HP-20 Diaion column and eluted with methanol/acetone, followed by 100 % methanol. The extracts were evaporated to dryness and chromatographed on Sephadex LH-20 (3 % methanol/dichloromethane)	Fotso et al. (2010)
Griseolutein B	*S. griseoluteus*	Acidified cell free broth (pH 2) extracted with butyl acetate, concentrated and crystallized with dioxane, which further purified by counter current distribution between phosphate buffer and ethyl acetate, the active fraction in acidic water crystallized (yellow colored) with pyridine-dioxane	Nakamura (1958)
Benthocyanin B and C	*S. prunicolor*	The mycelia free broth extracted with acetone and concentrated, where as the aqueous residue was extracted with ethanol. The residues fractionated in silica gel column packed in chloroform (chloroform–methanol, 10:1) and rechromatographed in same elution (20:1). The elluent on further gel filtered in Toyopearl HW-4OF (methanol) and Sephadex LH-20 (CHCW-MeOH, 1:1)	Shinya et al.(1993)
Phencomycin methyl ester	*S. fimicarius* ISP 5322	Cell free supernatant extracted in ethyl acetate, concentrated, resuspended in methanol and extensively extracted with cyclohexane.	Pusecker et al. (1997)

(continued)

Table 6.1 (continued)

Phenazine derivative	Organism	Purification method	Reference
Saphenamycin	*S. antibioticus,* strain tu 2706	Cell free supernatant extracted with methanol or methanol- acetone (1:1), the green crude extract concentrated and extracted with ethanol-acetone (pH 2–4) and further chromatographed on Sephadex LH-20 (Chloroform–Methanol,1:1)	Geiger and Schierlein (1988)
Saphenamycin methyl ester		Silica gel chromatography (hexane–acetone, 2:1)	
Saphenamycin Methyl Ether Methyl Ester		After saphenamycin eaxtraction further chromatographed on Sephadex LH-20 (Chloroform–Methanol, 1:1 and crystallization with acetone	
Saphenic acid		Silica gel chromatography with hexane- acetone (3:1) and fatty material get eluted, further chromatographed on Sephadex LH-20 (acetone–methanol 1:1)	
Saphenic Acid Methyl Ether		Silica gel chromatography (hexane–acetone, 2:1) and eluted yellow fraction was rechromatographed on Sephadex LH-20 with acetone elution	
Mixture of Fatty Acid Saphenyl Esters		First elution varied from hexane - acetone (3:1) to (1:1) and successively chromatographed on Sephadex LH-20 (Chloroform–methanol,1:1) and silica gel (hexane–acetone, 2:1)	
DC-86-Y DC-86-M	*S. luteogriseus* DO-86.	Acidified broth (pH 2) extracted with acetone and ethyl acetate (1:2.5) subsequently filtered with Celite where washed with water applied to a silica gel column, and chromatographed with chloroform	Takahashit et al. (1986)
DOB-41	*Pseudomonas* sp.	Culture broth added with methanol (50 %) and Hyflo Super-Cel (20 %), filtered and extracted with ethyl acetate (pH 3). After water wash 1 % sodium bicarbonate added to solvent and re-extracted with ethyl acetate (pH 3), further subjected to silica gel column (chloroform–methanol, 8:2) and re-chromatographed (chloroform–methanol, 19:1). Active fraction dissolved in ethyl acetate and washed with water (pH 3)	Shoji et al. (1988)
Phenazostatin A, B	*Streptomyces* sp.833	Extracted with ethanol and further purified using reserved phase HPLC with a solution of methanol–water (85:15)	Kim et al. (1997)

(continued)

Table 6.1 (continued)

Phenazine derivative	Organism	Purification method	Reference
Phenazostatin C	*Streptomyces sp.*	Concentrate the extraction of culture supernatant with an equal volume of hexane and chromatographed silica gel followed by elution with hexane-ethanol (4:1). Active fraction rechromatographed in Sephadex LH-20 column and eluted with methanol. The active eluate further chromatographed on C-18 column (80 % aqueous methanol)	Kim et al. (1999)
Phenazostatin D	*Pseudonocardia* sp.B6273	Culture filtrate extracted with ethyl acetate and concentrated. Latter subjected to vacuum flash chromatography on silica gel and eluted with cyclohexane and dichloromethane/methanol gradient. Likewise rechromatographed and eluted gradient of cyclohexane/ dichloromethane	Maskeya et al. (2003)
3′-O-Quinovosyl Saphenate 2′-O-Quinovosyl Saphenate	*Streptomyces* sp. CNB-253	The crude fermentation extract fractionated by vacuum flash silica chromatography using increasing amounts of ethyl acetate in isoodane. The active fraction further purified using HPLC (80 % ethyl acetate in isooctane)	Pathirana et al. (1992)
Aestivopheonin A, B	*S. purpeofuscus*	Acidified (pH 3) cell free culture broth extracted with ethanol and concentrated, followed by Silica gel column separation in n-hexame-ethanol (4:1) chloroform:ethanol (20:1). The elute further purified by HPLC (90 % methanol)	Shinya et al. (1995)
Phenazinolin A, B, C, D and E	*S. diastaticus* YIM DT26	The culture filtrate absorbed onto the Amberlite XAD-16 resin eluted with methanol and concentrated to slurry. The slurry further extracted with ethyl acetate and chromatographed successively on: (1) silica gel (chloroform–methanol gradient), (2) Sephadex LH-20 (methanol), and by (3) semi-preparative HPLC (methanol–water gradient)	Ding et al. (2011)
Palagiomicin A, B and C	*Plagiobacter variabilis*	Concentrated cell free filtrate washed with ethanol and extracted chloroform and subjected for concentration. The extract was concentrated to dryness and dissolved in phosphate buffer (pH 7) chromatographed on a preparative HPLC with gradient elution of acetonitrile in phosphate buffer (pH 7)	Imamura et al. (1997)

(continued)

Table 6.1 (continued)

Phenazine derivative	Organism	Purification method	Reference
Methano phenazine	*Methanosarcina mazei Go¨1*	Phenazine adsorbed membranes extracted with isooctane and purified by HPLC (cyclohexane and gradient of ethyl acetate 5–100 %)	Abken et al. (1998)
Strepto-phenazines A, B, C, D, E, F, G, H	*S. mediolani* LMG 20093T	The cell free culture broth extracted with equal volumes ethyl acetate, where as cells macerated in methanol and concentrated. Both extract subjected to Fast Centrifugation Partition Chromatography (n-heptane-ethyl acetate–methanol–water; 5.5:4.5:5.5:4.5). Further purified by gradient HPLC (water/acetonitrile)	Mitova et al. (2008)
Chromo phenazine A, B, C, D and F	*Streptomyces* sp. Ank 315	The culture broth mixed with Celite and filtered with a filter press, filtrate passed through an Amberlite XAD-16 and eluted with methanol. The methanol extract concentrated and the aqueous residue was extracted with ethyl acetate.	Zendah et al. (2012)
Geranyl phenazinediol Diacetylgeranyl phenazinediol	*Streptomyces* sp. strain LB173	The wet cell homogenized in ethanol organic supernatants concentrated and extracted with ethyl acetate, where the organic phases dried, which further fractionated on Sephadex LH-20 with methanol elution. Final purification by preparative RP-HPLC with acetonitrile−0.1 % Formic acid as mobile phase with a gradient of acetonitrile (80–100) %.	Ohlendorf et al. (2012)
Diazepinomicin/ECO-4601	Micromonospora strain DPJ15	Acidified (pH 3) culture broth stored overnight at −20 °C (overnight), further centrifuged then extracted with methanol, concentrated resuspended in methanol and filtered through Acrodisc GHP syringe (0.45 × 13 mm)	McAlpine et al. (2008)

and Bedmar 2001). Thus determination of R_f value of any test sample in correlation to standard phenazine can predict type of phenazine. Detection of phenazine on TLC have been tried alone and with developing agents. Perneel et al. (2007) have shown phenazine separation on Kieselgel 60 F_{254} and detected under UV-transilluminator. However, Upadhyay and Srivastava (2008) visualized phenazine by spraying with diazotized sulfanilic acid (DSA) on silica gel reverse-phase C18 TLC plate. Table 6.2, summarizes TLC separation scheme of different phenazines produced by respected organisms in different mobile phase. The specific R_f of each phenazine molecule can thus a database for identification of unknown phenazines.

6.3.2 High Pressure Liquid Chromatography

As like TLC, HPLC was most efficiently used for qualitative (analytical) and quantitative (preparative) estimation of phenazine. Characterisation can also be carried out by analytical HPLC using a C18 reversed-phase column. Retention times of standards can be compared with those of samples. To optimize the specific phenazine elution profile as well as retention time and different gradients need to optimized. Selin et al. (2010) analysed phenazines in isocratic solution of 45 % acetonitrile/35 % water/20 % methanol and product elution observed with AD20 detector at 225 nm, whereas Fotso et al. (2010) detected phenazine at 254 nm from Indonesian *Streptomyces* sp. by preparative HPLC (20 % Methanol/Water). Thus, changing the mobile phase, stationary phase as well as analysis method with type of phenazine, changes the interference. Table 6.3, summarizes some phenazine elution profile biosynthesized by various microbes, which clearly suggest variability of retention time with respect to mobile phase as well as type of phenazine.

6.3.3 UV–Visible Spectroscopy

Different color intensity and range of phenazines (blue, green, purple, yellow, red to brown) were reported earlier (Saha et al. 2008). This can be a unique id for identification of phenazines. In general pseudomonal yellow colored phenazine shows absorption maxima at 365 nm. However due to redox active nature absorption maxima of phenazines may vary depending upon the solvent conditions. E.g. Methanophenazines at oxidized state shows absorption maxima at 250 and 365 nm with shoulders at 300, 330, and 400 nm, whereas after reduction absorption at 250 nm increased and new peaks at 295 and 500 nm appeared (Soliev et al. 2011). Thus for determining the type of bacterial phenazines by UV–visible spectroscopy (1) colour of phenazine, (2) source of phenazine and (3) solvent condition have to be considered. Table 6.4, enlists details of absorption pattern of different phenazines with respect to their source and solvent conditions.

Table 6.2 TLC profile of different phenazines in different detection system

Phenazine derivative	Organism	Detection system	Rf	Reference
Phenazine-1-Carboxylic acid (PCA)	*P. aeruginosa*	Dichloromethane	0.17	Rane et al. (2007b)
	P. chlororaphis	Isopropanol:Ammonia: Water (8:1:1)	0.50	Kavitha et al. (2005)
	P. aureofaciens	Benzene:Acetic acid (95:5)	0.17	Pierson and Thoamashow (1992)
	Pseudomonas sp.	Ethyl acetate:Chloroform (5:5)	0.63	Sayed et al. (2008)
	P. aeruginosa	Chloroform :Methanol (90:10)	0.92	Lee et al. (2003)
	Pseudomonas sp. LBUM223	Methanol:Chloroform (1:20)	0.62	St-Onge et al. (2011)
	P. aeruginosa	Chloroform:Methanol (9:1)	0.40	Dakhama et al. (1993)
Pyocyanin	*P. aeruginosa*	Chloroform:Methanol (9:1)	0.33	
		Chloroform:Methanol (1:1)	0.57 –0.73	Baront and Rowe (1981)
		Ethyl acetate:Acetic acid:Water (3:2:1)	0.25–0.36	
2-Hydroxy PCA	*P.aureofaciens*	Benzene:Acetic acid (95:5)	0.19	Pierson and Thoamashow (1992)
Phenazine 1-carboxamide (PCN)	*P. chlororaphis* PCL 1391	Butanol:Acetone (9:1)	0.47	Chin-A-Woeng et al. (1998)
1-Hydroxy phenazine	*P. aeruginosa* TISTR 781	Dichloromethane:Methanol (1:1)	0.77	Saosoong et al. (2009)
	P. aeruginosa	Chloroform:Methanol (9:1)	0.72	Dakhama et al. (1993)
2-Hydroxy phenazine	*P. aureofaciens*	Benzene:Acetic acid (95:5)	0.04	Pierson and Thoamashow (1992)
Oxychlororaphine	*P. aeruginosa*	Chloroform:Methanol (9:1)	0.55	Dakhama et al. (1993)
Saphenic acid	*S. antibioticus* TU 2706	Hexane:Acetone (1:1)	0.32	Geiger and Schierlein (1988)
Phenazostatin D	*Pseudonocardia* sp. B6273	Dichloromethane	0.10	Maskeya et al. (2003)
		Dichloromethane/2 % Methanol	0.50	
Endophenazine A	*S. anulatus*	Chloroform:Methanol (9:1)	0.63	Krastel and Zeeck (2002)
Endophenazine B			0.24	
Endophenazine C			0.41	
Endophenazine D			0.10	
Chromophenazine A	Streptomyces sp. Ank 315	5 % Methanol/ Dichloromethane	0.36	Zendah et al. (2012)
Chromophenazine B			0.28	
Chromophenazine C			0.20	
Chromophenazine D			0.13	
Chromophenazine E			0.30	
Chromophenazine F			0.26	

Table 6.3 HPLC profile of different phenazines in various mobile phases

Phenazine derivative	Organism	Mobile phase	Rt	Reference
Phenazine-1-Carboxylic acid (PCA)	*P. aeruginosa*	Methano:Phosphate buffer (60:40)	3.25	Rane et al. (2007b)
	P. chlororaphis	Gradient: 8 % acetonitrile: (A) 25 mM Ammonium acetate (2 min) and (B) 80 % acetonitrile: 25 mM Ammonium acetate (25 min)	9.4	Mavrodi et al. (2001)
		Methanol: 5 mM Ammonium acetate (50 %:50 %)	3.2	Huang et al. (2011)
	Pseudomonas sp.	Gradient of (A) Acetonitrile (80 %) and (B) Sodium hypophosphite buffer (1 mM, pH4)	23.83	Sayed et al. (2008)
	P. fluorescens	30–70 % linear gradient of acetonitrile in water + 0.1 % trifluoroacetic acid	22.5	Thoamashow et al. (1990)
	P. fluorescens	Gradient: (A) 35 % Acetonitrile–0.1 % TFA in water (2 min) and (B) linear gradient to 100 % Acetonitrile (25 min)	14.1	Delaney et al. (2001)
	P. aeruginosa	Linear gradient 10–100 % Acetonitrile + TFA	17.1	Lee et al. (2003)
	P. aeruginosa	0.05 % Trifluoracetic acid (TFA) in water and 0.05 % TFA in Acetonitrile.	19.0	Denning et al. (2003)
Pyocyanin	*P. aeruginosa*	Gradient: (A) Acetonitrile/water/TFA (100:0:0.04, 5 min) and (B) Acetonitrile/water/TFA(60:40:0.04, 10 min)	10.4	Wilson et al. (1987)
		Gradient: (A) 8 % Acetonitrile: 25 mM Ammonium acetate (2 min) and 80 % Acetonitrile: 25 mM Ammonium acetate (25 min)	10.6	Mavrodi et al. (2001)
		0.05 % Trifluoracetic acid (TFA) in water and 0.05 % TFA in acetonitrile.	19.6	Denning et al. (2003)
Phenazine 1-carboxamide (PCN)	*P. aeruginosa* PAO1	Gradient: (A) 8 % acetonitrile: 25 mM Ammonium acetate (2 min) and 80 % acetonitrile: 25 mM Ammonium acetate (25 min)	16.4	Mavrodi et al. (2001)
	P. chlororaphis PCL 1391	Gradient: Acetonitrile in water, with 0.1 % TFA (18–80 %, vol/vol)	20	Chin-A-Woeng et al. (1998)
1-hydroxyphenazine (1-hp)	*P. aeruginosa*	Gradient: (A) 8 % Acetonitrile: 25 mM Ammonium acetate (2 min) and (B) 80 % acetonitrile: 25 mM Ammonium acetate (25 min)	18.2	Mavrodi et al. (2001)
		Gradient: (A) Acetonitrile/water/TFA (100:0:0.04, 5 min) and (B) Acetonitrile/water/TFA (60:40:0.04, 10 min)	12.6	Wilson et al. (1987)
		Gradient : (A) Solvent A:water:TFA (100:0.01) and (B) Solvent B:water:Acetonitrile:TFA (10:90:0.01)	20.4	Saosoong et al. (2009)
		0.05 % Trifluoracetic acid (TFA) in water and 0.05 % TFA in Acetonitrile	20.6	Denning et al. (2003)

(continued)

Table 6.3 (continued)

Phenazine derivative	Organism	Mobile phase	Rt	Reference
2-hydroxyphenazine	*P. chlororaphis*	Methanol: 5 mM Ammonium acetate (50 %:50 %)	16.5	Huang et al. (2011)
	P. fluorescens	Gradient: (A) 35 % Acetonitrile–0.1 % TFA in H_2O (25 min) and (B) Linear gradient to 100 % Acetonitrile	11.4	Delaney et al. (2001)
SB (212021)	*Streptomyces* sp.	0.05 M Ammonium acetate buffer (pH 6.5) in 30 % Methanol	9.6	Gilpin et al. (1995)
		27.5 % Methanol in 0.05 M Ammonium acetate buffer	18	
SB (212305)	*Streptomyces* sp.	Linear gradient of 100 % water to 50 % methanol (30 min)	19	
		0.05 M Ammonium acetate buffer (pH 6.5) in 30 % Methanol	6.5	
PD (116, 152)	*Streptomyces lomondensis*	Linear gradient: 0.025 M Borate buffer (pH 9.5)/Methyl cynide/Methanol (90:5:5) to 0.025 M borate buffer (pH 9.5)/Methyl cynide/Methanol (70:25:5)	3.5	Smitka et al. (1986)
Methanophenazine	Methanosarcina	(A) Cyclohexane, (B) ethyl acetate Gradient profile:Ethyl acetate concentration, 5 % at 0 min to 100 % at 10–20 min	4.5	Abken et al. (1998)

6.3.4 Fourier Transform Infrared Spectroscopy

To determine spectroscopic relevance and change, Infrared (IR) spectra is mostly preferred for neutral as well as positively and negatively charged phenazine (Mattioda et al. 2005). Due to structural relevance (three ring compound) phenazine resemblance to anthracene and flavin molecules, which creates ambiguity in interpretation when alone UV–Visible spectra considered for identification of phenazine. The functional group stretching get vary with different phenazine due to its structural feature. Aziz et al. 2012 have shown different bond stretching of phenazine viz. H–O (3475), C–H aromatic (3059), C=N (1627), C=C aromatic (1554), C=O (1469), C–O (1323), C–N (1284), ip O–H (1207), ip C–H aromatic (856), oop C–H aromatic (748). Thus phenazine like PCA, PNC, hydroxyl phenazine and other phenazines will differ according to their functional group i.e. –H–C=O (1735), $-NH_2$–C=O (1670), –OH (1200) respectively.

6.3.5 Nuclear Magnetic Spectroscopy (NMR—^{1}H and ^{13}C)

Nuclear magnetic resonance (NMR) spectroscopy has been proved for its usability for metabolic analyses and profiling (Reo 2002). NMR spectroscopy is one of the most widely used methods for analytical measurement of metabolic profiles due its (1) reliability, (2) reproducibility and (3) speed (Tulpan et al. 2011). NMR has been applied to metabolite-profiling studies in areas as diverse as plant metabolism (Want et al. 2005) human tissues extracts, body fluids (Beckonert et al. 2007) and microbial metabolite (Tulpan et al. 2011). Microbial phenazine has been structurally interrelated based on δ peaks corresponding to hydrogen and carbon atoms (Herbert and Holliman 1969; Gurusiddaiah et al. 1986). Nansathit et al. (2009) described Phenazine-1-Carboxylic acid by (1) ^{1}H NMR; δ at 7.30–9.00 and 15 ppm indicated the presence of 7 aromatic protons and carboxylic acid proton respectively and (2) ^{13}C NMR; δ at 124.95–143.95 and 165.86 ppm indicated the presence of 7 aromatic protons and carboxylic acid proton respectively. Likewise Table 6.6, summarizes ^{1}H and ^{13}C peak frequencies of different phenazines in produced by various bacteria.

6.3.6 Mass Spectroscopy

Metabolite identification by mass spectroscopy has been used in different living systems viz. (1) human (Ma et al. 2006), (2) plant (Allwooda and Goodacrea 2010) and microbes (Gräfe et al. 2001; Meyer et al. 2008) due to fast and sensible analysis. Based on protonated molecular ions $[M + H]^{+}$ corresponding molecular

Table 6.4 Absorption pattern of different bacteria phenazines

Phenazine derivative	Organism	UV–Visible absorption profile (nm)	Reference
Phenazine-1-carboxylic acid (Yellow)	*Pseudomonas* sp.	252, 354 (Sh), 365 (Methanol)	Sayed, et al. (2008)
	P. aeruginosa ID 4365	251, 370 (Dichloro methane)	Rane et al. (2007b)
	P. aeruginosa TISTR 781	250, 369 (Dichloro methane)	Nansathit et al. (2009)
	P. aeruginosa GC-B26	252, 365 (Methanol)	Lee et al. (2003)
	P. aeruginosa	368 (Methanol)	Jayatilake et al. (1996)
	P. fluorescens 2-79 (NRRL B-15132)	251, 361 (Chloroform)	Brisbane et al. (1987)
	P. aurantiaca PB-St2	250, 362 (Methanol)	Samina et al. (2009)
	S. antibioticus Tu 2706	252, 365 (Ethanol)	Geiger and Schierlein, (1988)
Phenazine-1-carboxamide	*P. aeruginosa*	366 (Ethanol)	Jayatilake et al. (1996)
1-Carboxymethyl phenazine	*S. fimicarius* ISP 5322T	366, 249, 214	Schneemann et al. (2011)
Compound 1	*Bacillus* sp.	240, 281, 289, 348, 364, 384 (Methanol)	Li et al. (2007)
Compound (2) (yellow solid)	*Streptomyces* sp.	364, 268, 247 (sh), 208	Fotso et al. (2010)
Compound (3) (yellow powder)		370, 206, 248 (sh), 274 (sh)	
Aeruginosin A (red)	*P. aeruginosa*	235, 282, 396, 515 (0.1 M phosphate buffer, pH 6.95); 235, 283, 396, 515 (0.l M acetate buffer, pH 3.87); 235, 283, 395, 515 (0.25 M glycine buffer, pH 9.91); 235, 285, 380, 538 (0.5 N HCl); 235, 275, 375, 525 (0.5 N NaOH)	Holliman (1969)
Aeruginosin B	*P. aeruginosa*	240, 290, 390, 522 (0.1 M phosphate buffer, pH 6.95); 240, 287.5, 392, 522 (0.l M acetate buffer, pH 3.87); 240, 290, 390, 522 (0.25 M glycine buffer, pH 9.91); 240, 295, 378, 542 (0.5 N HCl); 240, 295, 367.5, 530 (0.5 N NaOH)	Herbert and Holliman (1969)

(continued)

Table 6.4 (continued)

Phenazine derivative	Organism	UV–Visible absorption profile (nm)	Reference
Pyocyanin (Blue)	*P. aeruginosa* ID 4365	242, 279, 388	Rane et al. (2008)
	P. aeruginosa	201, 238, 318, 710, 886 (Methanol) 204, 242, 277, 387, 521 (0.1 N HCl)	Ohfuji et al. (2004)
	P. aeruginosa	242, 278, 365, 385, 520 (0.1 M HCl) 238, 316, 341, 368, 690 (Methanol)	Watson et al. (1986)
SB 212021 (2-hydroxy phenazine- l, 6-dicarboxylic acid)	*Streptomyces sp.*	210, 247, 294, 370, 520 (Methanol); 203, 248, 268, 369, 431 (Methanol + HCl); 210, 246, 298, 370, 520 (Methanol + NaOH)	Gilpin et al. (1995)
SB 212305 (Derivative of 2-hydroxy phenazine- l, 6-dicarboxylic acid.)		212, 249, 305, 380, 530 (D/W); 212, 251, 278, 378, 440 (D/W + HC1); 249, 305, 370, 515 (D/W +NaOH)	
1-hydroxyphenazine	*P. aeruginosa*	273, 363, 370, 383, 242 (0.1 M HCl); 264, 350, 360, 368, 237(Methanol)	Watson et al. (1986)
2-hydroxyphenazine	*P. aureofaciens*	367, 275, 229 (1 N NaOH); 388, 263, 217 (1 N HCl)	Levitch and Rietz, (1966)
	P. aurantiaca PB-St2	254, 358 (Methanol)	Samina et al. (2009)
Chlororaphin	*P. aeruginosa*	388, 278, 208 (0.2 N HCl); 327, 310, 245 ($CHCl_3$)	Kanner et al. (1978)
1,6-dihydroxyphenazine (Yellow)	*Streptosporangium sp.*	272, 373, 442 (Methanol) 262, 291 (0.1 N NaOH)	Patel et al. (1984)
1,6-dihydroxy-2-chlorophenazine (Purple)		275, 377, 440 (Methanol); 265, 296 (0.1 N NaOH)	
Endophenazine A (Yellow)	*S. cinnamonensis* DSM 1042T	371, 254, 214	Haagen et al. (2006)
	S. anulatus	255, 365 (Methanol)	Krastel and Zeeck (2002)

(continued)

Table 6.4 (continued)

Phenazine derivative	Organism	UV–Visible absorption profile (nm)	Reference
Endophenazine B (Violet)	*S. cinnamonensis* DSM 1042T	375, 256, 223	Haagen et al. (2006);
	S. anulatus	236, 283, 374, 516, 545 (sh) (Methanol); 272, 391, 482 (Methanol + HCl); 235, 283, 373, 516, 545 (sh) (Methanol + NaOH)	Krastel and Zeeck (2002)
Endophenazine C (Green)	*S. cinnamonensis* DSM 1042T	372, 249, 212	Haagen et al. (2006)
	S. anulatus	254, 410 (Methanol); 252, 426 (Methanol + HCl); 253, 387 (Methanol + NaOH)	Krastel and Zeeck (2002)
Endophenazine D (colourless solid)		293, 351 (Methanol)	
Di-phenazithionin (dark green powder)	*S. griseus* ISP 5236	260, 305, 375, 442	Hosoya et al. (1996)
Benthocyanin A	*S. prunicolor*	247,342,424, 622 (Methanol); 247, 344, 638 (Methanol + HCl); 247, 340, 407, 428, 616 (Methanol + NaOH)	Shinya et al. (1993)
		247, 342, 424, 622, and 675 sh (Methanol), 247, 344, 380, 418, 440, 638, 690sh (0.01 N HCl-Methanol); 247, 340, 364, 407, 428, 616, 660 sh (NaOH-Methanol).	Shinya et al. (1991)
Benthocyanin B	*S. prunicolor*	249, 344, 415, 430, 615 (Methanol)	Shinya et al. (1993)
		245, 335, 632, (Methanol + HCl); 250, 343, 415, 430, 615 (Methanol + NaOH)	
Benthocyanin C		246, 283, 445, 470, 570 (Metanol); 247, 280, 325, 410, 430, 665 (Methanol + HCl); 251, 335, 410, 440, 535 (Methanol + NaOH)	
Phencomycin (greenish yellow)	*Streptomyces* sp. B 8251	256, 368 (Methanol)	Pusecker et al. (1997)
5, 10-dihydrophencomycin methyl ester (brown)		216, 245, 450 (Methanol)	

(continued)

Table 6.4 (continued)

Phenazine derivative	Organism	UV–Visible absorption profile (nm)	Reference
Saphenamycin(yellow)	*S. antibioticus,* tu 2706	365, 255(Ethanol)	Geiger and Schierlein (1988)
Saphenamycin methyl ester (yellow)		365, 251(Ethanol)	
Saphenic acid (yellow)		365, 255 (Ethanol)	
Methyl Ester of Saphenic Acid Methyl Ether (yellow)		365, 252 (Ethanol)	
Mixture of Fatty Acid Saphenyl Esters		365, 254 (Ethanol)	
DC-86-Y (yellow)	*S. luteogriseus* DO-86	253, 367(Methanol)	Takahashit et al. (1986)
DC-86-M (yellow)		251, 363 (Methanol)	
DOB-41(yellow)	*Pseudomonas* sp.	255, 370 (Chloroform)	Shoji et al. (1988)
Phenazostatin A (yellow)	*Streptomyces* sp.833	252, 365(Methanol)	Kim et al. (1997)
Phenazostatin B (yellow)		253, 366 (Methanol)	
Phenazostatin C (yellow)	*Streptomyces* sp.	249, 365 (Methanol)	Kim et al. (1999)
Phenazostatin D	*Pseudonocardia* sp.B6273	385 (sh), 366, 350 (sh), 256 (Chloroform)	Maskey et al. (2003)
Aestivopheonin A (Orange)	*S. purpeofuscus*	229, 245, 296, 367, 490	Shinya et al. (1995)
Aestivopheonin B(Orange)		232, 245, 298, 368, 495	
Aestivopheonin C	*S. luridiscabiei* S63T	432(br), 327, 226	Kunigami et al. (1998)
	S. fulvorobeus LMG 19901T	432(br), 327, 226	Kunigami et al. (1998)
Aestivophoenin C derivative	*S. luridiscabiei* S63T	430(br), 325, 224	Kunigami et al. (1998)
	S. fulvorobeus LMG 19901T	430(br), 325, 224	Kunigami et al. (1998)

(continued)

Table 6.4 (continued)

Phenazine derivative	Organism	UV–Visible absorption profile (nm)	Reference
Phenazinolin A	*S. diastaticus* YIM DT26	235.1, 261, 291.8, 365.5 (Methanol)	Ding et al. (2011)
Phenazinolin B		236.3, 294.2, 365.5 (Methanol)	
Phenazinolin C		235.1, 259.9, 295.4, 362.3 (Methanol)	
Phenazinolin D		271.7, 382.3 (Methanol)	
Phenazinolin E		271.7, 382.3 (Methanol)	
Palagiomicin A (Reddish orange needles)	*Plagiobacter variabilis*	209, 265, 368 (Ethanol)	Imamura et al. (1997)
Senacarcin A	*S. fulvorobeus* LMG 19901T	370(br), 274, 224	Schneemann et al. (2011)
Streptophenazines A (yellow)	*S. mediolani* LMG 20093T	367, 363sh, 350sh, 252, 215	Mitova et al. (2008)
Streptophenazines B (yellow)		368, 364sh, 351sh, 252, 218	
Streptophenazines C (reddish)		371, 364sh, 354sh, 252, 213	
Streptophenazines E (yellow)		368, 364sh, 351sh, 252, 218	
Streptophenazines D (yellow)		368, 364sh, 351sh, 252, 218	
Streptophenazines F (yellow)		368, 364sh, 353sh, 252, 215	
Streptophenazines G (yellow)		368, 363sh, 351sh, 252, 214	
Streptophenazines H (reddish)		368, 363sh, 352sh, 252, 215	
Chromophenazine A	*Streptomyces* sp. Ank 315	229, 256, 271, 278, 308, 372, 463 sh, 490, 519 sh (Methanol)	Zendah et al. (2012)
Chromophenazine B		247 sh, 277 sh, 301, 309, 363, 491, 521 sh	
Chromophenazine C		224, 281, 361, 531	
Chromophenazine D		247, 301, 389, 491	
Chromophenazine E		251, 304, 394, 511	
Geranylphenazinediol	*Streptomyces* sp. LB173	204, 273, 354, 373, 458	Ohlendorf et al. (2012)
Diacetylgeranylphenazinediol		217, 259, 364, 410	

Table 6.5 IR profile of different phenazines in different bacteria

Phenazine derivative	Organism	IR frequencies (cm^{-1})	Reference
Phenazine-1-Carboxylic acid	*P. aeruginosa* ID 4365	3600, 3020 (Br), 2950 (Br), 2360 (Br), 1733, 1600, 1523, 1460	Rane et al. (2007a, b)
	P. aeruginosa TISTR 781	3446, 2664, 1741, 1472–1284	Nansathit et al. (2009)
	P. fluorescens 2–79	3040, 3020, 1740, 1625, 1605, 1565, 1525	Brisbane et al. (1987)
Compound 1	*Bacillus* sp.	3438, 2923, 2364, 1633, 1564, 1460, 1261, 1183, 798	Li et al. (2007)
Compound 2	*Streptomyces* sp.	2923, 1719, 1700, 1540, 1457, 1173, 1106, 764	Fotso et al. (2010)
Compound 3		2920, 2850, 1727, 1697, 1667, 1635, 1584,1518, 1463,1377, 1282, 1248	
Pyocyanin	*P. aeruginosa* ID 4365	3454, 3396, 2360, 2073, 1975, 1730, 1627, 1556, 1411	Rane et al. (2008)
SB 212021	*Streptomyces* sp.	3395, 1691, 1616, 1580, 1536, 1476, 1424, 1382, 1341, 1269, 1237, 1205, 1134, 1093,1049	Gilpin et al. (1995)
SB 212305	*Streptomyces* sp.	3389, 1690, 1622, 1580, 1533, 1506, 1472, 1433, 1403, 1313, 1232, 1206, 1147, 1118, 1053	Gilpin et al. (1995)
1,6-dihydroxyphenazine	*Streptosporangium sp.*	3400, 1640, 1530, 1522, 1485, 1440, 1207, 804	Patel et al. (1984)
1,6-dihydroxy-2-chlorophenazine		3400, 1635, 1610, 1555, 1525, 1485, 1200, 805, 750	
Benthocyanin A	*S. prunicolor*	3450, 1740, 1720	Shinya et al. (1993)
Benthocyanin B		3430, 1760, 1745	
Benthocyanin C		3430, 2180, 1735, 1670	
Saphenamycin Methyl Ester	*S. antibioticus*,tu 2706	3325, 1730, 1710, 1660, 1620	Geiger and Schierlein (1988)
Saphenamycin Methyl Ether Methyl Ester		1725, 1625, 1600 (br)	
DC-86-Y	*S. luteogriseus* DO-86	3410, 1728, 1700	Takahashit et al. (1986)
DC-86-M		3450, 1743, 1715	
DOB-41	*Pseudomonas* sp.	3530, 1742, 1470, 1193, 1130, 1065	Shoji et al. (1988)
Phenazostatin A	*Streptomyces* sp.833	1730, 1440, 1270, 1190, 1030, 760	Kim et al. (1997)
Phenazostatin B		1735, 1530, 1280, 1265, 1240, 1190, 1040, 750	
Phenazostatin C	*Streptomyces* sp.	1734, 1435, 1284, 1194, 1029, 754	Kim et al. (1999)

(continued)

Table 6.5 (continued)

Phenazine derivative	Organism	IR frequencies (cm^{-1})	Reference
Phenazostatin D	*Pseudonocardia* sp.B6273	2958, 2924, 2854, 1720, 1650, 1634, 1531, 1457, 1433, 1382, 1288, 1263, 1239, 1197, 1170, 1152, 1097, 1046, 1014, 856, 815, 797, 748, 709	Maskey et al. (2003)
3′-O-Quinovosyl Saphenate	*Streptomyces* sp. CNB-253	3357, 2975, 2931, 1726, 1566, 1269, 1058, 752	Pathirana et al. (1992)
2′-O-Quinovosyl Saphenate		3362, 2975, 2932, 1727, 1567, 1268, 1059, 753	
Aestivopheonin A	*S. purpeofuscus*	3450, 3330, 1680, 1260	Shinya et al. (1995)
Aestivopheonin B	*S. purpeofuscus*	3450, 3330, 1680, 1260	Shinya et al. (1995)
Phenazinolin A	*S. diastaticus* YIM DT26	3424, 2918, 2854, 1626, 1600, 1528, 1511, 1468	Ding et al. (2011)
Phenazinolin B		3426, 2920, 2852,1626, 1600, 1528, 1512, 1468	
Phenazinolin C		3432, 2923, 2854, 1627, 1601, 1530, 1472	
Phenazinolin D		3428, 3067, 2921, 2852, 1713, 1626, 1607, 1574, 1548, 1525, 1478, 1457, 1424	
Phenazinolin E		3420, 3065, 2920, 2853, 1712, 1624, 1607, 1573, 1548, 1525, 1478, 1456, 1423	
Palagiomicin A	*Plagiobacter variabilis*	3432, 2928, 1740, 1545, 1460, 1130, 1100	Imamura et al. (1997)
Chromophenazine A	*Streptomyces* sp. Ank 315	3429, 2923, 2854, 1583, 1544, 1460, 1377, 1321, 1233, 1161, 1055	Zendah et al. (2012)
Chromophenazine C		3446, 2928, 1736, 1718, 1654, 1541, 1459, 1384, 589	
Chromophenazine D		3447, 2925, 2373, 2080, 1836, 1560, 1495, 1292, 587	
Chromophenazine F		3429, 2923, 2853, 1641, 1544, 1496, 1446, 1384, 1275, 1128, 715	
Endophenazine A	*S. anulatus*	3440, 1737, 1532, 1462	Krastel and Zeeck (2002)
Endophenazine B		3436, 1735, 1628, 1595, 1548	
Endophenazine C		3435, 1651, 1508, 1472, 1254	
Endophenazine D		3344, 1665, 1596, 1488, 1457, 1266, 1094	

Table 6.6 NMR database of different phaenazines

Phenazine derivative	Organism	NMR frequency (δ)		Reference
		1H (ppm)	13C (ppm)	
Phenazine-1-Carboxylic acid	*P. aeruoginosa* TISTR 781	7.30–9.00	124.95–143.95	Nansathi et al. (2009)
	P. aeruoginosa GC-B26	8.57 (dd, 7.0, 1.5), 8.31–8.39 (m), 9.01 (dd, 8.7, 1.5), 7.99–8.08 (m), 8.31–8.39 (m), 8.31–8.39 (m), 7.99–8.08 (m) 15.61 (s)	125.2–166.1	Lee et al. (2003)
	P. fluorescens NRRL B-15132	15.579, 8.98, 8.7, 8.53, 8.310-8.380, 7.939-8.302	124.75–165.883	Gurusiddaiah et al. (1986)
Aeruginosin B	*P. aeruoginosa*	2.04, 2.46, 3.12, 3.98	–	Herbert and Holliman (1969)
Pyocyanin	*P. aeruoginosa* Pup 14B	6.68 *d* 8.7 (2H), 7.99 *t* 8.4 (3H), 6.66 *d* 8.4 (4H), 8.12 m (6H), 8.12 m (7H), 7.79 *t* 7.0 (8H), 8.38 *d* 8.4 (9H), 4.29, *s* (5-Me)	177.5 (1C),115.5 (2C), 147.3 (3C), 95.3 (4C), 136.4 (4aC), 134.4 (5aC), 116.8 (6C), 138.1 (7C), 128.0 (8C), 134.0 (9C), 138.5 (9aC), 146.7 (10aC), 36.7 (5-Me C)	Angell et al. (2006)
Phenazine 1-carboxamide	*P. aeruginosa* PUPa3	10.8 ($CONH_2$), 9.02 (dd, 8.2, 2.1- 2H), 7.97 (dd, 8.2, 8.4- 3H), 8.45 (dd, 8.2, 2.1- 4H), 8.31 (ddd, 2.4, 5.2, 8.2- 6H), 7.92 (dd, 8.2, 2.2- 7H), 7.94 (dd, 8.2, 2.2- 8H), 8.32 (ddd, 2.4, 5.2, 8.2- 9H)	166, 143.5, 141.5, 140.8, 134.3, 131.7, 131.0, 129.9, 129.1, 128.8.	Kumar et al. (2005)
1-hydroxy phenazine	*P. aeruginosa* SD 12	6.72 (2H), 7.26 (3H), 7.24 (4H) 7.36 (6H), 7.70 (7H), 7.32 (8H), 7.75 (9H)	151.7 (1C),108.9 (2C), 119.9 (3C), 131.9 (4C), 143.8 (4aC), 144.1 (5aC), 130.8 (6C), 129.2 (7C), 130.5 (8C), 129.7 (9C), 141.2 (9aC), 134.7 (10aC)	Dharni et al. (2012)

(continued)

Table 6.6 (continued)

Phenazine derivative	Organism	NMR frequency (δ)		Reference
		1H (ppm)	13C (ppm)	
Aestivophoenins A	*S. purpeofuscus* 2887-SVS2	7.08 (2H), 6.55 (3H), 6.35 (4H), 6.70 (6H), 7.00 (8H), 6.50 (9H), 7.68 (13,17H), 7.51 (14, 16H), 7.59 (15H) , 4.04 (18H), 5.08 (19H), 1.74 (21H), 1.69 (22H), 6.17 (1′H), 3.99 (2′H), 3.81 (3′H), 3.53 (4′H), 3.75 (5′H), 1.25 (6′H), 9.37 (10-NH)	109.7 (1C), 122.3 (2C), 121.6 (3C), 115.4 (4C), 136.7 (4aC), 135.5 (5aC), 112.9 (6C), 132.4 (7C), 127.2 (8C), 112.8 (9C), 139.1 (9aC), 140.5 (10aC), 194.5 (11C), 139.7 (12C), 129.9 (13, 17C), 129.0 (14, 16C), 132.2 (15C), 44.6 (18C), 119.5 (19C), 137.5 (20C), 25.8 (21C), 18.0 (22C), 166.8 (23C), 95.4 (1′C), 70.9 (2′C), 72.3 (3′C), 73.2 (4′C), 72.1 (5′C), 18.2 (6′C)	Shin-Ya et al. (1995)
Aestivophoenins B	*S purpeofuscus* 2887-SVS2	7.06 (2H), 6.53 (3H), 6.30 (4H), 6.59 (6H), 6.95 (8H), 7.67 (13, 17H), 7.50 (14, 16H), 7.59 (15H), 3.98 (18H), 5.05 (19H), 1.73 (21H), 1.65 (22H), 3.08 (14H), 5.22 (25H), 1.71 (27H), 1.71 (28H), 6.20 (1′H), 4.00 (2′H), 3.82 (3′H), 3.53 (4′H), 3.77 (5′H), 1.26 (6′H), 9.50 (10-NH)	109.6 (1C), 122.0 (2C), 121.6 (3C), 115.0 (4C), 136.7 (4aC), 135.3 (5aC), 111.4 (6C), 131.5 (7C), 127.4 (8C), 123.8 (9C), 136.7 (9aC), 140.5 (10aC), 194.6 (11C), 139.7 (12C), 129.9 (13, 17H), 128.9 (14, 16C), 132.2 (15C), 44.8 (18C), 119.6 (19C), 137.4 (20C), 25.7 (21C), 18 (22C), 167.1 (23C), 29.3 (24C), 120.6 (25C), 135.8 (26C), 25.8 (27C), 18.1 (28C), 95.4 (1′C), 70.9 (2′C), 72.2 (3′C), 73.2 (4′C), 72.1 (5′C), 18.2 (6′C)	Shin-Ya et al. (1995)
DC 86 M	S. luteogriseus DO-86	1.82 (3H, *d*, $J = 6.8$ Hz), 4.30 (2H, *d*, $J = 0.7$ Hz), 7.35 (1H, *q*, $J = 6.8$ Hz), 7.9′ 8.3 (4H, aromatic protons), 8.57 (1H, dd, $J = 1.7$, 8.8 Hz), 9.00 (1H, dd, $J = 1.7$, 7.1 Hz) and 15.49 (1H, br, COOH).	–	Takahash et al. (1986)

(continued)

Table 6.6 (continued)

Phenazine derivative	Organism	NMR frequency (δ)		Reference
		1H (ppm)	13C (ppm)	
DOB 41	*Pseudomonas* sp. DOB-41	1.73 (3H, *d*,$J = 6.5$ Hz); 3.36 (3H,*s*); 3.76 (1H,m); 3.71 (1H, m); 7.11 (1H, *q*, $J = 6.5$ Hz); 4.01 (1H,m)	2.76, *q*, 128 (Hz);57.4, *q*, 141.5 (Hz);62.0, *t*, 141 (Hz);67.7, *d*, 152 (Hz); 81.8, *d*, 146 (Hz) 127.2, *d*, 164 (Hz); 128.0, *d*, 168 (Hz); 130.6, *d*, 170 (Hz); 132.4, *d*, 166 (Hz); 133.6, *d*, 170 (Hz);134.3, *d*, 166 (Hz); 128.1, *s*; 139.5, *s*;140.1, *s*;140.2, *s*; 140.4, *s*;141.5, *s*	Shoji et al. (1988)
Endo phenazine A	*Bacillus* sp.	7.56 (2H br.*s*- 1, 6H), 7.23 (2Hdd, 8.3, 1.8- 3,8H), 7.46 (2Hd, 8.3- 4, 9H), 2.50 (6Hs- 11, 12H), 7.64 (2Hs- 2′, 3′H),	120.4 (1,6C), 142.4 (1a, 6aC), 134.9 (2, 7C), 127.7 (3, 8C), 110.1 (4, 9C), 148.9 (4a, 9aC), 21.5 (11, 12C), 160.9 (1′, 4′C), 123.5 (2′,3′C)	Li et al. (2007)
Phenazinolins A	*S. diastaticus* YIM DT26	8.88 (*d*, 8.6- 4H), 7.87 (dd, 7.5, 8.6- 5H), 7.47 (*t*, 7.5- 6H), 7.88 (*d*, 7.5- 7H), 7.54 (*d*, 9.8- 9H), 7.00 (*d*, 9.8- 10H), 10.06 (*s*- 11OH), 7.04 (*d*, 7.6- 12H), 7.58 (*t*, 10.1- 13H), 7.35 (d, 8.3- 14H), 3.59 (dd, 17.7, 5.5-16H), 3.15 (dd, 17.7, 0.6- 16H), 3.70 (m- 17H), 4.62 (dd, 4.0, 3.1- 18H), 6.15 (br s- 18Oh), 6.23 (dd, 4.0, 2.8- 9H)	180.1 *s* (1C), 109.4 *s* (2C), 131.7sb (2aC), 130.3 *s* (3aC), 116.4*d* (4C), 132.7*d* (5C), 124.1*d* (6C), 130.5*d* (7C), 135.1 *s* (7aC), 146.4sc (8aC), 133.0 *d* (9C), 135.3*d* (10C), 153.1 *s* (11C), 111.5*d* (12C), 131.3*d* (13C), 117.9*d* (14C), 142.0 *s* (14aC), 152.7 *s* (15aC), 33.7*t* (16C), 30.2*d* (17C), 62.8*d* (18C), 56.9*d* (19C), 146.4Se (19aC), 131.6Sb (20aC)	Ding et al. (2011)

(continued)

Table 6.6 (continued)

Phenazine derivative	Organism	NMR frequency (δ)		Reference
		1H (ppm)	13C (ppm)	
Phenazinolins B	*S. diastaticus* YIM DT26	8.68 (*d*, 8.5- 4H), 7.92 (mf- 5H), 7.49 (t, 7.9f- 6H), 7.91 (d, 7.9f-7H), 7.55 (d, 9.8- 9H), 7.00 (d, 9.8- 10H), 7.95 (d, 8.0f- 11H), 7.78 (m-12H), 7.72 (t, 8.6-13H), 7.93 (d, 8.6f- 14H), 3.65 (dd, 17.7, 5.0- 16H), 3.70 (*t*, 5.0- 17H), 4.63 (m- 18H), 6.11 (*d*, 2.4- 18OH), 6.34 (m- 19H)	180.1 *s* (1C), 109.4 *s* (2C), 131.6 *s* (2aC), 130.3 *s* (3aC), 115.6*d* (4C), 132.4*d* (5C), 124.0*d* (6C), 130.5de (7C), 135.1 s (7aC), 146.4 s (8aC), 133.0*d* (9C), 135.3*d* 10C), 28.1*d* (11C), 130.6de (12C), 129.5*d* (13C), 128.5*d* (14C), 140.3 *s* (14aC), 152.6 *s* (15aC), 33.4*t* (16C), 30.3*d* (17C), 62.7*d* (18C), 56.6*d* (19C), 148.9 *s* (19C)	Ding et al. (2011)
Phenazinolins C	*S. diastaticus* YIM DT26	8.02 (*d*, 8.8- 4H), 7.85 (m- 5H), 7.52 (mb-6H), 7.98 (*d*, 8.0- 7H), 7.54 (mb- 9H), 6.94 (*d*, 9.6- 10H), 10.34 (*s*- 11OH), 7.03 (*d*, 7.6- 12H), 7.49 (mb – 13H), 7.27 (*d*, 8.4- 14H), 3.93 (dd, 19.2, 5.9-16H), 3.46 (ddc- 16H), 5.40 m (17H), 4.60 (dd, 4.4, 3.3- 18H), 6.18 (d 3.3-18OH), 4.96 (dd, 4.4, 2.3- 19H)	178.9 *s* (1C), 106.4 *s* (2C), 132.3 *s* (2aC), 130.5 *s* (3aC), 114.2 *d* (4C), 133.2 *d* (5C), 124.3 *d* (6C), 131.0 *d* (7C), 135.6 *s* (7aC), 146.7 *s* (8aC), 132.7 *d* (9C), 135.5 *d* (10C), 153.2 *s* (11C), 111.4 *d* (12C), 129.9 *d* (13C), 117.7 *d* (14C), 141.2 *s* (14aC), 150.6 *s* (15aC), 34.6 *t* (16C), 52.5 *d* (17C), 62.8 *d* (18C), 39.7 *d* (19C), 151.4 *s* (19aC), 131.8 *s* (20aC)	Ding et al. (2011)
Phenazinolins D	*S. diastaticus* YIM DT26	8.02 (*d*, 8.8- 4H), 7.85 (m- 5H), 7.52 (mb-6H), 7.98 (*d*, 8.0- 7H), 7.54 (mb- 9H), 6.94 (*d*, 9.6- 10H), 10.34 (*s*- 11OH), 7.03 (*d*, 7.6- 12H), 7.49 (mb- 13H), 7.27 (*d*, 8.4- 14H), 3.93 (dd, 19.2, 5.9-16H), 3.46 (ddc- 16H), 5.40 m (17H), 4.60 (dd, 4.4, 3.3- 18H), 6.18 (*d* 3.3-18OH), 4.96 (dd, 4.4, 2.3- 19H)	178.9 *s* (1C), 106.4 *s* (2C), 132.3 *s* (2aC), 130.5 *s* (3aC), 114.2 *d* (4C), 133.2 *d* (5C), 124.3 *d* (6C), 131.0 *d* (7C), 135.6 *s* (7aC), 146.7 *s* (8aC), 132.7 *d* (9C), 135.5 *d* 10C), 153.2 *s* (11C), 111.4 *d* (12C), 129.9 *d* (13C), 117.7 *d* (14C), 141.2 *s* (14aC), 150.6 *s* (15aC), 34.6 *t* (16C), 52.5 *d* (17C), 62.8 *d* (18C), 39.7 *d* (19C), 151.4 *s* (19aC), 131.8 *s* (20aC)	Ding et al. (2011)

(continued)

Table 6.6 (continued)

Phenazine derivative	Organism	NMR frequency (δ)		Reference
		1H (ppm)	13C (ppm)	
Phenazinolins E	*S. diastaticus* YIM DT26	7.90 (*s*-2H), 7.28 (*d*,7.9 h- 6H), 7.57 (*t*, 7.9- 7H), 7.08 (*d*, 7.3- 8H), 10.48 (*s*-9OH), 10.62 (*s*- 11OH), 7.27 (*d*, 7.3 h-12H), 7.62 (*d*, 8.6- 13H), 5.60 (dd, 3.4,2.1- 16H), 4.78 (m- 17H), 6.16 (*d*, 2.4- 17OH), 4.82 (m- 18H), 3.86 (dd, 17.4, 5.2- 19H), 3.44 (dde- 19H), 14.71 (br s- 21OH)	127.1 *s* (1C), 127.9*d* (2C), 151.0 *s* (3C), 122.4 *s* (4aC), 40.0 *s* (5aC), 142.7 *s* (6C), 117.8df (7C), 111.6dg (8C), 153.6 *s* (9C), 132.7 *s* (9aC), 136.9 s (10aC), 153.2 s (11C), 111.6dg (12C), 132.5*d*(13C), 117.8df (14C), 139.1 *s* (14aC), 152.8 *s* (15aC), 76.0*d* (16aC), 62.6*d* (17C), 30.9*d* (18C), 34.5*t* (19C), 146.4 *s* (19aC), 135.1 *s* (20aC), 165.1 *s* (21C)	Ding et al. (2011)
Phenazine L-Quinovose Esters I	*Streptomyces* sp. CNB 253	8.26 (dd, 1.5, 6.9- 2H), 7.9 (m- 3H), 8.38 (dd, 1.5, 8.8- 4H), 7.87 (m- 7H), 7.87 (m- 8H), 8.15 (dd, 2.0, 8.0- 9H), 5.85 (*q*, 6.5- 12H), 1.75 (*d*, 6.5- 13H), 5.55 (*d*, 3.5- 1′H), 5.18 (dd, 3.5, 9.5- 2′H), 4.21 (*t*, 9.5- 3′H), 3.43 (*t*, 9.5- 4′H), 4.06 (dq, 6.5, 9.5 -5′H), 1.41 (*d*, 6.5-6′H)	130.5 (C-1C), 133.3 (CH- 2C), 121.2 (CHb- 3C), 133.2 (CH- 4C), 134.0 (Cc- 4a), 141.4 (Cc- 5aC), 143.7 (C-6C), 127.6 (CH- 7C), 129.3 (CHb-8C), 132. 1 (CH- 9C), 141.6 (Cc-9aC), 142.8 (Cc- 10a), 167.3 (C- 11a), 66.9 (CH- 12C), 23.6 (CH3-13C), 90.6 (CH- 1′C), 76.9 (CH- 2′C), 71.3 (CH-3′), 75.5 (CH- 4′), 67.7 (CH- 5′), 17.7 (CH3- 6′)	Pathirana et al. (1992)
Phenazine L-Quinovose Esters II	*Streptomyces* sp. CNB 253	8.25 (dd, 1.5, 6.9- 2), 7.87 (m- 3H), 8.38 (dd, 1.5, 8.8- 4H), 7.9 (m- 7H), 7.9 (m-8H), 8.17 (dd, 2.0, 8.0- 9H), 5.90 (*q*, 6.5- 12H), 1.75 (*d*, 6.5- 13H), 5.32 (*d*, 4.0- 1′H), 3.81 (dd, 4.0, 8.0- 2′H), 5.58 (*t*, 9.5- 3′H), 3.45 (*t*, 9.5- 4′H), 4.19 (dq, 6.5 9.5- 5′H), 1.41 (*d*, 6.5- 6′H)	131.3 (C- 1C), 132.5 (CH- 2C), 127.2 (CHb- 3C), 133.2 (CH- 4C), 139.4 (Cc- 4a), 141.5 (Cc- 5aC), 143.3 (C-6C), 127.4 (CH- 7C), 129.5 (CHb-8C), 132. 0 (CH- 9C), 141.8 (Cc-9aC), 143.0 (Cc- 10aC), 168.5 (C- 11C), 67.2 (CH- 12C), 23.7 (CH3- 13C), 92.3 (CH- 1′C), 70.9 (CH- 2′C), 79.7 (CH- 3′C), 73.9 (CH- 4′C), 67.5 (CH-5′C), 17.5 (CH3- 6′C)	Pathirana et al. (1992)

(continued)

Table 6.6 (continued)

Phenazine derivative	Organism	NMR frequency (δ)		Reference
		1H (ppm)	13C (ppm)	
Phenazine L-Quinovose Esters III	*Streptomyces* sp. CNB 253	8.27 (dd, 1.4, 6.8- 2H), 7.9 (m- 3H), 8.43 (dd, 1.4, 8.6- 4H), 7.9 (m- 7H), 7.9 (m- 8H), 8.21 (dd, 2.0, 8.2- 9H), 5.87 (*q*, 6.5- 12H), 1.75 (*d*, 6.5- 13H), 4.79 (*d*, 7.9- 1′H), 5.09 (dd, 7.9, 9.4- 2′H), 3.85 (*t*, 9.5- 3′H), 3.43 (*t*, 9.5- 4′H), 4.06 (dq, 6.5, 9.5- 5′H), 1.41 (*d*, 6.5- 6′H)	130.7 (C- 1C), 132.9 (CH- 2C), 127.3 (CHb- 3C), 133.8 (CH- 4C), 133.8 (Cc- 4a), 141.4 (Cc- 5a), 142.7 (C- 6C), 127.3 (CH- 7C), 129.3 (CHb- 8C), 132. 1 (CH- 9C), 141.6 (Cc- 9aC), 143.9 (Cc- 10aC), 167.6 (C- 11C), 66.9 (CH- 12C), 23.6 (CH3- 13C), 94.6 (CH- 1′C), 76.1 (CH- 2′C), 72.8 (CH- 3′C),75.0 (CH-4′C), 67.7 (CH- 5′C), 17.7 (CH3- 6′C)	Pathirana et al. (1992)
Phenazine L-Quinovose Esters IV	*Streptomyces* sp. CNB 253	8.25 (dd, 1.4, 6.8- 2H), 7.9 (m- 3H), 8.36 (dd, 1.0, 8.6 -4H), 7.9 (m- 7H), 7.9 (m- 8H), 8.16 (dd, 2.9, 8.2- 9H), 5.83 (*q*, 6.5- 12H), 1.75 (*d*, 6.5- 13H), 4.82 (*d*, 7.9- 1′H), 3.58 (dd, 7.9, 9.4- 2′H), 5.29 (*t*, 9.4- 3′H), 3.47 (*t*, 9.4- 4′H), 3.65 (dq, 6.5, 9.4- 5′H), 1.41 (*d*, 6.5- 6′H)	131.2 (C- 1C), 132.5 (CH- 2C), 127.2 (CHb- 3C), 133.2 (CH- 4C), 139.3 (Cc-4aC), 141.2 (Cc- 5aC), 143.3 (C- 6C), 127.5 (CH- 7C), 129.4 (CHb- 8C), 132. 0 (CH- 9C), 141.8 (Cc- 9a), 143.0 (Cc-10aC), 168.3 (C- 11C), 67.2 (CH- 12C), 23.7 (CH3- 13C), 96.4 (CH- 1′C), 73.0 (CH- 2′C), 81.5 (CH- 3′C), 73.6 (CH- 4′C), 71.8 (CH- 5′C), 17.5 (CH3- 6′C)	Pathirana et al. (1992)

(continued)

Table 6.6 (continued)

Phenazine derivative	Organism	NMR frequency (δ)		Reference
		1H (ppm)	13C (ppm)	
Phenazostatins C	*Streptomyces* sp. 833	8.18 (1H, ma- 2H), 7.68 (1H, dd, 8.1, 6.9- 3H), 8.02 (1H, dd, 8.1, 0.9- 4H), 7.71 (1H,m- 7H), 7.77 (1H,dd, 8.5, 7.0- 8H), 8.17 (1H, m- 9H), 7.04 (1H, q, 7.1- 12H), 2.00 (3H, d, 7.1- 13H), 4.10 (3H,s- H11- OCH3), 8.11 (1H, d, 7.3- 2′H), 7.58 (1H, d, 7.3- 3′H), 7.97 (1H, d, 7.8- 6′bH), 7.75 (1H, dd, 7.8, 7.0- 7′cH), 7.82 (1H, dd, 8.4, 7.0- 8′cH), 8.29 (1H, d, 8.4- 9′bH), 4.01 (3H, *s*- 11′-OCH3H)	131.0 *s* (1C), 132.0 *d* (2C), 127.8 *d* (3C), 134.0 *d* (4C), 142.0 *s* (4aC), 142.2 *s* (5aC), 145.5 *s* (6C), 128.3 *d* (7C), 130.7 *d* (8C), 128.6 *d* (9C), 144.0 *s* (9aC), 140.5 *s* (10aC), 167.2 *s* (11C), 34.0 *d* (12C), 21.0q q (13C), 52.7 *q* (11- CH3H), 129.2 s (1′C), 132.2 *d* (2′C), 126.0 *d* (3′C), 151.0 s s (4′C), 141.2 *d* (4′aC), 142.2 *d* (5′aC), 130.0 *d* (6′bC), 130.5 *d* (7′cC), 130.8 *d* (8′cH), 130.2 *d* (9′bH), 143.3 *s* (9′aH), 141.1 *s* (10′aH), 167.3 *s* (11′H), 52.6 *q* (11′-OCH3C)	Kim et al. (1999)
Phenazostatins A	*Streptomyces* sp. 833	8.31 (2H), 7.92 (3H), 8.49 (4H), 7.98 (7H), 7.80 (8H), 8.25 (9H), 7.04 (12H), 1.96 (13H), 4.13 (11-OCH3H), 7.28 (1′H), 7.72 (3′C), 8.15(4′C), 7.99 (6′b -H), 7.72 (7′c -H), 7.72 (8′c -H), 8.16 9′b -H),	130.9 *s* (1C), 132.2 *d* (2C), 128.8*d* (3C), 133.9*d* (4C), 141.9 *s* (4aC), 140.7 *s* (5aC), 140.6 *s* (6C), 126.7 *d* (7C), 130.5 *d* (8C), 129.8 *d* (9C), 143.6 *s* (9aC), 140.8 *s* (10aC), 167.2 *s* (11C), 72.2 *d* (12C), 23.2 *q* (13C), 52.7 *q* (11-OCH3C), 107.1 *d* (1′C), 159.2 *s* (2′C), 126.9 *d* (3′C), 130.4 *d* (4′C), 140.6 *s* (4′aC), 143.1 *s* (5′aC), 129.5 *d* (6′bH), 130.4 *d* (7′cC), 129.1 *d* (8′cC), 129.7 *d* (9′bC), 141.8 *s* (9′aC), 144.9 *s* (10′aC),	Kim et al. (1999)

(continued)

Table 6.6 (continued)

Phenazine derivative	Organism	NMR frequency (δ)		Reference
		1H (ppm)	13C (ppm)	
Phenazostatins D	*Pseudonocardia* sp. B6273	8.22 (2H), 7.78 (3H), 8.01 (4H), 7.66 (7H), 7.70 (8H), 8.03 (9H), 5.06 (1′H), 1.32 (2′H), 4.10 (OMeH)	131.2 (1C), 131.9 (2C), 128.3 (3C), 133.9 (4C), 141.7 (4aC), 142.6 (5aC), 145.5 (6C), 128.0 (7C), 130.7 (8C), 127.6 (9C), 143.7 (9aC), 140.4 (10aC), 38.0 (1′C), 19.1 (2′C), 167.3 (CO C), 52.6 (OMe C)	Maskey et al. (2003)
Pelagiomicin A	*Pelagiobacter variabilis*	–	124.9 (1C), 137.0 (2C), 131.0 (3C), 134.6 (4C), 142.6 (4aC), 142.7 (5aC), 125.1 (6C), 132.9 (7C), 107.6 (8C), 154.4 (9C), 56.6 (9-OCH3C), 132.8 (9aC), 138.4 (10aC), 165.9 (11C), 62.8 (12C), 174.1 (1′C), 62.6 (2′C), 70.9 (3′C), 25.1 (3 -CH3C), 26.2 (3′-CH3C)	Imamura et al. (1997)
Pelagiomicin B	*Pelagiobacter variabilis*	–	126.8 (1C), 135.1 (2C), 131.4 (3C), 133.7 (4C), 142.0 (4aC), 142.0 (5aC), 124.2 (6C), 133.7 (7C), 108.6 (8C), 154.3 (9C), 56.8 (9-OCH3C), 133.1 (9aC), 138.2 (10aC), 165.6 (11C), 63.1 (12C), 168.9 (1′C), 57.3 (2′C), 29.4 (3′C), 18.0 (3 -CH3C), 17.4 (3′-CH3C)	Imamura et al. (1997)
Pelagiomicin C	*Pelagiobacter variabilis*	–	125.7 (1C), 134.8 (2C), 130.8 (3C), 133.5 (4C), 141.4 (4aC), 141.3 (5aC), 124.0 (6C), 132.2 (7C), 108.1 (8C), 153.4 (9C), 56.3 (9-OCH3C), 132.3 (9aC), 137.5 (10aC), 165.0 (11C), 62.0 (12C), 167.2 (1′C), 39.5 (2′C)	Imamura et al. (1997)

(continued)

Table 6.6 (continued)

Phenazine derivative	Organism	NMR frequency (δ)		Reference
		1H (ppm)	13C (ppm)	
PD 116	*Streptomyces* sp. NRRL 15783	2.04 (3H, *s*), 3.86 (3H, *s*), 6.61 (1H, *d*, $J = 8.0$ Hz, 3-H), 8.03 (1H, *d*, $J = 8.0$, 2-H), 9.83 (1H, *s*)	8.14, 52.10, 105.22, 110.35, 113.05, 119.96, 130.90, 133.62, 133.95, 137.94, 139.90, 156.25, 157.33, 166.20, 170.85, 194.24	Smitka et al. (1986)
Bethocyanin A	*S. prunicolor*	6.96 (1H), 6.76 (4H), 7.53 (6H), 7.66 (7H), 8.30 (8H), 7.81 (14, 18H), 7.48 (15, 17H), 7.38 (16H), 4.87 (19H), 5.16 (20H), 2.13 (22H), 2.13 (23H), 5.00 (24H), 1.94 (26H), 1.57 (27H), 1.52 (28H)	102.9 (1C), 158.6 (2C), 140.2 (3C), 92.5 (4C), 133.3 (4aC), 132.2 (5aC), 117.8 (6C), 131.0 (7C), 128.4 (8C), 125.3 (9C), 134.6 (9aC), 150.2 (10aC), 112.0 (11C), 168.3 (12C), 131.4 (13C), 128.1 (14, 18C), 129.1 (15,17C), 128.9 (16C), 47.1 (19C), 115.9 (20C), 143.6 (21C), 39.7 (22C), 26.5 (23C), 123.1 (24C), 132.8 (25C), 17.4 (26C), 25.8 (27C), 17.9 (28C), 166.3 (29C)	Shin-Ya et al. (1993)
Bethocyanin B	*S. prunicolor*	6.71 (4H), 7.43 (6H), 7.67 (7H), 7.42 (8H), 7.80 (9H), 7.77 (14, 18 H), 7.46 (15, 17 H), 7.37 (16 H), 4.92 (19H), 5.15 (20H), 2.12 (22H), 2.12 (23H), 4.98 (24H), 1.94 (26H), 1.55 (27H), 1.51 (28H)	101.4 (1C), 161.1 (2C), 140.2 (3C), 92.4 (4C), 132.4 (4aC), 131.7 (5aC), 113.7 (6C), 132.7 (7C), 125.1 (8C), 127.9 (9C), 128.0 (9aC), 148.3 (10aC), 110.9 (11C), 167.4 (12C), 130.8 (13C), 128.7 (14, 18C), 128.9 (15, 17C), 127.9 (16C), 46.8 (19C), 115.7 (20C), 143.2 (21C), 39.4 (22C), 26.1 (23C), 122.9 (24C), 132.7 (25C), 17.1 (26C), 25.6 (27C), 17.7 (28C), 162.1 (29C)	Shin-Ya et al. (1993)

(continued)

Table 6.6 (continued)

Phenazine derivative	Organism	NMR frequency (δ)		Reference
		1H (ppm)	13C (ppm)	
Bethocyanin C	*S. prunicolor*	6.25 (4H), 7.04 (6H), 7.31 (7H), 7.15 (8H), 7.30 (9H), 7.50 (14, 18H), 7.44 (15, 17H), 7.43 (16H), 4.39 (19H), 4.95 (20H), 1.97 (22H), 1.97 (23H), 4.97 (24H), 1.39 (26H), 1.64 (27H), 1.56 (28H), 14.54 (10-NH H), 15.70 (29-OH H)	95.2 (1C), 180.3 (2C), 139.7 (3C), 101.3 (4C), 130.3 (4aC), 130.4 (5aC), 113.5 (6C), 128.4 (7C), 123.6 (8C), 119.1 (9C), 123.9 (9aC), 148.8 (10aC), 110.8 (11C), 120.9 (12C), 135.4 (13C), 129.7 (14, 18C), 129.5 (15, 17C), 129.1 (16C), 45.9 (19C), 116.1 (20C), 142.4 (21C), 39.2 (22C), 26.2 (23C), 123.1 (24C), 132.2 (25C), 16.6 (26C), 25.7 (27C), 17.7 (28C), 172.1 (29C)	Shin-Ya et al. (1993)

nH hydrogen ion position; *nC* carbon ion position

Table 6.7 Mass profile of phenazine in various bacteria

Phenazine derivative	Organism	Mass (m/z)	Ionization/ Analytical techniques	Reference
Phenazine -1 – Carboxylic acid	*P. aurantiaca*	225	MALDI-TOF Vertical MS (Analyzer)	Samina et al. (2009)
	S. anulatus	225	LC MS	Saleh et al. (2012)
	Pseudomonas spp.	224.06	ESI MS	Sayed et al. (2008)
	P. aeruginosa	224.06	HR EI-MS	Lee et al. (2003)
	P. aeruginosa	224.1	ESI MS	Denning et al. (2003)
Pyocyanin	*P. aeruginosa*	212	Desorption EI/ MS	Wilson et al. (1987)
		210	Thermospray LC/ MS	Watson et al. (1986)
		211.08	Mass Spectrometry	Rada et al. (2008)
Phenazine 1-carboxamide (PCN)	*P. chlororaphis* PCL 1391	223	Single quadrupole MS	Chin-A-Woeng et al. (1998)
	P. aeruginosa	223.3	FI-MS	Shanmugaiah et al. (2010)
		223	–	Kumar et al. (2005)
1-hydroxy phenazine	*P. aeruginosa*	197	HPLC/MS, Thermospray (TSP) MS	Wilson et al. (1987)
		196	GC/EI MS Desorption EI/ MS	Watson et al. (1986)
		196	EI MS	Kerr et al. (1999)
		196	EI MS	Saosoong et al. (2009)
DOB-41	*Pseudomonas spp.*	37	EI-MS	Shoji et al. (1988)
2-hydroxy phenazine	*P. aurantiaca*	197	MALDI-TOF Vertical MS	Samina et al. (2009)
PD (116,152)	*Streptomyces lomondensis*	328.0704	EI-MS m/z	Smitka et al. (1986)
SB (212305)	*Streptomyces sp.*	459	Ion Spray MS	Gilpin et al. (1995)

(continued)

Table 6.7 (continued)

Phenazine derivative	Organism	Mass (m/z)	Ionization/ Analytical techniques	Reference
Chromophenazine A	*Streptomyces sp. Ank 315*	329.16498	HRESIMS	Zendah et al. (2012)
Chromophenazine B		373.15459		
Chromophenazine C		308.13960		
Chromophenazine D		398.16241		
Chromophenazine E		501.18201		
Chromophenazine F		524.19472		
1,6-dihydroxy phenazine	*Streptosporangium* sp.	212.05	EI-MS	Patel et al, 1984
Endophenazine A	*S. anulatus*	292.12	EIMS	Krastel and Zeeck (2002)
		293	LC MS	Saleh et al. (2012)
Endophenazine B		323	HPLC–ESI–MS	Saleh et al. (2012)
Endophenazine C		309	DCI-MS	Krastel and Zeeck (2002)
Endophenazine D		284.0797	EI-MS	Krastel and Zeeck (2002)
Endophenazine E	*S. coelicolor* M512	421.186790	HR MS	Saleh et al. (2012)
Phenazinolin A	*S. diastaticus* YIM DT26	409.12	HR-ESI–MS	Ding et al. (2011)
Phenazinolin B		393.13		
Phenazinolin C		409.12		
Phenazinolin D		469.11		
Phenazinolin E		469.11		
Benthocyanin A	*S. prunicolor*	493.2071	HRFAB-MS	Shin-ya et al. (1993)
Benthocyanin B		493.2106		
Benthocyanin C		492.2279		
Phenazostatin D	*Pseudonocardia* sp. B6273	531	DCI-MS	Maskeya et al. (2003)
		530	EI-MS	
		530.19	HREIMS	
Saphenic acid	*S. antibioticus* TU 2706	268.27	EIMS	Geiger and Schierlein (1988)
Saphenamycin		402.41		
Griseolutein A	*S. griseoluteus*	342.3	–	Nakamura (1958)
Griseolutein B		344.31		
Phenazine derivative Compound (2)	*Streptomyces* sp. ICBB8198	467.1850	HRESIMS	Fotso et al. (2010)
Phenazine derivative Compound (3)	*Streptomyces* sp. ICBB8198	436.16326	HREIMS	
DC-86-M	*S. luteogriseus* DO-86	326	EI-MS	Takahashit et al. (1986)

(continued)

Table 6.7 (continued)

Phenazine derivative	Organism	Mass (m/z)	Ionization/ Analytical techniques	Reference
Griseoluteic acid	*Streptomyces* strain P510	284.09	HR-ESI MS	Wang et al. (2011)
Diphenazithionin	*S. griseus* ISP 5236	611.0873	HR- FAB- MS	Hosoya et al. (1996)
Pelagiomicin A	*Pelagiobacter variabilis*	400. 1509	HR-FAB-MS	Imamura et al. (1997)
3′-O-Quinovosyl Saphenate	*Streptomyces* sp. CNB-253	415.1473	HRCIMS	Pathirana et al. (1992)
2′-O-Quinovosyl Saphenate		415	LRCIMS	
Methanophenazine	*Methanosarcina* sp.	538.3930	EI-HRMS	Abken et al., 1998
Phenazine derivative	*Bacillus* sp.	291.1143	HRESIMS	Li et al. (2007)
Aeruginosins 98 A	*M. aeruginosa* NIES-98	609.32	–	Ishida, et al. (2009)
Aeruginosins B		575.40		

mass for a secondary metabolites have been studied earlier (Smedsgard and Frisvad 1996). Similarly bacterial phenazines were studied for mass analysis using different ioniozation techniques in mass spectroscopy. Table 6.7, enlists mass profile of different phenazines in corresponding producing bacteria by various ionizing techniques.

6.4 Conclusion

The application of electrophoretic and spectroscopic analysis is a useful tool to identify and indicate phenazine biosynthesis during growth phase of different bacteria. The analysis of phenazine is useful to determine the biodiversity of metabolite with different bacteria in different environmental conditions as well as to isolate and determine the biologically active metabolite. The data summarized herewith is mostly focused on phenazine isolation and characterization by *Pseudomonas* sp. and *Streptomyces* sp. however; other bacterial species like Methanobacteria and *Bacillus* sp. have also been explored. To our knowledge this is the first complete compiled report on different bacterial phenazines. Thus unrelated phenazine to above information may possibly represent new phenazine.

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Chapter 7
Roles and Regulation of Phenazines in the Biological Control Strain *Pseudomonas chlororaphis* 30-84

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Abstract Pseudomonads are well known for the production of diverse secondary metabolites, including phenazines that are essential for the control of plant diseases. Emerging evidence demonstrates that phenazines play multiple roles in the ecological fitness of the producing microbe. It is now understood that phenazines modify cellular redox state, act as electron shuttles altering electron flow patterns, contribute to biofilm formation and architecture, act as cell signals that regulate patterns of gene expression, and contribute to the survival of the producer. When associated with eukaryotic hosts, phenazines modify numerous host cellular responses. Of particular interest in defining their functional impact are the observations that bacterial species may produce different types of phenazines and many produce more than one phenazine derivative. The amount of each derivative produced may change during growth or in response to the intracellular or extracellular environment. Furthermore, environmental conditions influence the redox state of the derivatives, altering their functionality. The regulatory complexity governing phenazine production likely reflects the complexity of the roles they play for the producing microbe. Here, we highlight briefly some of the roles phenazines play in the biological control activity of pseudomonads belonging to the *Pseudomonas fluorescens* subgroup, with special emphasis on *Pseudomonas chlororaphis* 30-84. We also review the regulatory network governing phenazine production in

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P. chlororaphis 30-84, with special emphasis on how the different regulatory controls function in terms of the signals to which they respond and the suites of genes they control. Despite the recognized roles phenazines play in bacterial fitness and biological control, growing evidence suggests that phenazine non-producing phenotypic variants defective in the GacS/GacA two component regulatory system are common components of plant-associated bacterial communities. We provide additional discussion on why these phenotypic variants should not be overlooked.

7.1 Phenazine Producers

Phenazines are produced by a variety of Eubacteria including members of the genera *Brevibacterium, Burkholderia, Erwinia, Nocardia, Pantoea, Pseudomonas, Pelagiobacter, Sorangium, Streptomyces*, and *Vibrio* (see Mavrodi et al. 2006; Mavrodi et al. 2010; Mentel et al. 2009; Turner and Messenger 1986; Choi et al. 2009). Additionally, a member of the Archaea, *Methanosarcina*, was shown to produce a phenazine derivative (Abken et al. 1998). The fluorescent pseudomonads comprise the most well-documented group of phenazine-producers, of which two distinct subgroups have been studied: the *Pseudomonas aeruginosa* subgroup and the *Pseudomonas fluorescens* subgroup containing Pseudomonas chlororaphis subspecies *aureofaciens* and other closely related species. *P. aeruginosa* is characterized as an opportunistic pathogen and the *P. aeruginosa* subgroup includes both clinical and environmental isolates. Phenazine production by *P. aeruginosa* is discussed in detail in other chapters (see Chap. 3). Several members of the *P. fluorescens* subgroup have been studied extensively due to their beneficial properties as biological control agents and are the focus of this chapter.

Biological control pseudomonads are known to produce a diversity of phenazine structures and many of these bacteria produce more than one phenazine derivative. Bioinformatic analysis of phenazine biosynthesis clusters from multiple genera and species indicate that the basic phenazine biosynthetic 'core' is comprised of five highly conserved genes (Mavrodi et al. 2006; Mentel et al. 2009; Gross and Loper 2009), and that these genes may have become distributed among diverse bacterial genera via horizontal transmission (Mavrodi et al. 2010). In most bacteria, the regions flanking the five core biosynthetic genes contain one or more additional genes that encode enzymes that subsequently modify the basic phenazine heterocyclic three-ring structure by adding different side groups that alter the properties of the final phenazine derivative. Thus, differences in the biological activities of the phenazines may be responsible in part for the varied biological control attributes of the different phenazine producing strains.

7.2 Roles of Phenazines in Biological Control

Emerging evidence that phenazines play multiple roles in the ecological fitness of the producing bacterium has been discussed previously in several excellent reviews (Chin-A-Woeng et al. 2003; Mavrodi et al. 2006, 2010, 2013; Pierson and Pierson 2010) as well as elsewhere within the chapters of this book. Phenazine production among biological control strains contributes to multiple aspects of disease control including pathogen inhibition, rhizosphere competence, and biofilm formation. These roles are discussed briefly below.

7.2.1 Pathogen Inhibition

Phenazines are diffusible compounds that in laboratory studies may be observed to become attached to or taken up by eukaryotic hosts including target fungi and even plant roots (Pierson et al. unpublished), and more recently to bind to extracellular DNA (Das et al. 2013). Although the mechanisms for the action of phenazines in antifungal activities may vary depending on the type of phenazine, a major mechanism is thought to be the generation of toxic reactive oxygen species (ROS) in other organisms or specific tissues, which has been demonstrated using *P. aeruginosa* (Turner and Messenger 1986; Majahan et al. 1999; Lau et al. 2004). This ability has been attributed to the capacity of the aromatic ring structure of the phenazine molecule to accept and donate electrons. Early studies on cellular respiratory chains demonstrated the ability of phenazines to uncouple oxidative phosphorylation by shunting electrons from similar pathways in mammalian cells (Stewart-Tull and Armstrong 1971) and in *Bacillus subtilis* (Bisschop et al. 1979). Several subsequent reviews describe the formation of ROS and oxidative stress by phenazines, their roles in virulence and competition (Laursen and Nielsen 2004; Mavrodi et al. 2006), and how the structure of phenazine derivatives affects their function as redox active natural products (Beifuss and Tietze 2005). Phenazine production by soil-borne bacteria contributes to antifungal activity against a diversity of plant pathogenic fungi (Chin-A-Woeng et al. 2003; Mavrodi et al. 2006; Pierson and Pierson 2010). Although biocontrol agents may share the ability to produce phenazines, they differ in the types of phenazines they produce, the pathogens they inhibit, and the hosts they colonize and protect. For example, *P. fluorescens* 2-79 produces phenazine-1-carboxylic acid (PCA) and was shown to limit the ability of the actinomycete fungus *Gaeumannomyces graminis* var. *tritici* to cause to take all disease on wheat (Thomashow and Weller 1988). P.chlororaphis strain 30-84, also used to control take all on wheat, produces three phenazines, PCA, 2-hydroxyphenazine-1-carboxylic acid (2OHPCA), and 2-hydroxyphenazine (2OHPZ) (Pierson and Thomashow 1992). In contrast to *P. fluorescens* 2-79, the genome of *P. chlororaphis* contains *phzO*, a gene that encodes a monooxygenase, located immediately downstream of the core genes

(Delaney et al. 2001). The presence of PhzO converts a small amount (~10–30 %) of the yellow PCA into the bright orange 2OHPCA. Additionally, a third minor derivative, 2OHPZ, is generated spontaneously from 2OHPCA (Delaney et al. 2001; Maddula et al. 2006). Another biological control strain, *P. chlororaphis* PCL1391, isolated from tomato, was shown to produce phenazine-1-carboxamide (PCN) and some PCA (Chin-A-Woeng et al. 1998). *P. chlororaphis* PCL1391 and *P. aeruginosa* PAO1 contain *phzH*, encoding a putative transamidase that converts a portion of PCA into PCN, enabling these strains to produce both PCA and PCN (Chin-A-Woeng et al. 2001). PCN effectively controlled *Fusarium oxysporum* f. sp. *radicis lycopersici*, the causative agent of tomato foot and root rot. The production of both PCA and PCN by *P. aeruginosa* PNA1 was shown to contribute to the control of *Pythium myriotylum*, the causative agent of root rot of cocoyam (Tambong and Höfte 2001). Additionally, pyocyanin (PYO) produced by *P. aeruginosa* LEC1 was effective in the inhibition of *Septoria tritici* of wheat (Flaishman et al. 1990). Two genes in *P. aeruginosa* PAO1, *phzM*, encoding a methyltransferase (Parsons et al. 2007), and *phzS*, encoding a flavin-containing monoxygenase, together are responsible for the conversion of PCA to PYO (Greenhagen et al. 2008; Mavrodi et al. 2001) whereas PhzS alone can facilitate conversion of PCA to 1-hydroxyphenazine (1OHPZ).

There is growing evidence supporting the hypothesis that the activity and specificity of phenazines in antifungal interactions is related to the type and relative proportion of the phenazine derivatives produced as well as their charge or redox state in the environment. For example, Maddula et al. (2006) demonstrated that changes in the relative amounts of the phenazine derivatives produced by *P. chlororaphis* 30-84 significantly altered pathogen inhibition. Since the phenazine modifying enzyme encoded by the gene *phzO* is responsible for the conversion of PCA to 2OHPCA, this alteration was brought about by varying the level of *phzO* expression. Two derivatives of 30-84 were constructed. The first derivative (30-84PCA) was mutated in *phzO* and produced only PCA, whereas the second derivative (30-84O*) contained additional copies of *phzO in trans* and overproduced 2OHPCA (Maddula et al. 2008). Comparison of wild type 30-84 to 30-84PCA and 30-84O* demonstrated that the 2OHPCA overproducing derivative and the derivative producing only PCA were significantly more and less effective in pathogen inhibition in vitro than the wild type, respectively (Maddula et al. 2008). Subsequently, using 30-84PCA, it was demonstrated that the expression of alternative terminal modifying genes (e.g., *phzS, phzM, phzH*) from *P. aeruginosa* introduced separately *in trans* altered the spectrum phenazines produced by 30-84 and the range of fungal pathogens 30-84 inhibited in laboratory assays (Yu et al. 2012). These reports reinforce the importance of the type and relative proportion of the phenazines produced for the antifungal activity of the phenazine-producing microbe.

Intracellular and extracellular environmental conditions play roles in the amount of phenazines produced as well as their activity. It is well established that in *P. fluorescens* and P. chlororaphis the amount of phenazines produced is influenced by growth medium and soil conditions including pH, temperature,

carbon and nitrogen source, the concentration of oxygen and various minerals, and other biologically active compounds (e.g., fusaric acid) (Mavrodi et al. 2006; Park et al. 2011; van Rij et al. 2004, 2005). An interesting caveat to these observations is the demonstration by Chin-A-Woeng et al. (1998) that environmental conditions such as pH affect the antifungal activity of specific phenazine derivatives produced, presumably by altering which ions are present. As discussed by Chin-A-Woeng et al. (2003), this finding suggests that the ionic form or even redox state may influence antifungal activity directly or via phenazine solubility in water or the plant associated matrix. Recently, Chen et al. (2013) examined the effects of substituent groups on the energetic and electrochemical behaviors of several phenazine derivatives using quantum chemical calculations. Their findings revealed that the redox potentials and the free energy of the reactions were affected by the location of substituent groups on the phenazine ring structure and by the surrounding protonated "water clusters" in aqueous culture, i.e., electron-donating and electron-withdrawing groups interacted differently. These observations are supported by recent studies showing that the environmental pH influences the relative antimicrobial activity of the different phenazine derivatives a microbe produces. For example, Cezairliyan et al. (2013) showed that although *P. aeruginosa* PA14 produces three different phenazine derivatives (PCA, 1OHPZ, and PYO) capable of killing nematodes, the three phenazines differ in terms of the pH range over which the phenazines are toxic to nematodes. They showed that PCA, the primary toxic agent against *Caenorhabditis elegans*, was most detrimental when the growth medium was acidified, whereas PYO was not toxic under acidic conditions and 1OHPZ was produced at concentrations too low to kill *C. elegans*.

7.2.2 Phenazines, Rhizosphere Competence, and Biofilm Formation

The production of phenazines confers a selective advantage to phenazine producing strains competing with other microorganisms in the rhizosphere (Mazzola et al. 1992). The contribution of phenazines to rhizosphere competence was demonstrated by comparing the survival of wild type *P. chlororaphis* 30-84 and *P. fluorescens* 2-79 to that of mutants deficient in phenazine production over repeated wheat harvest cycles. Phenazine production was strongly correlated with bacterial persistence in natural soil in the presence of the indigenous microbial community (Mazzola et al. 1992). In contrast, no significant differences were observed in the sizes of wild type and phenazine mutant populations over time in sterile soil. Although the exact reasons for this advantage are still unclear, these results are consistent with the commonly held belief that phenazines function primarily as antimicrobial compounds and hence confer the greatest advantage in the presence of a competitive rhizosphere community. However, evidence from numerous studies suggests that phenazines typically are ineffective at inhibiting the growth of co-occurring rhizosphere colonizing bacteria, probably their most

immediate competitors, although this varies with the type of phenazine produced (Fernando et al. unpublished; Beifuss and Tietze 2005; Mavrodi et al. 2006; Dwivedi et al. 2009). The observation that antibiotic-producing and non-producing bacteria appear to harbor resistance to phenazines supports the general hypothesis nicely stated by O'Brien and Wright (2011) that over evolutionary time, antibiotics probably provided producers with a strong selective advantage in nutrient poor environments and thus the 'resistome' (sensu D'Costa et al. 2006) present today is "highly evolved, widespread, and probably ancient."

More recent studies suggest that the ability to produce phenazines has a more pronounced effect on rhizosphere competence under dryland agriculture. These studies revealed that rhizosphere *Pseudomonas* populations containing phenazine biosynthesis genes dominated on dryland winter wheat, whereas under irrigation only 8–50 % of plants harbored pseudomonads containing phenazine genes (Mavrodi et al. 2012b, 2013). In related papers (Mavrodi et al. 2012a, 2013), the authors showed that the amount of phenazine-1-carboxylic acid produced by the rhizosphere population was quite high (34.6–557.6 ng/g root) in dry soils, providing further evidence that phenazine production was favored under these conditions.

An important aspect of rhizosphere competence is the ability to rapidly colonize and persist on plant roots. The formation of bacterial biofilms, i.e., bacterial aggregates contained within a self-formed polysaccharide matrix, on plant surfaces is an essential component of bacterial persistence (Davey and O'Toole 2000; Ramey et al. 2004). Maddula et al. (2006) were the first to demonstrate that phenazines also contribute to biofilm formation and structure. Using fluorescence microscopy and microscopic chambers known as flow cells, they compared biofilm formation by wild type P. chlororaphis 30-84 to that of mutants deficient in phenazine production. Derivatives of strain 30-84 defective in phenazine production included those with disruptions of the phenazine biosynthetic operon as well as disruptions of the quorum sensing genes *phzR* and *phzI*, known to regulate phenazine production. Both types of derivatives of 30-84 were unable to establish biofilms even after 6 days. The expression of additional functional copies of *phzR/phzI* introduced *in trans* did not restore the ability of the phenazine structural mutant to adhere or develop biofilms. In contrast, constitutive expression of the genes encoding the phenazine biosynthetic operon resulted in extensive biofilm formation prematurely (e.g., after 1–3 days) in both mutants, indicating that phenazines and not quorum sensing *per se* were responsible for the deficiency in the capacity to form biofilms. Furthermore, addition of purified phenazines to the growth medium in a static biofilm assay restored biofilm formation to the phenazine biosynthetic mutant. Quorum sensing (described further under regulation) has been shown to play critical roles in many microbe–microbe and microbe-host interactions, both pathogenic and beneficial (reviewed in Fuqua et al. 2001). Although many Gram-negative bacteria are dependent on quorum sensing for normal biofilm development, the work by Maddula et al. (2006) definitively identified which quorum sensing-regulated traits were contributing.

In subsequent experiments (Maddula et al. 2008), comparison of wild type 30-84 to 30-84PCA and 30-84O* demonstrated that surface attachment to glass by the 2OHPCA overproducer (30-84O*) occurred more quickly and uniformly compared to the derivative producing only PCA (30-84PCA) or the wild type (44 % total coverage compared to 1 % by 30-84PCA or the wild type after 45 min). The 2OHPCA overproducer also formed thicker biofilms than the wild type, although the total cell biovolume was similar. In contrast, the rate of surface attachment by 30-84PCA was similar to that of the wild type, but 30-84PCA produced a thicker biofilm with a 4-fold higher biovolume of cells than either the wild type or 30-84O*. Interestingly, counts of cells dispersing from the biofilm revealed that significantly fewer cells dispersed from the 30-84O* biofilm than the 30-84PCA or 30-84 biofilm, suggesting that phenazine-mediated differences in surface attachment, reproduction and survival, and dispersal may contribute to biofilm architecture. These observations on the differential roles of specific phenazine derivatives in antifungal activity and biofilm formation support the hypothesis that bacteria may produce different phenazine structural derivatives in specific concentrations due to the diverse functions they serve in the producing organism.

7.3 Regulation

The production of secondary metabolites, including phenazines, is dependent on intracellular metabolic conditions including the availability of metabolic precursors and other inducers, bacterial growth rate, and developmental stage, as well as extracellular conditions, which in turn influence the intracellular environment. Almost all studies on phenazine regulation to date have focused on pseudomonads. These studies show that different pseudomonads utilize combinations of conserved regulatory systems integrated into sensory networks to control phenazine production (reviewed in Mavrodi et al. 2006; Mentel et al. 2009; Gross and Loper 2009; Pierson and Pierson 2010). The regulatory complexity governing phenazine production likely reflects the complexity of the functions the types of phenazines produced play for the producing microbe (Pierson and Pierson 1996). Further, since the roles phenazines play for different producers may not be the same, the regulatory hierarchy controlling them would be expected to differ as well. Thus, comparative analysis of gene regulation in more than one or two experimental systems is required to gain a more complete picture of how the sensory network functions to regulate phenazines in response to environmental conditions and in a manner consistent with their roles in bacterial fitness. A general description of the regulation of phenazine synthesis with special emphasis on *P. aeruginosa* is provided in Chap. 3 of this volume. In the following section, we review the regulatory network governing phenazine production in *P. chlororaphis*, with special emphasis on how the different regulatory controls function in terms of the signals to which they respond and the suites of genes they control.

7.3.1 PhzR/PhzI

Phenazine biosynthesis in P. chlororaphis and *P. fluorescens* is regulated directly by the PhzR/PhzI quorum sensing system (Chin-A-Woeng et al. 2001; Wood and Pierson 1996; Mavrodi et al. 1998; Khan et al. 2007). PhzR is a member of the LuxR family of transcriptional regulators and it activates the expression of the phenazine biosynthetic genes in response to the accumulation of an *N*-acyl-homoserine lactone (AHL) signal. Mutants defective in *phzR* are deficient in phenazine biosynthesis, and the addition of multiple copies of *phzR* expressed *in trans* significantly increased phenazine production in the wild type (Pierson et al. 1994; Khan et al. 2007). PhzI is a LuxI homolog and encodes an AHL synthase. In *P. fluorescens* 2-79, *P. chlororaphis* 30-84 and PCL 1391, *phzI* and *phzR* are located immediately upstream of the phenazine biosynthetic operon. In *P. fluorescens* 2-79, PhzI is responsible for the synthesis of six AHL signals (Khan et al. 2007). It was originally reported that *P. chlororaphis* strains 30-84 and PCL 1391 produced and responded predominantly to the signal *N*-hexanoyl-homoserine lactone (HHL) (Chin-A-Woeng et al. 2001; Wood et al. 1997). In subsequent work, it was shown that *phzI* in *P. fluorescens* 2-79, *P. chlororaphis* 30-84, and *P. chlororaphis* PCL1391 produce nearly the same AHL signals, with the 3-hydroxyhexanoyl (OHHL) signal being the most predominant (Khan et al. 2007). Furthermore, although the PhzR protein of 30-84 appeared to respond equally to HHL and OHHL, only OHHL was produced at levels high enough to activate PhzR under the laboratory conditions examined. Although the availability of signal-specific precursors is thought to be the mechanism determining the types and amounts of the specific signal derivatives produced, it is unknown how the metabolic state of the cell or other intracellular or extracellular conditions factors into the specificity of signal production. AHLs effectively activate phenazine biosynthetic gene expression in 30-84 and 2-79 at 1 nM concentration (Wood et al. 1997; Khan et al. 2007). AHL accumulation is also important for phenazine expression on wheat roots; inactivation of *phzI* in 30-84 resulted in a 1,000-fold decrease in *phzB* expression on roots (Wood et al. 1997). *P. chlororaphis* 30-84 also has a second quorum sensing system, CsaR/CsaI, that contributes to exoprotease production and cell aggregation, but plays a minor role in phenazine regulation under laboratory conditions (Zhang and Pierson 2001).

Phenazine production on wheat roots is influenced by signal production from other members of the microbial community. This was first demonstrated using a genomic reporter of 30-84 containing a disruption of the *phzI* gene and the ice nucleation reporter gene *inaZ* fused to one of the phenazine biosynthetic genes (e.g., *phzB::inaZ* fusion) (Pierson et al. 1998). When grown in a mixture with a second derivative of 30-84 that could produce signal but did not have ice nucleation activity, AHL production by the signal donor was sufficient to induce ice nucleation activity by the reporter on plant roots. These results demonstrated that signal exchange between bacterial populations could activate phenazine gene expression on plant surfaces. Using this reporter to screen a collection of isolates

from wheat plants collected from three geographically separate locations, it was revealed that approximately 8 % of the isolates were capable of producing signals that complemented the signal deficiency of the reporter (Pierson et al. 1998). Some of these isolates, all *Pseudomonas* species, were included in assays that demonstrated their ability to restore phenazine gene expression by the reporter to wild type levels on wheat roots. Additionally, a second subset of approximately 7 % of the isolates from the same rhizosphere collection prevented or reduced phenazine production by 30-84 in vitro (Morello et al. 2004). Some of these strains produced diffusible products that could be collected in cell-free supernatants and negatively affected phenazine production. For example, 30-84 grown in conditioned medium containing diffusible products from several different strains produced lower amounts of phenazines (1.5–9-fold) compared to growth in conditioned media from an AHL nonproducing derivative of strain 30-84. Growth of the *phzB::lacZ* reporter strain 30-84Z in this conditioned medium resulted in decreased *lacZ* expression (4 to 9-fold) as compared to growth in conditioned medium from the control strain, indicating that inhibition of *phzB* occurred at the level of gene expression (Morello et al. 2004). These studies confirmed the importance of quorum sensing signaling for phenazine production in the rhizosphere and the potential for other members of the rhizosphere community to positively or negatively influence phenazine gene expression. The observations that PhzI is capable of producing multiple signal derivatives in varying amounts and that PhzR is differentially responsive to these signals highlight the potential for modulating the production of phenazines and other secondary metabolites controlled by quorum sensing via population and community signal production.

7.3.2 GacS/GacA

In *Pseudomonas,* GacS/GacA regulate the expression of genes involved in communication (e.g., quorum sensing), the production of secondary metabolites (including phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, hydrogen cyanide, and the phytohormone indole-3-acetic acid), extracellular enzymes, and several carbon storage compounds as well as an oxidative stress response and other functions important for biological control or plant pathogenicity (reviewed in Hassan et al. 2010). In P. chlororaphis 30-84, mutations in *gac* resulted in loss of phenazine, hydrogen cyanide, exoprotease, lipase, gelatinase, and AHL production as well as deficiencies in pathogen inhibition, biofilm formation, and rhizosphere competence (Chancey et al. 1999; Maddula et al. 2006). Global regulation by Gac in many *Pseudomonas* species operates through the small RNA-binding proteins RsmA and RsmE, members of the RsmA/CsrA family (Reimmann et al. 2005; Lapouge et al. 2008). These small RNA-binding proteins act as negative regulators of gene expression by binding to 5′-GGA-3′ motifs located in the 5′ leading sequence of target mRNAs (Blumer et al. 1999; Heeb and Haas 2001; Heeb et al. 2002). The binding of these proteins reduces target protein levels either by a

reduction in mRNA translation (by blocking the recruitment of the 30S ribosomal subunit), by a reduction in mRNA stability (by targeting messages for degradation), or both (Dubey et al. 2003). Gac also is required for the expression of the small regulatory non-coding RNAs (ncRNAs), *rsmX*, *rsmY*, and *rsmZ* (Humair et al. 2010; Lapouge et al. 2008). These ncRNAs have multiple stemloop structures that interact with RsmA and RsmE, sequestering them and relieving post-transcriptional repression (Babitzke and Romeo 2007). Despite the significant research on Gac/Rsm regulation in *Pseudomonas*, its role in the regulation of phenazine biosynthesis and its relationship to other regulatory pathways controlling phenazines are less understood.

In recent work (Wang et al. 2013), the functionality of major components of the Gac/Rsm signal transduction pathway in the regulation of phenazine production by strain 30-84 was identified and characterized (Fig. 7.1). Analysis of the genomic sequences of *rsmX*, *rsmY*, and *rsmZ* in *P. chlororaphis* 30-84 revealed the presence of a conserved palindromic consensus sequence that was typical of GacA-controlled ncRNA genes and a conserved −10 hexamer (5′-TAATCT-3′) that was

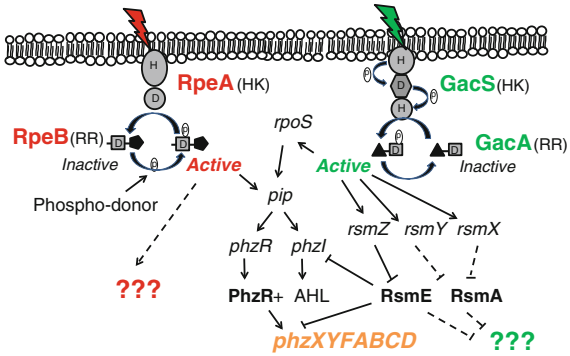

Fig. 7.1 Proposed model for the regulation of phenazine biosynthesis by the Gac-Rsm system in *P. chlororaphis* strain 30-84. The model shows the relationship between the RpeA/RpeB and GacS/GacA systems and other known regulators of phenazine production. GacS is predicted to be a transmembrane protein based on amino acid sequences. Amino acids H (histidine) and D (aspartate) involved in phosphorylation are indicated. *Solid arrows* point to genes that are regulated by other genes. *Dashed arrows* indicate unknown or as yet uncharacterized regulatory pathways. GacA positively controls the expression of *rsmX*, *rsmY*, and *rsmZ*. In turn, *rsmZ* activates phenazine production, probably by titrating the translation suppressor RsmE. Although the transcript abundance of *rsmA* was significantly higher and the transcript abundances of *rsmY* and *rsmX* were significantly lower in the *gacA* mutant compared to wild type 30-84, neither RsmA nor these two ncRNAs appeared to play a role in the regulation of phenazines under the experimental conditions tested. It is currently unknown what other genes, if any, RsmA controls. A functional GacA is also required for increased expression of other phenazine regulatory genes including *rpoS*, *pip*, and *phzR/phzI*. RpeA is predicted to be a transmembrane protein. RpeB positively controls the expression of *pip*, which in turn activates phenazine production through the PhzI/PhzR quorum sensing system. RpeA has net phosphatase activity on RpeB when growing in liquid medium. In the absence of RpeA, RpeB is possibly over-phosphorylated by small phospho-donors such as acetyl phosphate, resulting in the increased expression of the *pip*, *phzR/phzI* and the phenazine biosynthetic genes. It is currently unknown what other genes, if any, RpeB controls. *HK* histidine kinase, *RR* response regulator, *P* phosphoryl group, ??? unknown traits

identical to that found in *P. protegens* Pf-5 (Humair et al. 2010). Consistent with this finding, a mutation in *gacA* resulted in lower abundance of the three ncRNAs. Furthermore, using a genetic reporter consisting of the *rsmZ* promoter fused to a promoterless green fluorescent protein gene, Wang et al. (2013) demonstrated that GacA is required for *rsmZ* promoter activity. Of significance, these results also demonstrated that constitutive expression of *rsmZ* from a non-*gac* regulated promoter resulted in complete restoration of AHL and phenazine production as well as the expression of other *gac*-dependent secondary metabolites in *gac* mutants. Bypassing GacA control of *rsmZ* also restored antifungal activity to a *gacA* mutant. In contrast to *rsmZ*, constitutive expression of *rsmX* (confirmed via quantitative RT-PCR) did not rescue phenazine production. Constitutive expression of *rsmY* by the *tac* promoter (a hybrid *E. coli* promoter, de Boer et al. 1983) appeared to be lethal in *gacA* mutants, since no transformants were recovered. This was the first report of *rsmZ* functioning differently than the other two ncRNAs in Gac-mediated regulation. However, given that transcript abundances of both *rsmX* and particularly *rsmY* are significantly lower in a *gacA* mutant, it is likely that they are involved in Gac-mediated regulation of yet unidentified targets. To determine the roles of RsmA and RsmE in the regulation of phenazine production, multiple copies of *rsmA* and *rsmE* were introduced separately into the wild type strain. Interestingly, overexpression of *rsmE*, but not *rsmA*, resulted in decreased AHL production and phenazine gene expression in *P. chlororaphis* 30-84, and only a mutation in *rsmE* bypassed the requirement for GacA in phenazine gene expression. The observation that *rsmE* and *rsmZ* (but not *rsmA* or the other two ncRNAs) are involved specifically in phenazine gene regulation demonstrates the potential for the fine tuning of the regulation of secondary metabolites under Gac/Rsm control via differences in the function of the Rsm components. Other genes with demonstrated roles in phenazine production in *P. chlororaphis* encode for the general stress response regulator RpoS, the phenazine inducing protein (Pip), and the RpeA/RpeB two component regulators (Girard et al. 2006a; Wang et al. 2012b). In order to determine the hierarchic role of the Gac/Rsm pathway on the regulation of phenazines in 30-84, a combination of whole genome transcriptomic and genetic analyses were used (Wang et al. 2013). Consistent with previous findings, the transcript abundances of *phzXYFABCD* and *phzO* were significantly lower in a *gacA* mutant compared to wild type, as were the transcript abundances of the phenazine quorum sensing regulators *phzI* and *phzR*. The transcription abundances of other phenazine regulatory genes such as *rpoS* and *pip* also were decreased, whereas the transcript abundances of *rpeB* and *rpeA* were unchanged. Significantly, constitutive expression of the *phzR/phzI* quorum sensing system in multiple copies *in trans* did not rescue phenazine production in the *gacA* mutant. These observations demonstrate the direct post-transcriptional control by Gac of the phenazine biosynthetic genes. As expected, constitutive expression of *pip* and *rpoS* also failed to rescue a *gacA* mutant. Pip, a homolog of the TetR family of transcriptional regulators, was shown previously to promote phenazine production in 30-84 and *P. chlororaphis* PCL1391 by enhancing *phzI* and *phzR* expression (Wang et al. 2012b; Girard et al. 2006a, b). In both strains, the expression of *pip* is

regulated by the sigma factor RpoS, which in turn is regulated by GacA. In 30-84, *pip* is also regulated by the RpeA/RpeB two component system (Wang et al. 2012b). The significance of this in phenazine regulation is discussed in the next section. The effect of *gacA* mutation on *rpoS* and *pip* expression suggests that GacA also influences the expression of phenazines and additional phenotypes via RpoS and Pip.

7.3.3 RpeA/RpeB

RpeA and RpeB comprise another two component signal transduction system shown to be important in the regulation of phenazine biosynthesis in *P. chlororaphis* 30-84. The role of RpeA was first identified by characterization of a transposon mutant of P. chlororaphis 30-84 that produced high levels of phenazines especially when grown in minimal medium (Whistler and Pierson 2003). The altered gene, named repression of phenazine expression (*rpeA*), encodes a protein that is similar in amino acid sequence to a group of putative two component regulatory systems found in other pseudomonads (Wang et al. 2012b). RpeA mutants produced significantly more phenazines at lower cell densities in minimal medium than did the wild type, despite the strains producing similar amounts of AHL (Whistler and Pierson 2003). However, RpeA negatively regulated *phzR* expression in minimal medium, suggesting that RpeA modulates phenazine production under certain nutrient conditions by regulating PhzR levels. The putative cognate response regulator *rpeB* was identified four nucleotides upstream of *rpeA* and was transcribed from the same strand of genomic DNA (Wang et al. 2012b). In contrast to RpeA, loss of RpeB resulted in decreased phenazine and AHL production and decreased *phzR* expression. Similarly, an *rpeA/rpeB* double mutant produced lower amounts of phenazines and AHLs than the wild type, supporting the hypothesis that although the RpeA/RpeB two component system as a whole positively contributes to phenazine production, the two genes regulate phenazine production under certain nutrient conditions by modulating the level of AHL and PhzR. Additionally, quantitative real-time PCR analysis of the transcript abundance of other known phenazine regulatory genes revealed that expression of *pip* was significantly reduced in an *rpeB* mutant, whereas the transcript abundances of *rpoS, gacS*, and *gacA* were unchanged. Complementation assays showed that overexpression of *pip in trans* rescued phenazine production in an *rpeB* mutant, whereas multiple copies of *rpeB* genes were unable to restore phenazine production in a *pip* or *phzR* mutant. These results indicate that RpeA and RpeB differentially regulate phenazine production and act upstream of Pip and PhzR in the phenazine regulatory network. Further, the results indicate that RpoS and RpeB/RpeA independently regulate phenazine production, potentially using *pip* as a common regulatory intermediate. Consistent with these findings, expression of *rpeB* and *rpeA* was not altered by mutation of *gacA*, indicating that RpeA/RpeB and GacS/GacA do not regulate phenazines in a hierarchic manner.

Differential regulation of phenotypes by the cognate regulator and sensor components of other two component sensory transduction systems, as was observed for RpeA and RpeB in 30-84, has been observed in pseudomonads and other genera (Nicastro et al. 2009; Wang et al. 2009, 2011, 2012a). It is assumed that this behavior is the result of the well-documented ability of the sensor kinase protein to have both kinase and phosphatase activities (reviewed in Krell et al. 2010), and that the switch from a kinase to a phosphatase depends on environmental signals. In 30-84, Wang et al. (2012b) speculated that the RpeA protein may have a net phosphatase activity resulting in the dephosphorylation of RpeB under laboratory conditions; thus RpeB proteins would be more highly phosphorylated in the absence of RpeA, leading to the over-production of phenazines. Under conditions such as those encountered in the rhizosphere, RpeA may have a net kinase activity, and positively contribute to phenazine production by phosphorylating RpeB. In this case, RpeA and RpeB would act synergistically as positive regulators of phenazine production, although this hypothesis needs to be verified.

Data on RpeA/RpeB regulation of phenazines in 30-84 support the hypothesis that RpeA and RpeB modulate phenazine production in response to the metabolic state or stress condition of the cell and act upstream of Pip and PhzR (Whistler and Pierson 2003; Wang et al. 2012b). Furthermore, a recent study showed that under certain stress conditions *pip* overexpression in PCL1391 is detrimental to cells (Girard and Rigali 2011), supporting the hypothesis that Pip also modulates biological functions (such as phenazine production) in response to stress. It is interesting that homologs of *pip*, originally characterized in P. chlororaphis strain PCL1391 as the 'phenazine inducing protein,' are also found in phenazine non-producing biocontrol pseudomonads such as *P. protogens* Pf-5 (95 % predicted amino acid sequence similarity to the PCL1391 *pip*). Furthermore *pip* homologs (sharing >80 % predicted amino acid sequence similarity with PCL1391 *pip*) are present in the same subset of *Pseudomonas* strains that have *rpeB* homologs with high predicted amino acid sequence similarity to the 30-84 *rpeB*. It remains to be determined whether these regulatory homologs are functional and if they interact as do RpeA/RpeB and Pip in 30-84. However, since the majority of these *Pseudomonas* species do not produce phenazines, it is logical that a functional Pip would regulate traits other than phenazines.

7.3.4 Final Comments on Phenazine Regulation

The preceding discussion provides important clues toward understanding the interrelationship among important regulatory elements participating in the complex network controlling phenazine production in 30-84, especially the degree of functional overlap in terms of the signals to which the regulatory elements respond and the suites of genes they control. All phenazine producing pseudomonads exhibit direct control of phenazine biosynthesis via quorum sensing (e.g., PhzR/

PhzI). As described in this chapter and other publications (Dulla and Lindow 2008; Loh et al. 2002; Waters and Bassler 2005), quorum sensing regulation enables cells to coordinate gene regulation in response to the availability of AHL signal molecules, which in turn may be influenced by the number of isogenic and non-isogenic signal producers present as well as the diffusivity of the signals and the presence of other factors that may affect the availability of the signals or the cell's regulatory response. Furthermore, plant hosts are able to contribute to the conversation via the secretion of substances that mimic bacterial signals affecting bacterial behaviors (Gao et al. 2003; Teplinski et al. 2000). Previous studies demonstrated that mutations in quorum sensing have far-reaching effects on the transcript abundance of numerous genes, e.g., 6–10 % of the genome of *P. aeruginosa* is believed to be under QS control (Wagner et al. 2003; Schuster et al. 2003). However, as demonstrated for *P. chlororaphis* 30-84, the most profound effects of *phzR/phzI* mutations are the direct result of loss of phenazine production. These observations are supported by preliminary transcriptomic analysis of mutants with defects in the phenazine biosynthetic genes or *phzR/phzI* quorum sensing, which revealed a significant overlap in the genes that were altered in transcript abundance relative to the wild type (Pierson et al. unpublished). As demonstrated for P. chlororaphis 30-84 and PCL 1391, quorum sensing represents an important control point for other regulatory genes such as RpeA/RpeB and RpoS that appear to regulate phenazine production in a non-hierarchic manner through Pip control of quorum sensing. In contrast, the Gac/Rsm system mediates the regulation of numerous secondary metabolites including phenazines and other genes important for bacterial fitness. Recent transcriptomic work comparing *gacA* mutants and the wild type in *P. protogens* Pf-5 and *P. chlororaphis* 30-84 revealed that approximately 10–13 % of the genes were differentially regulated by *gacA* compared to wild type (635 of 6,147 and 771 of 5,967 genes, respectively; Hassan et al. 2010; Wang et al. 2013). However, study of phenazine regulation in *P. chlororaphis* 30-84 demonstrated that the Gac/Rsm regulatory system controls phenazine gene expression directly as well as through control of quorum sensing, via mechanisms not yet identified. Given that quorum sensing autoinduction of *phzI* and *phzR* causes an exponential increase in the transcript abundance of *phzI, phzR* and the phenazine biosynthetic genes, the ability to rapidly reduce and modulate transcript abundance post-transcriptionally via RsmE and the ncRNA *rsmZ* would provide tighter regulation of the timing and amount of phenazine produced. These studies also demonstrated the potential for the differential regulation of secondary metabolites under Gac/Rsm control via differences in the function of the Rsm components.

7.4 To Produce Phenazines or not to Produce Phenazines, that is the Question

Despite the importance of phenazine production for bacterial fitness and biological control capability, as reviewed here and in other chapters, it is interesting that all phenazine-producing pseudomonads studied to date exhibit phenotypic variation in secondary metabolite production via spontaneous mutation of the *gacS/gacA* regulatory system. Previous studies demonstrated that the genetic stability of *gacS/gacA* is influenced by growth conditions. High mutation frequencies of *gacS* and *gacA* have been observed in liquid culture (Chancey et al. 1999, 2002; Driscoll et al. 2011; Laville et al. 1992). In one study, *gacS/gacA* mutants were found in 192 separate cultures and comprised up to 61 % of the total bacteria in each culture (Duffy and Défago 2000). In *P. fluorescens* CHA0, instability of both genes was correlated with high electrolyte concentrations and was reduced by certain mineral amendments (Duffy and Défago 2000). Rhizosphere populations of *Pseudomonas* often contain variants with defective Gac systems (Chancey et al. 2002; Schmidt-Eisenlohr et al. 2003). Chancey et al. (2002) demonstrated that after inoculation of wheat seedlings with P. chlororaphis 30-84, *gacS/gacA* mutants were recovered from 97 % (29/31) of inoculated wheat plants and these comprised up to 36 % of the total bacterial populations, even though no mutants were detected in the inoculum. Southern hybridization analysis of the recovered *gac* mutants did not indicate a conserved mutational mechanism. Reversibility of *gac* mutations has not been demonstrated in many well-studied biological control strains with the exception of *Pseudomonas* sp. PCL1171, where reversibility was correlated with the expression of MutS-dependent mismatch repair mechanisms (Van den Broek et al. 2005). Recently, we recovered for the first time revertants of a spontaneous 30-84 *gacA* mutant from the wheat rhizosphere (Pierson et al. unpublished).

The prevalence of *gac* mutants in laboratory and natural environments (e.g., in the rhizosphere) suggests that despite the lack of production of phenazines and other secondary metabolites, there is some benefit to the *gac* mutant (Gac^-) phenotype and thus selection at some level to maintain it. One hypothesis, best stated by Driscoll et al. (2011) is that Gac^- variants are 'cheaters' that do not produce their share of external products, but benefit from the external products produced by their cooperative counterparts. Cheaters thus may increase rapidly by out-reproducing their counterparts, as seen in laboratory situations such as in liquid culture. An alternative hypothesis is that the interaction between wild type (Gac^+) and Gac^- phenotypes is mutualistic in nature, and thus maintained by selection.

These hypotheses were tested by Chancey et al. (2002) in work that showed that in mixed rhizosphere populations, the outcome of the interaction between wild type and mutants is very different from that observed in rich medium, with both Gac^+ and Gac^- phenotypes benefiting. A replacement series design was used to measure the rhizosphere competence and competitive fitness of wild type and *gac* mutants. A replacement series refers to the process of producing inoculation treatments

composed of mixed populations in which one strain 'replaces' the other while keeping the total population density constant. These experiments revealed that although the Gac$^-$ population partially displaced the Gac$^+$ population in sterile soil, it did not do so in natural soil. Interestingly, in natural soil, the wild type 30-84 population was larger in treatments where it was co-inoculated with the *gacA* mutant than when it was inoculated alone, demonstrating a selective advantage to the wild type Gac$^+$ population when coexisting with the Gac$^-$ variant. These results indicate that although *gacS/gacA* mutants survive in natural rhizosphere populations, they do not typically displace wild type populations. The better survival of the wild type in mixtures with mutants suggests that mutants arising *de novo* or introduced within the inoculum benefit the survival of wild type populations in the rhizosphere.

More recently, Driscoll et al. (2011) examined whether the beneficial effect of *gac* mutants on 30-84 was related to the ability of mixed populations to form biofilms. As determined previously, 30-84 *gac* mutants do not produce phenazines essential for biofilm formation (Maddula et al. 2006). Thus, as expected, 30-84 *gacA* mutants did not form biofilms in the absence of the wild type. And as seen previously on roots, both 30-84 and the 30-84GacA attained higher biovolumes in mixed biofilms than either strain grown separately. In contrast, the biovolume of the wild type was reduced when grown with a phenazine-deficient derivative of 30-84 containing a mutation in the phenazine biosynthetic genes, suggesting competition between these strains and a potential reason why mutants containing defects in the phenazine biosynthetic genes (as compared to Gac$^-$ mutants, which also do not make phenazines) are never recovered.

Recent transcriptomic work with *gacA* mutants and wild type strains of *P. protogens* Pf-5 and P. chlororaphis 30-84 revealed that approximately 30–45 % of the genes differentially regulated by *gacA* were expressed at a higher level in the *gacA* mutant compared to the wild type (238 of 635 and 220 of 771 genes, respectively; Hassan et al. 2010; Wang et al. 2013). The most obvious groups of genes included those involved in iron uptake and protein translation, however about 25 % of the genes more highly expressed in the *gacA* mutant compared to wild type were hypothetical or unknown genes in both strains, indicating that there is much to learn about the role of the Gac$^-$ phenotype in niches outside the laboratory. Given the importance of phenazine production for the fitness and biological control capability of phenazine-producing strains, it is intriguing to speculate on which traits expressed singly or collectively in the Gac$^-$ phenotype enable the cells to "suffer the slings and arrows of outrageous fortune," potentially brought about by the lack of phenazine production, and ultimately to provide a selective advantage to populations having both Gac$^+$ and Gac$^-$ phenotypes (metaphor from *Hamlet* by William Shakespeare).

7.5 Final Comments

Phenazines constitute a fascinating and diverse group of redox active molecules that play multiple and varied roles contributing to the fitness of the producing microbe as well as influencing microbe–microbe and microbe-host interactions. Of interest are the observations that phenazine-producing microbes produce a diversity of phenazines, that many produce more than one derivative, and that the type of phenazines produced, their relative abundances, and their patterns of expression influence their biological impact and specificity. These differences in phenazine production ultimately help define the ecological niche the microbe occupies. While it has been known that phenazines interact with DNA for decades, recent observations that phenazines interact with extracellular DNA demonstrate there is still much to learn about the roles phenazines play for the producing microbe. Furthermore, emerging evidence that phenazines contribute to plant competence in specific environmental niches such as dryland agriculture reinforces the importance of the environment in delineating the roles of phenazines in ecological interactions. Future work should consider the specific functions each derivative serves for the producing microbe, including considerations of how the environment may affect and in turn be influenced by the relative abundance and physical, chemical and biological properties of each phenazine derivative produced.

The complexity of the regulatory network governing phenazine production reflects the complexity of the functions phenazines serve. Ongoing studies of regulatory circuits controlling phenazine production are providing insights into specific environmental cues that determine the amounts and types of phenazines produced. Comparison of regulatory networks in multiple phenazine-producing bacteria is needed to fully understand the selective pressures that have shaped how the regulatory genes controlling phenazine production have become integrated. Modern approaches such as transcriptomics are proving useful for examining the linkages between primary and secondary metabolism and the hierarchic relationship between phenazine-specific and more general metabolite-regulating genes.

Despite the importance of phenazine production for microbial fitness and biological control, the presence of phenazine non-producing Gac^- phenotypic variants in plant-associated communities may increase the overall fitness of the phenazine-producing strain. Of interest are the observations that the Gac^- phenotype results not only in the reduction in expression of many secondary metabolites, but an increase in the expression of other metabolites and bacterial traits, as well as many genes of currently unknown function. Ongoing research using transcriptomic comparisons of gene expression patterns of Gac^+ and Gac^- phenotypes *on plants* will provide insights into candidate genes and traits contributing to the beneficial characteristics of the Gac^- phenotype.

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Chapter 8
Role of Phenazine-1-Carboxamide Produced by *Pseudomonas chlororaphis* PCL1391 in the Control of Tomato Foot and Root Rot

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Abstract Tomato foot and root rot (TFRR) is a tomato root disease caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*). No chemicals are available which efficiently suppress TFRR. In this chapter we show that the bacterium *Pseudomonas chlororaphis* strain PCL1391 is able to suppress the disease. To this end it uses antibiosis as its (major) disease-suppressing mechanism. The produced antibiotic was identified as phenazine-1-carboxamide (PCN). In contrast to the PCN-producing bacterium, pseudomonads which produce the PCN biosynthetic precursor phenazine-1-carboxylic acid (PCA) as their major phenazine were not active in disease suppression. However, when PCA was converted to PCN by complementing these strains with the *phzH* gene, which encodes an amidotransferase, the complemented strains produced PCN and controled TFRR. In order to be effective in disease control, strain PCL1391 should be able to produce PCN under a variety of environmental conditions. We therefore studied the regulation of PCN production under various environmental factors, by regulatory genes, by the plant, and by the pathogenic fungus. Special attention was paid to the secondary metabolite fusaric acid secreted by the fungus. Fusaric acid is detected by the bacterium as a chemo-attractant to reach the fungus, to colonize its surface and to finally use it as a food source. Conversely, fusaric acid is used by the fungus to inhibit the production of PCN and to reduce the bacterial growth rate. It is clear that during disease control the PCN-producing bacterium wins this battle. The result of the evaluation of the described studies is that we can understand in quite some detail how *P. chlororaphis* strain PCL1391 acts as a disease control agent and also why it is not active under all environmental conditions.

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Abbreviations

CAS	Casamino acids
CLSM	Confocal laser scanning microscopy
C6-HSL	*N*-hexanoyl-L-homoserine lactone
Forl	*Fusarium oxysporum* f. sp. *radicis-lycopersici*
gfp	Gene encoding green fluorescent protein
PCA	Phenazine-1-carboxylic acid
PCN	Phenazine-1-carboxamide
TFRR	Tomato foot and root rot

8.1 Introduction

Tomato foot and root rot (TFRR) is a root disease caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*). TFRR is also called crown and root rot of tomato. TFRR is a serious problem for field and greenhouse crops (Jarvis 1988). Chemicals do not efficiently suppress TFRR (Benhamou et al. 1994). In contrast, some bacteria are fortunately able to reduce TFRR (Haas and Défago 2005; Lugtenberg and Kamilova 2009; Pliego et al. 2011). One of these bacteria is the phenazine-1-carboxamide (PCN) producing bacterium *Pseudomonas chlororaphis* strain PCL1391 (Chin-A-Woeng et al. 1998), which was isolated and studied extensively in our laboratory. In this chapter we describe the isolation of this strain, the requirement of PCN for disease control, the differential effect of strains producing PCN and PCA on disease suppression, the very complex regulation of PCN synthesis by both genetic and environmental factors, and finally draw conclusions on the role of PCN in various steps of the disease control process. Previously reviews have been published on the role of phenazines in biocontrol (Thomashow and Weller 1988; Chin-A-Woeng et al. 2003a, b) and on their biosynthesis and regulation (Mavrodi et al. 2006).

8.2 Isolation of *Pseudomonas chlororaphis* PCL1391 and Its Characterization as a Biocontrol Agent

P. chlororaphis strain PCL1391 was isolated from a tomato plant (provided by Prof. José Olivares) grown in a commercial field near Granada, Andalucia, Spain (Chin-A-Woeng et al. 1998). After removal of bulk soil, the root material and adhering rhizosphere soil particles were shaken with water, and dilutions of the suspension were spread on solid KB medium supplemented with carbenicillin, chloramphenicol and cycloheximide. After incubation at 28 °C, seventy of the colonies were tested for inhibition of *Forl* growth. To this end, a 0.5 × 0.5 cm

agar plug containing the fungus was stabbed in the middle of an LB agar plate, followed by inoculating bacterial strains (six per plate) at a distance of 3 cm from the fungus as described by Geels and Schippers (1983). Of these strains, *P. chlororaphis* strain PCL1391 appeared to form the largest fungal growth inhibition zone and was therefore chosen for further study.

In a plate assay, *P. chlororaphis* strain PCL1391 also appeared to inhibit the in vitro growth of a range of fungi, including the phytopathogens *Alternaria dauci*, *Botrytis cinerea*, *Pythium ultimum*, *Rhizoctonia solani*, and *Verticillium albo-atrum*. The strain secretes a hydrophobic compound, identified as PCN, as well as HCN, chitinase(s), lipase(s), protease(s) and siderophore(s). It also appeared to colonize tomato roots as efficiently as the then best-known colonizer in our collection, *P. fluorescens* WCS365 (Lugtenberg and Dekkers 1999; Lugtenberg et al. 2001).

Disease control experiments were carried out using tomato seeds dipped in a suspension of bacteria (10^9/ml) in 1 % methylcellulose. After drying, the seeds were sown in potting soil containing *Forl* spores (3×10^6 spores per kg soil). Disease symptoms were scored after 3 weeks. Controls contained either no bacteria or no fungal spores. *P. chlororaphis* strain PCL1391 appeared to control TFRR efficiently, in contrast to *P. fluorescens* strains F113 (Shanahan et al. 1992) and WCS374 (Leemans et al. 1995), strains both known to control diseases caused by other fungi (Chin-A-Woeng et al. 1998).

Biocontrol of TFRR appeared to depend on at least two factors, namely PCN production and tomato root colonization. The need for PCN was concluded from the fact that a PCN biosynthetic mutant did not show significant disease control (Chin-A-Woeng et al. 1998). The need for root colonization was shown by testing competitive root colonization-negative mutants. Mutants impaired in each one of three known colonization traits, namely motility, prototrophy for amino acids, and the presence of a site-specific recombinase (Lugtenberg et al. 2001) all appeared to be negative in disease control (Chin-A-Woeng et al. 2000). *P. chlororaphis* strain PCL1391, like other pseudomonads, forms micro-colonies or biofilms on part of the root surface (Bloemberg et al. 1997; Bloemberg and Lugtenberg 2004).

The process of attachment of *Forl* to the root and subsequent invasion was visualized using (mutants of) the gene encoding green fluorescent protein (*gfp*) labelled *Forl* and confocal laser scanning microscopy (CLSM) (Fig. 8.1a–d). The tomato root is autofluorescent. The infection process starts with attachment of fungal hyphae to root hairs (Fig. 8.1a) followed by colonization of the grooves between the junctions of the epidermal cells (Fig. 8.1b), penetration of the root cells (Fig. 8.1c) and overgrowth of the internal root (Fig. 8.1d).

Pseudomonas bacteria were labelled in the same way. Their interaction with the root starts with colonizing the grooves between plant cells (Fig. 8.1e), the same sites as colonized by fungus. Subsequently the bacteria form micro-colonies or biofilms on part of the root (Fig. 8.1f). The bacteria in the biofilm are covered by a mucoid layer (see Fig. 8.1g, which is a detail of Fig. 8.1f, h, a scanning microscopy picture in which the mucoid layer is broken open). A biofilm creates ideal

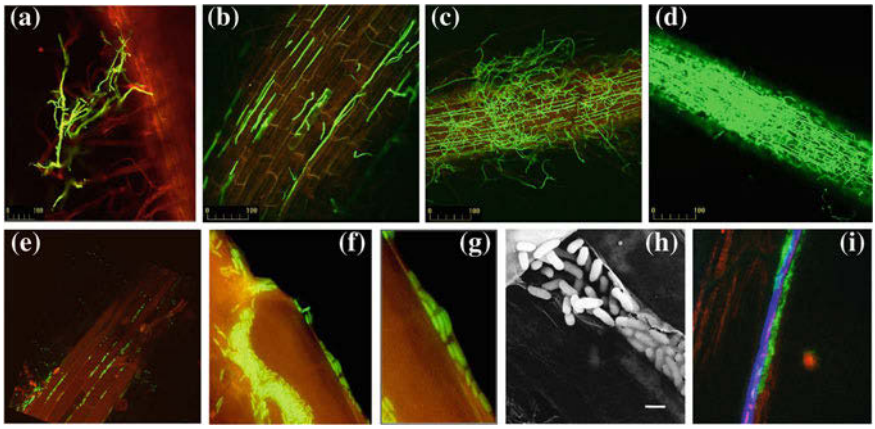

Fig. 8.1 Visualization of plant–microbe and microbe–microbe interactions during biocontrol. CLSM (**a**–**g** and **i**) and scanning electron microscopy (**h**) were used to visualize control of TFRR caused by Forl (*Fusarium oxysporum* fsp. *radicis-lycopersici*) by *Pseudomonas* biocontrol bacteria. For explanation, see text. Panels **a**, **c**, and **d** were reproduced from Lagopodi et al. 2002, panel **b** from Bolwerk et al. 2003, and panel **h** from Chin-A-Woeng et al. 1997. Panel **e** is from Bolwerk, Lagopodi and Bloemberg, *unpublished*. Panels **f** and **g** are from Bloemberg et al. 1997; Copyright © American Society for Microbiology

conditions for quorum sensing and processes dependent on quorum sensing, such as F-mediated DNA transfer, and the syntheses of antibiotics (e.g. PCN) and exoenzymes. The bacteria also attack the fungus directly by colonizing the hyphae extensively (Fig. 8.1i).

8.3 Comparison of the Roles of PCN and PCA in the Control of TFRR

Some biocontrol strains, e.g. *P. fluorescens* 2-79 (Thomashow and Weller 1988) and *P. aureofaciens* 30-84 (Pierson and Thomashow 1992), produce phenazine-1-carboxylic acid (PCA) but not PCN. In our hands, these strains were inactive in suppressing TFRR. In an attempt to understand this difference, the growth-inhibiting activities of equimolar amounts of PCN and PCA were compared as a function of the pH. At pH values of 5 and lower, both compounds were inhibitory but PCA was slightly more active. At pH values between 5.7 and 7.0, PCN was superior whereas PCA was even inactive at values between 5.9 and 7.0 (Chin-A-Woeng et al. 1998). It is likely that the difference in pH dependence of the antifungal activities of the two compounds is an important factor in the outcome of their disease control activities.

To determine the role of the amino group in biocontrol, the *phzH* gene, present in strain PCL1391 but not in the two PCA-producing strains, was identified and characterized. A *phzH* mutant appears to accumulate PCA instead of PCN. The

deduced PhzH protein shows homology with asparagine synthetases, which belong to class II glutamine amidotransferases. These results indicate that the conversion of PCA to PCN takes place via a transamidase reaction catalysed by PhzH. A *phzH* mutant of *P. chlororaphis* strain PCL1391 is unable to control TFRR. Transfer of the *phzH* gene to the PCA producing strains *P. fluorescens* 2-79 and to *P. aureofasciens* 30-84 enabled these strains to control TFRR (Chin-A-Woeng et al. 2001b). It must therefore be concluded that the amino group of PCN is crucial for the control of TFRR.

8.4 Regulation of PCN Synthesis by Quorum Sensing and Environmental Factors

The expression of the biosynthetic *phzABCDEFGH* operon (Chin-A-Woeng et al. 2001b) is regulated by quorum sensing (Bassler 1999). The *luxI* and *luxR* homologues of strain PCL1391, *phzI* and *phzR*, regulate the expression of the biosynthetic operon. PhzI produces *N*-hexanoyl-L-homoserine lactone (C_6-HSL) as the main autoinducer, whereas smaller amounts of C_4-HSL and C_8-HSL are also produced (Chin-A-Woeng et al. 2001a). The autoinducers supposedly activate the transcriptional activator PhzR by binding to it (Fig. 8.1). Activated PhzR is thought to turn on the biosynthetic *phz* genes. Quorum sensing is dependent on population density. Gene expression studies have shown that the culture supernatant confers positive regulation of *phzI*, not only by autoinducers but also by at least one unknown factor (Chin-A-Woeng et al. 2001b). Production of *N*-acyl homoserine lactones seems to be essential and necessary for PCN biosynthesis, as no condition (environmental or genetic change—except *phz* genes) has been found so far under which PCN is produced in the absence of autoinducer, and vice versa.

We studied the influence of environmental conditions relevant for plant growth in detail, using growth at 28 °C in Vogel-Bonner medium amended with 0.05 % CAS (casamino acids) and 30 mM glucose as the basic medium, which was subsequently modified to study the effects of various factors (Van Rij et al. 2004). PCN production starts at the end of the exponential growth phase and continues to increase until the cells reach the stationary phase. Growth on the carbon sources glucose, L-pyroglutamic acid and glycerol results in the highest PCN levels (Van Rij et al. 2004). Omitting 0.05 % CAS from the growth medium dramatically reduces the levels of PCN production. Addition of extra CAS increases PCN levels. Testing of individual amino acids showed that all tested individual amino acids increase PCN levels at least 2-fold and that the largest increase is caused by phenylalanine (23 times) and tyrosine (13 times). A remarkable finding was that the addition of 1 mM phenylalanine causes the PCN production to start earlier in the growth curve (at an OD_{620} nm value of 1.0 instead of 2.0). Replacement of $(NH_4)_2SO_4$ by the same amounts of nitrogen from urea or $NaNO_3$ results in a decrease in PCN production. Testing of other ions showed that low Mg^{2+} increases

PCN levels and that salt stress (but not osmotic stress), and low concentrations of ammonium, ferric, phosphate, and sulphate ions reduce PCN levels (Van Rij et al. 2004).

At temperatures of 21, 28 and 31 °C, PCN production is similar but at 16 °C the level drops to practically zero. This may be related to the remarkable reduction in growth rate by 80 %. Reducing the O_2 level to 1 % results in a substantial increase in PCN production. The pH of the growth medium has a strong influence on the PCN level. Starting pH values of 7.0 and 8.0, resulting in final pH values of 6.5 and 7.1, respectively, result in normal PCN levels but a starting pH of 6.0, which results in a final pH of 4.2, abolishes PCN production (Van Rij et al. 2004).

Other relevant facts are the following: (1) attempts to find synergism between conditions that result in high PCN levels failed; (2) comparison of our results with literature data showed that some environmental factors have similar effects on other studied *Pseudomonas* strains but that other environmental factors have opposite effects in other strains; (3) analyses of autoinducer levels under conditions of high and low PCN production demonstrated that, under all tested conditions, PCN levels correlate with autoinducer levels, indicating that the regulation of PCN levels by environmental factors takes place at or upstream of autoinducer production (Van Rij et al. 2004).

8.5 Genetic Regulation of PCN Synthesis by the Bacterium

Considering the complex regulation of PCN production by environmental factors (see above), by the plant and by the fungus (see the following sections), it must be expected that many genes and regulatory cascades are involved in the regulation of PCN production. This indeed appears to be the case.

The previously mentioned quorum-sensing genes *phzI* and *phzR* are located at the end of a complex and not yet completely understood genetic regulatory cascade (Fig. 8.2). Every gene that has been shown to influence phenazine production by strain PCL1391 (except the *phz* genes themselves) was correlatively shown to affect autoinducer synthesis.

A two-component system including a membrane-associated sensor (GacS) and a response transcriptional regulator (GacA) activates the *phz* operon under all conditions tested so far. It responds to a yet-unknown environmental factor and is at the top of the PCN regulatory cascade. In *Pseudomonas* species, the *gac* genes are global regulators of secondary metabolism that are situated upstream of many regulatory cascades. They function as master regulators involved in the control of a substantial set of genes and traits such as the production of antibiotics, HCN and extracellular enzymes (Haas and Défago 2005).

Between GacS/GacA and the quorum-sensing system, at least three genes are responsible for modulating PCN synthesis. Firstly, *Pseudomonas* sigma regulator (*psrA*) regulates its own expression by negative feed-back (Chin-A-Woeng et al. 2005)

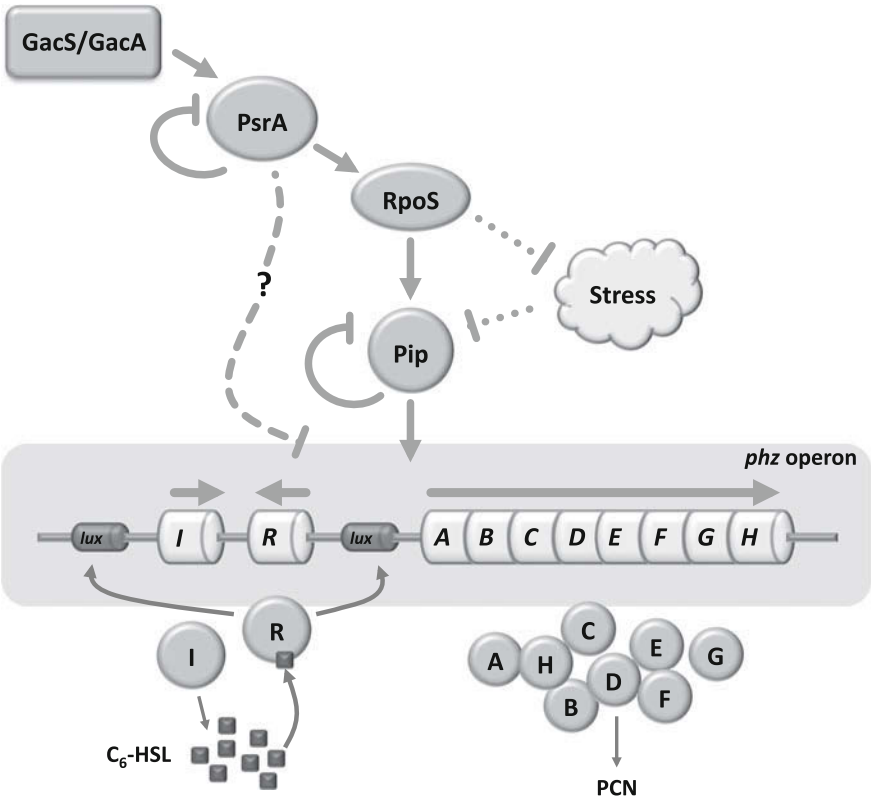

Fig. 8.2 Three main genetic pathways for the regulation of PCN synthesis. All pathways are downstream of the master regulatory system GacS/GacA and upstream of the quorum-sensing system PhzI/PhzR. *Dashed lines*: in rich medium, PsrA negatively regulates the *phz* operon via unknown genes. *Plain lines*: in minimum medium, PsrA positively regulates PCN production via RpoS and Pip. *Dotted lines*: in minimum medium, under several types of stress conditions (including the *Forl* toxin fusaric acid), PCN synthesis is switched off to give priority to another RpoS-regulated pathway: stress resistance. See Sect. 8.5 for more details

and stimulates expression of the second regulatory gene, *rpoS* (alternative sigma factor) (Girard et al. 2006a). It was shown that *psrA* negatively affects PCN production in rich medium (Chin-A-Woeng et al. 2005), but positively in poor medium (Girard et al. 2006a). In rich medium, *rpoS* does not have a significant influence on PCN production, but clearly stimulates it under poor conditions (Girard et al. 2006a). These differences reflect the complexity of the *phz* operon regulation by the environment and indicate that genes other than *rpoS* must be present downstream of *psrA* in the regulatory cascade. Thirdly, the *pip* gene—encoding the phenazine inducing protein Pip—was also found to have a negative auto-regulatory role and, just downstream of *rpoS*, a positive effect on PCN synthesis in poor medium (Girard et al. 2006b).

Interestingly, experiments involving sub-inhibitory concentrations of various stress factors in poor medium also link Pip to stress response by *P. chlororaphis*. We propose that Pip would be downstream of the stress sigma factor RpoS, a 'decision' point for attributing the use of energy to either PCN production (under favourable growth conditions) or stress resistance (Girard and Rigali 2011).

8.6 Regulation of PCN Synthesis by the Plant

Since *P. chlororaphis* strain PCL1391 is PCN-dependent for biocontrol (see Sect. 8.2), PCN is likely to be produced on the plant root. This turns out to be indeed the case. Using a derivative, designated as strain PCL1119, which harbours promoterless *luxAB* genes inserted in the *phzB* gene of the phenazine biosynthetic operon, Chin-A-Woeng et al. (1998) showed expression of the phenazine biosynthetic operon on the tomato root.

Growth on the carbon sources glucose, L-pyroglutamic acid and glycerol results in high PCN levels (7.9 μM PCN produced per OD_{620} value for glucose), whereas growth on the five most common carbon sources found in tomato root exudate (citric acid, malic acid, lactic acid, succinic acid and pyruvic acid; Lugtenberg and Bloemberg 2004) results in limited PCN levels (only 1.0, 0.05, 1.2, 0.13, and 0.17 μM PCN per OD_{620} value, respectively). From these results it was concluded that the tomato root exudate composition is far from optimal for PCN production by *P. chlororaphis* strain PCL1391 (Van Rij et al. 2004).

Some exudate components indirectly contribute to PCN production by functioning as chemo-attractants for *Pseudomonas* cells. De Weert et al. (2002) reported the results of assays measuring chemotaxis of biocontrol strain *P. fluorescens* WCS365 towards individual tomato root exudate components. They found positive chemotaxis towards some organic acids and some amino acids but not towards sugars. Comparison of the minimal active concentrations with the concentrations estimated to be present in exudate led to the conclusion that malic acid and citric acid are the major chemo-attractants in the tomato rhizosphere (De Weert et al. 2002).

8.7 Regulation of PCN Synthesis by the Pathogen

Killing of the pathogenic fungus *Forl* by the PCN-producing *P. chlororaphis* strain PCL1391 is not as easy as it may look since it was found that the fungus has developed a smart defence strategy. It appears that the secondary metabolite fusaric acid, secreted by *Fusarium*, plays a crucial role in the interaction between fungus and bacterium. *Forl* and many other *Fusarium* strains produce the fungal

toxin fusaric acid (Notz et al. 2002). At a fusaric acid concentration of 0.3 mM, PCN production levels of the bacterium start to decrease, while 1.5 mM fusaric acid decreases PCN production by as much as 97 %. This fusaric acid concentration also decreases the growth rate of the bacterium by 25 % (Van Rij et al. 2004). It is conceivable that such conditions can be reached on the root. Since *P. fluorescens* strain WCS365 is chemotactically attracted towards fusaric acid secreted by *Forl* (De Weert et al. 2003), we consider this likely to also be the case for *P. chlororaphis* strain PCL1391. These observations suggest a dual role of fusaric acid in biocontrol: on the one hand it is used by the bacterium as a guide towards hyphae to use them as a food source, whereas on the other hand it disarms the biocontrol bacterium by inhibiting the production of PCN, its major weapon against the fungus, as well as by inhibiting growth. Successful biocontrol apparently depends on the ratio of the activities of the bacterial and fungal metabolites PCN and fusaric acid, respectively.

In attempts to understand the role of fusaric acid in more detail, we performed a number of genetic studies. Since fusaric acid also represses the production of the quorum-sensing signal C_6-HSL, it is clear that inhibition of PCN synthesis by fusaric acid occurs at or before the level of C_6-HSL synthesis (Van Rij et al. 2004). Further studies indicated that PCN repression by fusaric acid is maintained even during PCN production-stimulating growth conditions such as the presence of additional phenylalanine, additional ferric iron ions, and a low Mg^{2+} concentration. In contrast, constitutive expression of *phzI* or *phzR* increases C_6-HSL levels and stops the repression of PCN production by fusaric acid (Van Rij et al. 2005). Transcriptome analysis confirmed that fusaric acid represses expression of the biosynthetic *phz* operon as well as of the quorum sensing regulatory genes *phzI* and *phzR*. Fusaric acid does not affect the expression of *gacS*, *rpoS* and *psrA*, genes which have been shown to regulate the synthesis of PCN (Girard et al. 2006a; Chin-A-Woeng et al. 2005). These results show that reduction of PCN synthesis by fusaric acid is the result of a direct or indirect repression of *phzR* and *phzI*. An interesting observation is that fusaric acid not only represses the production of the antibiotic PCN but also of another antibiotic involved in biocontrol of plant diseases, namely 2,4-diacetylphloroglucinol, produced by biocontrol strain *P.fluorescens* CHA0 (Duffy and Défago 1999). Therefore, and because this strain does not produce acyl-homoserine lactones, it is likely that fusaric acid interferes with PCN synthesis by indirect repression of *phzR* and *phzI*, at least partially via the Pip regulator (see Fig. 8.2, Girard et al. 2011).

Transcriptome analysis also showed that genes that are highly up-regulated by fusaric acid also are up-regulated by iron starvation in *P. aeruginosa* (Ochsner et al. 2002; Palma et al. 2003; Ghysels et al. 2004), suggesting an overlapping stress response to fusaric acid and iron starvation (Van Rij et al. 2005).

8.8 Conclusions on the Role of PCN in Various Steps of the Biocontrol Process

In biocontrol experiments, spores of the fungal pathogen *Forl* are mixed with the soil while the beneficial PCN-producing bacterium *P. chlororaphis* strain PCL1391 is coated on the tomato seeds. Upon germination of the seeds, the bacterium is attracted to the root by root-exudate compounds, which are also utilized for multiplication. The bacterium colonizes the root, first as single cells, later as microcolonies or biofilms (Fig. 8.1; Bloemberg et al. 1997). The majority of the bacteria are found at the grooves along the junctions of the epidermal cells. The bacterium reaches the plant root earlier than the fungal hyphae (Bolwerk et al. 2003).

Roots of seedlings secrete components that allow the fungal spores to germinate (Kamilova et al. 2005; Steinkellner et al. 2005) and attract the hyphae to the root. Using *gfp*-labelled *Forl*, the process of tomato root infection by *Forl* was analysed. The first step is attachment of hyphae to the root hairs. This is followed by root colonization of the grooves along the junctions of the epidermal cells. Finally, the hyphae penetrate the plant cells, overgrow the root and cause the death of the plant (Lagopodi et al. 2002).

In the case of disease control, the bacterium has reached the root first and controls fungal growth. It out-competes the fungus for growth on exudate components and does not allow the hyphae to penetrate the plant root. Instead, the bacterium colonizes the fungal hyphae, weakens them, and eventually uses them as food (Bolwerk et al. 2003; De Weert et al. 2003).

Analyses of molecular details of the plant-bacterium-fungal interaction have revealed the following. (1) Exudate compounds (especially malic acid and citric acid; De Weert et al. 2002) are used as chemo-attractant to guide the bacterium to the root where it uses major exudate compounds (including citric, malic, lactic, oxalic, pyruvic and succinic acids; Kamilova et al. 2006; Lugtenberg et al. 2001; Lugtenberg and Bloemberg 2004; Lugtenberg and Kamilova 2009) for multiplication. (2) In vitro interference contrast microscopy experiments show that PCN negatively affects hyphal growth and branching of the fungus, which presumably negatively affects the colonization and infection abilities of the fungus (Bolwerk et al. 2003). (3) The hyphae secrete fusaric acid, which is used by the bacterium as a chemo-attractant to find the hyphae, colonise them, and use them as food. Fusaric acid is used by the fungus to inhibit the biosynthesis of PCN, the major weapon of the bacterium. One can predict that in the case of successful disease control the PCN-producing bacterium has been more successful than the fusaric acid-producing fungus. It is likely that the following factors contribute to the success of disease control. (a) The fact that bacterium and fungus colonize the same niche, which allows the bacterium to optimally attack the fungus. (b) The timing by the bacterium, which reaches the grooves on the plant root first and builds up high numbers of cells and a high PCN concentration before the fungus arrives. (c) The relative concentrations and efficiencies of the weapons PCN and fusaric acid on the

battlefield. (d) Some of the observations reported in this chapter negatively impact application of phenazine-producing strains in biocontrol. Firstly, the pH of some soils has a strong effect on the *efficacy* by which PCA, but much less PCN, inhibits fungal growth. Secondly, many environmental factors influence the level of PCN *production.* Therefore it can be predicted that biocontrol by *P. chlororaphis* strain PCL1391 will not be effective in all soils and not under all environmental conditions.

The result of the evaluation of the studies described here is that we can understand in quite some detail how the PCN-producing *Pseudomonas chlororaphis* strain PCL1391 acts as a disease control agent and also why it is not active under all environmental conditions.

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Chapter 9
Genomic Features and Regulation of Phenazine Biosynthesis in the Rhizosphere Strain *Pseudomonas aeruginosa* M18

Yuquan Xu

Abstract The genome of the rhizosphere strain *Pseudomonas aeruginosa* M18 contains specific accessory regions that benefit survival in rhizosphere niches in addition to the conserved core genome. The M18 genome contains two homologous *phz* gene clusters, *phzA1-GI* (*phz1*) and *phzA2-G2* (*phz2*), each of which encodes essential enzymes responsible for phenazine-1-carboxylic acid (PCA) production. There exists a regulatory feedback loop of the two *phz* gene clusters, which are oppositely controlled directly by RsmA-mediated activity. Expression of the two *phz* gene clusters also is oppositely regulated by temperature: the *phz2* cluster is more highly expressed at 28 °C, while the *phz1* cluster is expressed more highly at 37 °C. The gene *phzM,* involved in the conversion of PCA to pyocyanin (PYO), is less efficiently expressed at 28 °C in a temperature-dependent and strain-specific manner, resulting predominantly in the production of PCA rather than PYO in M18. The genome was systematically engineered based on genetic regulation of the *phz* gene clusters and fermentation was optimized using surface response methodology. PCA yield in the modified strain can reach more than 5 g per liter during a 72 h fermentation. A new antifungal pesticide, Shenqinmeisu, with PCA as one of its main components, was first registered in China in 2011 and has been widely applied in commercial farm fields to protect rice and vegetables against diseases caused by *Rhizoctonia solani*, *Fusarium oxysporum*, and other phytopathogens. Sales in China have reached over 2.2 million US dollars during the last 2 years.

9.1 General

Much attention has been paid and there have been many publications over the past 100 years describing studies of strains of *Pseudomonas aeruginosa* of nosocomial origin, including investigations of their genetics, physiology and biochemistry,

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diversity and adaptation, and opportunistic pathogenicity against humans and animals. However, *P. aeruginosa* also is ubiquitous in the environment, including in water and soil (Selezska et al. 2012), and several *P. aeruginosa* strains that inhabit rhizosphere niches have been found to show excellent plant growth-promoting properties and to display effective biological control against various phytopathogens (Buysens et al. 1996; Anjaiah et al. 2003; Bano and Musarrat 2003; Kumar et al. 2005; Park et al. 2012). None of these environmental strains has been investigated in detail with regard to genome structure and major biocontrol compounds, especially in relation to the regulation and expression of the redundant *phz* gene clusters involved in phenazine biosynthesis.

P. aeruginosa strain M18 was isolated in 1996 by our group from the rhizosphere of a sweet melon plant growing in a Shanghai suburb of China. Strain M18 previously was called *Pseudomonas* sp. M18 because of its paradoxical features. For example, its 16S ribosomal DNA as well as several global regulatory genes including *gacA*, *rpoS*, *qscR*, *vqsR*, *lasI*, *rhlI,* and *rsmA* share high sequence similarity to homologous genes of the nosocomial strain *P. aeruginosa* PAO1 (Ge et al. 2004, 2007; Hu et al. 2005; Zhang et al. 2005; Yan et al. 2007; Huang et al. 2008; Wang et al. 2008; Chen et al. 2008). However, strain M18 has several unusual features that distinguish it from other *P. aeruginosa* strains, and indeed make it more similar to soil-inhabiting biocontrol *Pseudomonas* spp. such as *P. fluorescens* 2-79 (Gurusiddaiah et al. 1986) and Pf-5 (Howell and Stipanovic 1980; Nowak-Thompson et al. 1999). First, the predominant phenazine produced by strain M18 is phenazine-1-carboxylic acid (PCA) rather than pyocyanin (PYO). PCA is considered to be a major biocontrol compound (Mavrodi et al. 2006), while PYO is not necessary for fungal killing (Gibson et al. 2009). It has been determined that much more PCA can be produced by strain M18 than by *P. aeruginosa* PAO1 (Huang et al. 2009). Second, M18 is the first strain reported to produce both PCA and pyoluteorin (Plt). The combination of PCA and Plt can extend the activity of the strain because they have different antifungal spectra and can function synergistically against various phytopathogens. The Plt biosynthesis gene cluster, of approximately 30 kb (Huang et al. 2004, 2006) including its flanking regulatory region, is highly homologous to that of the biocontrol strains *P. fluorescens* Pf-5 and CHA0, and can be expressed in these strains. In contrast, an inactivated frameshift mutation in *pltB* has been found in the nosocomial Liverpool Epidemic Strain *P. aeruginosa* LESB58 (Winstanley et al. 2009). Thirdly, the regulation of synthesis of PCA and Plt in M18 differs from that in other strains of *P. aeruginosa*. For example, PCA is negatively regulated and Plt is positively regulated by the global regulator GacA (Ge et al. 2004) and by RsmA (Zhang et al. 2005; Ren et al. 2013). Other studies have been conducted on the differential regulation of PCA and Plt production by quorum sensing (QS) signaling *N*-acyl homoserine lactones (AHLs) secreted by M18 (Yan et al. 2007; Chen et al. 2008). The interrelationship between the QS *las* and *rhl* systems in strain M18 differs from that in *P. aeruginosa* PAO1 (Lu et al. 2009a). The differentially regulated production of PCA and Plt in M18 might enable the strain to produce variously weighted combinations of these two compounds, thereby generating versatile

antifungal activities under different environmental conditions. However, based on the recently published complete genome sequences of M18, genomic sequences of other *Pseudomonas* spp. in GenBank, and the phylogenetic tree generated by maximum parsimony from 58 phylogenetically useful genes in *P. aeruginosa* (Mathee et al. 2008), the M18 genome is most similar to that of the Liverpool Epidemic Strain *P. aeruginosa* strain B58 (LESB58) except for the presence of specific accessory regions of the M18 genome. Thus, M18 is indeed a novel *P. aeruginosa* strain originating from the rhizosphere and we now designate it as the rhizosphere-originating strain *P. aeruginosa* M18 (Wu et al. 2011).

The rhizosphere-originating strain *P. aeruginosa* M18 is an effective biocontrol agent with an excellent broad antimicrobial spectrum and strong activity against phytopathogens including fungi like *Rhizoctonia solani*, *Sclerotina sclerotiorum*, *Fusarium oxysporum*, *Colletotrichum gossypii*, *Mycosphaerella melonis*, *Phytophthora capsici*, *Ralstonia solanacearum* and so on, Gram-negative bacteria like *Xanthomonas oryzae* pv. *oryzae* and Gram-positive bacteria like *Bacillus cereus*, and also nematodes.

Our group has concentrated on studies of the genomic structure and the synthesis and regulation of phenazines in strain M18 for decades. In this chapter, we summarize and highlight some recent, exciting insights into genome features and the regulation and expression of the two *phz* gene clusters in this strain, emphasizing the features that distinguish it from its clinical counterparts and exploring its development as a cell factory to produce the useful secondary metabolite PCA for agricultural purposes.

9.2 The Conserved Core and the Specific Accessory Regions of the M18 Genome

The genome of the rhizosphere strain *P. aeruginosa* M18 has been completely sequenced, assembled, and deposited in GenBank with the accession number CP002496. The genome contains a conserved core region that shares homology with other sequenced *P. aeruginosa* strains except for the presence of several specific accessory regions (Wu et al. 2011). The average GC content of the M18 genome is 66.5 %, which is remarkably similar to that of the previously sequenced *P. aeruginosa* strains PAO1, PA14, LESB58, and PA7. The M18 genome is comprised of a single chromosome of 6,327,754 bp, which is slightly larger than the 6,264,404 bp of strain PAO1 (Stover et al. 2000) but smaller than the 6,537,648 bp of strain PA14 (Lee et al. 2006), 6,601,757 bp of LESB58 (Winstanley et al. 2009), and 6,588,339 bp of strain PA7 (Roy et al. 2010). The M18 genome contains 5,684 open reading frames (ORFs) that represent 89 % of the total genomic DNA, and 80 RNA genes. Using mGenomeSubtractor online software (Shao et al. 2010), we found that these five *P. aeruginosa* strains of clinical or environmental origin contain a highly conserved core genome encompassing more

than 90 % of ORFs, with several accessory regions of less than 10 % of ORFs (except for the taxonomic outlier PA7) that vary considerably depending on the environmental niche of the various strains. Among the five genomes, only 93 ORFs in the M18 genome, 109 in PAO1, 256 in PA14, 380 in LESB58, and 929 in PA7 were strain specific at the level of 0.6 identity or 0.7 match length/query length.

Analyses of the complete M18 genome sequence and its temperature-dependent transcriptional profiles have revealed several important features that distinguish M18 from sequenced nosocomial *P. aeruginosa* strains. First, although the M18 core genome is most similar to that of *P. aeruginosa* strain LESB58, 14 mammalian virulence-related genes are absent or truncated in M18; M18 is more sensitive to antibiotics including tetracycline, kanamycin, gentamycin, and ofloxacin; and is easier to eradicate in a mouse acute lung infection model than LESB58.

Second, the M18 genome contains several specific accessory regions of genomic plasticity which differ markedly from all sequenced nosocomial isolates. The accessory regions in the M18 genome contain seven genomic islands (GIs), including two novel prophage clusters and five specific non-phage islands. Each prophage contains a gene coding for chitinase, which can break down glycoside bonds and is essential for the degradation of chitin, a major component of the fungal cell wall. A copy of the *capB* gene was identified in prophage II in addition to three others in the M18 genome. The *capB* gene is known to encode a cold shock protein involved in adaptation to cold stress. The non-phage GIs also contain various genes encoding enzymes responsible for complete Type I and Type III DNA restriction-modification systems, pyoluteorin (Plt) synthesis, environmental substance degradation, and so on. Importantly, all mammalian and human pathogenicity-related GIs and prophages contained in other sequenced nosocomial strains of *P. aeruginosa* are absent or inactivated in the M18 genome.

Third, compared with other *P. aeruginosa* sequences available in GenBank, the genome of strain M18 contained the fewest insertion sequences (ISs) (only three) and the most (three) clustered regularly interspaced short palindromic repeats (CRISPRs). Other completely sequenced *P. aeruginosa* strains, PAO1, PA14, PA7, and LESB58, carry 12, 10, 15, and 8 IS elements, respectively. To our knowledge, the IS elements are postulated to be important drivers of genome evolution in many bacteria such as *Xanthomonas* (Monteiro-Vitorello et al. 2005). Aside from serving as vectors for lateral gene transfer, IS elements can generate other types of genome modification including rearrangements, inversions, and deletions, any of which can increase genome instability. Therefore, the comparatively small number of IS elements in the genome of strain M18 is consistent with the relative stability of the strain in rhizosphere niches. The different spacer sequences in CRISPR loci are known to be critical for bacteria to resist foreign phage invasion (Brouns et al. 2008; Marraffini and Sontheimer 2008) and the three CRISPR loci in the genome indicate that strain M18 has developed efficient features to combat phage invasion in the rhizosphere.

Fourth, based on microarray profiles, M18 has developed specific temperature-dependent gene expression patterns to meet the requirements of surviving and thriving in the rhizosphere. In particular, none of the genes in the GIs was

expressed more highly at 37 °C than at 28 °C, but 24 genes in GIs and prophages were up-regulated at 28 °C, a temperature closer to that in rhizosphere niches (Wu et al. 2012).

In summary, we may draw the conclusion that strain M18 has developed a specific genome structure and temperature-dependent gene expression patterns to meet the requirements for fitness and survival under the selective pressure imposed on it in the rhizosphere habitat.

9.3 Phenazine Production and Regulation in Strain M18

Phenazines are important heterocyclic nitrogen-containing compounds produced mainly by some strains of fluorescent *Pseudomonas* spp. and a few other bacterial genera. The biological functions of phenazine compounds have been investigated extensively, including as signaling molecules and in antibiosis and extracellular electron shuttling, which can mediate mineral acquisition and the reoxidation of NADH under high-density oxygen-limited conditions such as those that occur in mature biofilms. Ultimately, phenazines contribute to microbial behavior and ecological fitness in competitive environments (Hernandez et al. 2004; Price-Whelan et al. 2006; Dietrich et al. 2006, 2008; Maddula et al. 2008; Pierson 2010).

More than one kind of phenazine compound can be produced by a single *Pseudomonas* species, and the predominant phenazine differs in various species of *Pseudomonas* of environmental origin. Environmental and clinical isolates of *P. aeruginosa* carry conserved phenazine (*phz*) biosynthesis genes responsible for PCA production, and the PCA molecules can be converted into various phenazine derivatives including PYO, phenazine-1-carboxamide (PCN), and others. Cystic fibrosis isolates of *P. aeruginosa* often overproduce PYO (Finnan et al. 2004; Fothergill et al. 2007; Selezska et al. 2012). The blue-colored PYO is used for phenotypic identification of the organism and represents an important virulence factor (Smirnov and Kiprianova 1990; Pirnay et al. 2009; Selezska et al. 2012). In constrast, PCA and PCN are major phenazines produced by *Pseudomonas* spp. of rhizosphere origin and their crucial roles in plant root disease suppression have been well documented in studies with biological control strains such as *Pseudomonas fluorescens* 2-79 and the *P. chlororaphis* strains 30-84 and PCL1391. The *chlororaphis* strains convert PCA into 2-hydroxyphenazine-1-carboxylic acid (2-OHPCA) and PCN, respectively (Thomashow et al. 1990; Mazzola et al. 1992; Mavrodi et al. 1998; Chin-A-Woeng et al. 2001). PCA and 2-OHPCA contribute significantly to the biocontrol activity of fungal phytopathogens such as *Gaeumannomyces graminis* var. *tritici* (Thomashow and Weller et al. 1998, 1990; Pierson et al. 1994). Chromosomal insertion of genes coding for PCA biosynthesis enhances the efficacy of damping-off disease control by *P. fluorescens*. Phenazine-deficient strains of *P. fluorescens* 2-79 and *P. chlororaphis* 30-84 have reduced survival rates and a diminished ability to compete with the resident microflora (Mazzola et al. 1992; Chin-A-Woeng et al. 2000; Timms-Wilson et al. 2000).

9.3.1 The Predominant Phenazine in Strain M18 is PCA Rather than Pyocyanin

Interestingly, the predominant phenazine compound produced by the *P. aeruginosa* rhizosphere strain M18 is PCA rather than PYO, the major product of *P. aeruginosa* strains of nosocomial origin. When strains M18 and PAO1, of rhizosphere and nosocomial origin, respectively, were inoculated onto KMB plates and incubated at 28 or 37 °C for 1 to 2 days, the two strains produced distinct pigments, as shown in Fig. 9.1a, b. At 28 °C, strain M18 produced a yellow-reddish pigment, while strain PAO1 produced a yellow pigment with a little blue color. However, when the plates were incubated at 37 °C, strain M18 still produced the yellow-reddish pigment, whereas strain PAO1 produced a blue-green pigment. To assay fungal inhibition by the two strains, the fungus *F. oxysporum* var. *cucumerinum* was co-cultured with strains M18 and PAO1 on potato dextrose agar at 28 °C. Mycelium growth of the fungus was totally inhibited by strain M18, but strain PAO1 showed almost no fungal killing activity (Fig. 9.1c). It is well known that phenazine compounds are the major contributors to the colony color of various pseudomonads. PYO produces a characteristic blue-green color, whereas PCA generates a yellow-reddish color. The different colony colors in Fig. 9.1 indicate that different phenazines are produced by the two strains in a temperature-dependent and strain-specific manner. The predominant phenazines produced by strains M18 and PAO1 were determined and found to differ at the different temperatures. The optimal temperature for PCA production by strain M18 was 28 °C, at which temperature the major phenazine produced was PCA at a concentration 9-fold greater than that produced by PAO1. The quantitative ratio of PCA to PYO was very high, reaching up to 105:1 at 28 °C and was 5:1 even at 37 °C. PYO production by PAO1 was greatest at 37 °C, with a quantitative ratio of PYO to PCA of 2:1 (Fig. 9.2).

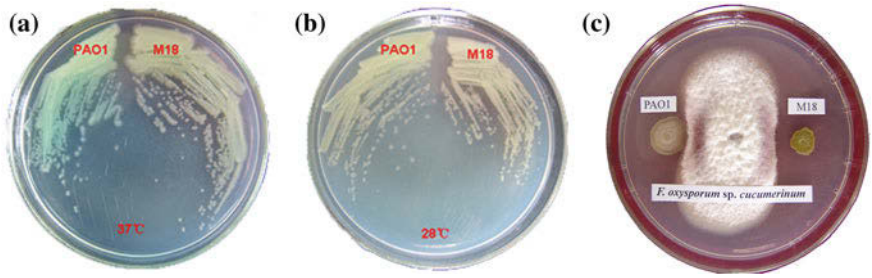

Fig. 9.1 Different phenotypes of *P. aeruginosa* M18 and PAO1. **a** Pigments produced by strains M18 and PAO1 grown for 16–24 h on KMB plates at 37 °C and **b** 28 °C. **c** A 5 mm plug of the fungus *F. oxysporum var. cucumerinum* was placed in the center of a PDA plate, and cultures of *Pseudomonas* strains M18 and PAO1 were inoculated 25 mm from the center on each side of the plate. Strain M18 strain showed stronger inhibitory activity than did PAO1 against *F. oxysporum* (Huang et al. 2009)

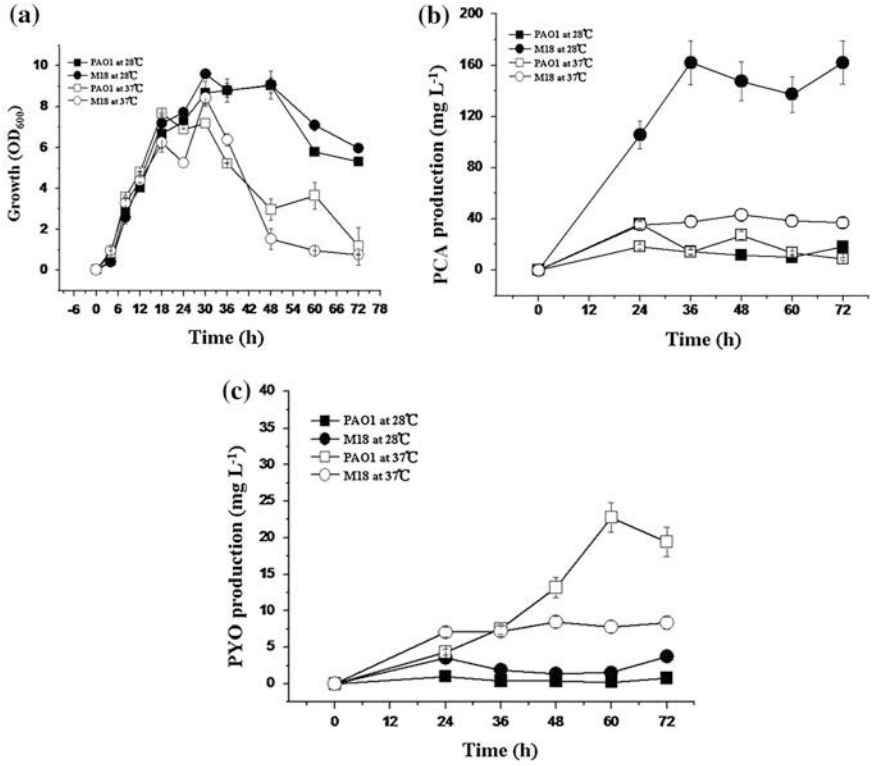

Fig. 9.2 Dynamic growth curves and differential accumulations of PCA and PYO in strains *P. aeruginosa* M18 and PAO1. **a** The dynamic growth curves and **b** PCA'and **c** PYO production of *P. aeruginosa* strain M18 (*circles*) and PAO1 (*squares*) grown in PPM broth at 28 °C (*solid symbols*) and 37 °C (*open symbols*). Values are the means and standard deviations of triplicate cultures (Huang et al. 2009)

9.3.2 A Regulatory Feedback Loop Controls Expression of the Two phz *Gene Clusters in Strain M18*

The extensive diversification that has occurred during microbial evolution is manifested in the distribution of phenazine genes in bacteria originating from various environmental niches (Klockgether et al. 2010; Mavrodi et al. 2010, 2012). The core phenazine biosynthetic gene cluster, *phz*, responsible for the production of PCA, a precursor of various phenazine derivatives, is highly conserved among various pseudomonads (Mavrodi et al. 2006, 2012). The core *phz* gene cluster has been shown to move between genera via horizontal gene transfer, thereby driving genetic diversity in phenazine-producing bacteria (Fitzpatrick 2009). The pseudomonads can be divided into two categories based on the copy number of the core *phz* gene cluster contained within the chromosomal genome. The first category is

composed of those *Pseudomonas* spp. strains containing only one *phz* gene cluster and includes *P. fluorescens* 2-79, *P. chlororaphis* 30-84, PCL1391, and others (Mavrodi et al. 1998; Delaney et al. 2001; Chin-A-Woeng et al. 2001). These particular pseudomonads are known as plant growth-promoting rhizobacteria (PGPR). They inhibit soilborne phytopathogenic fungi and are beneficial to plant growth and crop production (Dwivedi and Johri 2003). Moreover, they have evolved a high degree of ecological dependence on the plant environment, and cross-species infection of animals or humans has been extremely rare.

Two nearly identical core *phz* gene clusters, termed *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*), respectively, but with different promoters and flanking regions, are found in the second category of pseudomonads, which includes the most extensively studied phenazine producer, *P. aeruginosa* (Mavrodi et al. 2001). *P. aeruginosa* is a well-characterized opportunistic pathogen of animals and humans, and also is ubiquitously distributed throughout soil and aquatic habitats (Berg et al. 2005; Mavrodi et al. 2010, 2012). The location of the two *phz* gene clusters and their flanking genes has been well characterized in various *P. aeruginosa* genomes. Two *phz* gene clusters are also found in the genome of the rhizosphere-originating strain *P. aeruginosa* M18, which shares 99 % homology with *P. aeruginosa* PAO1. The distance between the two *phz* gene clusters is ~2.7 MB in *P. aeruginosa* M18, a little longer than the ~2.6 MB in strains PAO1 and PA14. The gene arrangements in M18 that flank the two *phz* gene clusters are the same as those in other strains of *P. aeruginosa* except for a spacer of 520 bp of unknown function in M18 between *phzG2* and the downstream ORF PAM18_3137. In PAO1, PA14, PA7, and LESB58 this intergenic sequence is 88, 174, 255, and 318 bp long, respectively. These intergenic sequences consist of clustered interspaced short palindromic repeats of unknown function, the length of which seems to be strain specific. In contrast, the intergenic region directly downstream of the *phz1* gene cluster between *phzG1* and the downstream gene *phzS* is almost the same in all *P. aeruginosa* strains.

The relationship between regulation and expression of the two *phz* gene clusters involved in PCA synthesis was investigated in detail in *P. aeruginosa* M18 (Li et al. 2011). A feedback loop regulating expression of the two *phz* gene clusters was identified (Fig. 9.3). PCA produced by the wild-type strain was compared with that produced by the chromosomally inactivated mutants M18ΔP1 and M18ΔP2, in which the expression of the *phz1* and *phz2* gene clusters, respectively, were inactivated. PCA synthesized by M18ΔP1 was only about 20 mg/L, or one-quarter of that from strain M18, and PCA production from M18ΔP2 was almost completely abolished, suggesting that little PCA could be produced from the *phz1* gene cluster in the absence of PCA originating from the *phz2* gene cluster. These results suggested that the *phz2* gene cluster could only produce a small amount of PCA and that the majority of PCA was produced from the *phz1* cluster. The results further suggested that PCA produced from the *phz2* cluster could activate expression of the *phz1* gene cluster to produce more PCA in strain M18. We also observed that both of the *phz* gene clusters could respond to exogenous synthetic PCA to produce much more PCA. We deduced that the expression of the two *phz*

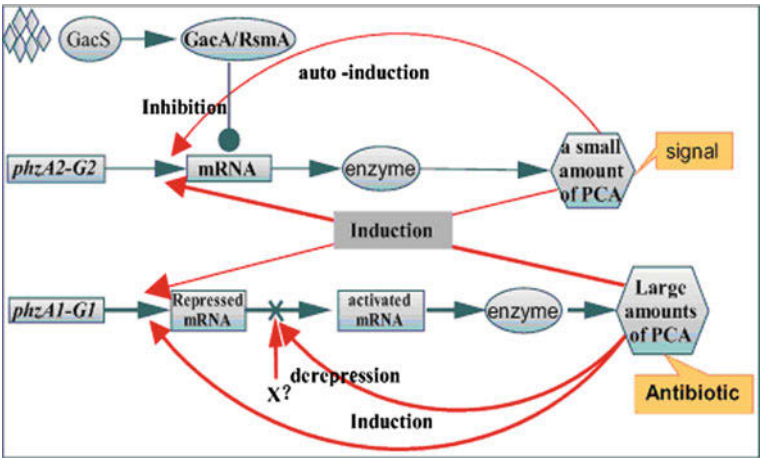

Fig. 9.3 Integrative relationship of the Gac/Rsm signal transduction pathway and expression of two *phz* operons in *P. aeruginosa* M18. Low efficiency expression of the *phzA2-G2* gene cluster produces a small amount of PCA to autoinduce its own transcription and expression of the *phzA1-G1* gene cluster (*red fine lines*). High transcriptional expression of *phzA1-G1* is blocked in the 5′-UTR region and can be partially relieved at the post-transcriptional level by PCA or some unknown factor(s) (*red thick lines*). The interactions of autoinduction and induction of the two *phz* clusters result in large amounts of PCA that acts as an antibiotic for biocontrol. Negative control of *phzA2-G2* expression by GacA occurs mainly at the post-transcriptional level in response to environmental signals. Symbols: *solid circle*, inhibition; *solid arrow*, activation; *diamond*, environmental signals; X, unknown factor(s) (Li et al. 2011)

gene clusters represents a feedback amplification pattern such that the *phz1* gene cluster is mostly repressed without induction by PCA produced from the *phz2* gene cluster. Thus, a small amount of PCA produced from the *phz2* cluster functions as a signal to induce self-expression and to activate the expression of the *phz1* cluster, thereby generating a large amount of PCA in wild-type M18.

A hierarchical cascade pattern of the LasIR and RhlIR quorum sensing (QS) systems that involves small diffusible signaling molecules in response to environmental changes is a well-documented cell density-dependent mechanism of intercellular communication in *P. aeruginosa* PAO1 (Latifi et al. 1996). In contrast to the various N-acyl-homoserine lactones (AHLs) that mediate complicated QS systems in other *P. aeruginosa* strains, we found that a single phenazine molecule, PCA, produced in different quantities by the two *phz* gene clusters, acts as the functional mediator involved in the regulatory feedback loop governing expressions of the two *phz* gene clusters in *P. aeruginosa* strain M18.

Several distinguishing features have been characterized in this regulatory feedback loop. First, each of the two *phz* gene clusters plays a distinctive role to produce different amounts of PCA. It has previously been reported that several antibiotics are able to function as molecular signals at subinhibitory concentrations and to enhance potentially adaptive characteristics to support microbial survival

(Latifi et al. 1996; Linares et al. 2006; Fajardo and Martinez 2008; Mlot 2009). Phenazine has been demonstrated to play a dual role, both as a signaling molecule at a lower concentration and as an antibiotic at a relatively higher one. Notably, we found in this study that the two *phz* clusters play different roles to meet the requirements of producing different amounts of PCA molecules: the less efficiently expressed *phz2* gene cluster produces a small amount of PCA which can then act as a signal to induce the expression of the *phz1* cluster and many other genes, while the more highly expressed *phz1* cluster is responsible for producing a larger amount of PCA that can then act as an antibiotic to combat fungal or bacterial phytopathogens in rhizosphere niches. Comparison of microarray-based transcriptome assays of wild-type strain M18 and the *phz1* mutant M18ΔP1 showed that the small amounts of PCA produced from the *phz2* cluster could function as a signal molecule to activate the expression of six genes, including *mexGHI* (a general phenazine transporter) in addition to *phz1* and *phz2*, and to inhibit expression of 18 ORFs including 11 of unknown function and seven genes related to twitching motility.

The feedback loop of the two *phz* gene clusters is regulated efficiently and flexibly to control the amounts of PCA produced, presumably to prevent the potential toxic effect of elevated phenazine concentrations on cell growth. We found that the expression of the *phz1* gene cluster could potentially be more highly transcribed, but that the corresponding transcript was blocked by the presence of two cis-acting elements located, respectively, from about +1 to +90 nt and from +255 to +337 nt in the 5′-noncoding region of *phz1* transcripts. Secondary structure analysis of these elements downstream of the transcriptional start site indicated the presence of a potential riboswitch in the region between +230 and +337 nt. Riboswitches are complex folded RNA domains that directly bind a specific metabolite, such as an amino acid, and then control gene expression by exploiting changes in the RNA structure to influence transcription elongation or translation initiation (Mandal and Breaker 2004; Winkler and Breaker 2005). To date, all riboswitches appear to function as repressors of gene expression in response to dynamic primary metabolites (Brantl 2004). Recent studies have made it clear that riboswitches represent a diverse and widespread form of regulation. Here, for the first time, we describe a predicted riboswitch located in the +230 to +337 nt of 5′-UTR of *phz1* mRNA in strain M18 that may be involved in the regulation of *phz* gene expression, a region responsible for the biosynthesis of the secondary metabolite PCA. We hypothesize that such an on–off switch serves as a defensive countermeasure and could easily change secondary structure in response to various environmental cues, although the detailed mechanism remains unknown. What is clear from the current data is that the two *phz* gene clusters have developed specific interactive regulatory features under the evolutionary selective pressure imposed on *P. aeruginosa* M18. We suspect that the PCA molecule, other unknown factor(s), and RNA-mediated regulation are involved in the ability to turn on or off the expressions of the two *phz* gene clusters, allowing for dominant and dynamic regulation of PCA biosynthesis by using this regulatory feedback loop through 5′-UTRs in *P. aeruginosa* M18.

We also found that PCA production is negatively controlled by GacA in *P. aeruginosa* M18 at 28 °C (Ge et al. 2004) in contrast to synthesis of PCA and PYO in *P. aeruginosa* PAO1, which is positively regulated by GacA at 37 °C (Reimmann et al. 1997). The reduced PCA production in strain M18 occurred mainly at the post-transcriptional level in the *phz2* gene cluster (Li et al. 2011). PCA production was increased in the inactivated *gacA* mutant M18G, and reduced in the *rsmA*-inactivated mutant M18RA (Zhang et al. 2005) (see below), a result opposite to PYO production in other *P. aeruginosa* strains (Heurlier et al. 2004; Burrowes et al. 2006). However, the detailed mechanism(s) by which the Gac/Rsm signal transduction system regulates the expression of the two *phz* gene clusters remains to be investigated.

Finally, it was found that the LysR family transcriptional regulator PqsR is an activator of PCA biosynthesis in *P. aeruginosa* M18 (Lu et al. 2009b).

9.3.3 *The Two* phz *Gene Clusters are Regulated Oppositely by RsmA in Strain M18*

The Gac/sRNA/Rsm signal transduction system, a well-documented mechanism involved in post-transcriptional regulation in prokaryotes, acts by modulating translation initiation at target transcripts. The two-component regulatory system GacS/GacA is conserved across a wide range of Gram-negative bacteria and plays a key role in mediating successful adaptation to changing environments (Heeb et al. 2001). The system consists of a sensor kinase, GacS, and a response regulator, GacA. GacS is located on the cell membrane and receives an unknown signal(s) to phosphorylate the response regulator GacA, which then positively controls the transcription of small regulatory RNAs (sRNAs) such as RsmY and RsmZ. Expression of these sRNAs is thought to adjust the rate of translational initiation by sequestering RNA-binding proteins of the RsmA family (Liu et al. 1998; Majdalani et al. 2005; Storz et al. 2005; Babitzke and Romeo 2007; Lapouge et al. 2007). The RsmA regulon in *P. aeruginosa* PAK has recently been reported to cover over 500 genes, of which approximately two-thirds are affected positively in an *rsmA* mutant and the rest are affected negatively by RsmA inactivation. RsmA acts directly as a negative translational regulator to competitively bind the SD sequence of mRNA targets, while the positive effects of RsmA are achieved indirectly by RsmA-mediated interference with translation of specific regulatory factors (Brencic and Lory 2009). To date, the RsmA homolog CsrA has been shown to activate gene expression by stabilizing *flhDC* mRNA in *Escherichia coli* (Wei et al. 2001), but positive and direct RsmA-mediated regulation of gene expression has not previously been found in *Pseudomonas* spp. Surprisingly, we found that expression of the two *phz* gene clusters in *P. aeruginosa* M18 was oppositely regulated by RsmA through direct binding of its targets: the *phz1* transcript was negatively regulated and the *phz2* transcript was positively regulated (Ren et al. 2013).

Based on previously published reports, four modification genes, *phzM*, *phzS*, *phzH,* and *phzO,* are involved in conversion of PCA into various phenazine derivatives by *Pseudomonas* spp. (Stover et al. 2000; Chin et al. 2001; Mavrodi et al. 2006; Parsons et al. 2007; Greenhagen et al. 2008). PCA can be converted to PYO by *phzM*, a methyltransferase, and *phzS*, a monooxygenase, encoded by genes that flank the *phz1* gene cluster and are known to play a critical role in the conversion of PCA into PYO in *P. aeruginosa* PAO1 (Mavrodi et al. 2001). The enzyme encoded by *phzH* converts PCA to PCN and the corresponding gene in the wild-type M18 genome is inactivated by a frame-shift mutation. The gene *phzO*, which is involved in the conversion of PCA to 2-hydroxyphenazine-1-carboxylic acid (2-OHPCA), does not exist in the M18 genome (Wu et al. 2011).

To investigate the role of RsmA in control of *phz* gene expression, the triple-mutant strains M18ΔMSP1 and M18ΔMSP2 were first constructed in which *phzM, phzS,* and gene clusters *phz1* or *phz2* were deleted or inactivated. The quadruple mutants M18ΔMSAP1 and M18ΔMSAP2 were then constructed in which *rsmA* was inactivated in the triple mutants. These four mutant strains were used to accurately measure PCA production from *phz1* or *phz2* as influenced by RsmA without interference of PhzM and PhzS. PCA production by the quadruple mutant M18ΔMSAP1 was only one-third of that from the triple-mutant M18ΔMSP1, indicating that the expression of the *phz2* gene cluster was positively regulated by RsmA, whereas PCA production from M18ΔMSAP2 was 3-fold greater than that from M18ΔMSP2, indicating that expression of the *phz1* gene cluster was negatively regulated by RsmA. The inverse regulation of the two *phz* gene clusters by RsmA was not temperature-sensitive in strain M18.

The target sites bound by RsmA are conserved and related to the SELEX-derived consensus sequence 5′-($^{A}/_{U}$)CANGGANG($^{U}/_{A}$)-3′, where N is any nucleotide (Lapouge et al. 2008). We found that the *phz1* RsmA-binding site (RABS) 5′-CUCGGAGG-3′ within the 5′-UTR overlapped with the predicted ribosome binding sequence (SD), whereas the predicted RABS 5′-UAUGGAUG-3′ within the *phz2* transcript was located 18 nucleotides upstream of the predicted SD sequence. The leader sequence of the *phz1* transcript favors the adoption of a conformation for competitive binding of RsmA and the 30S ribosome subunit (Lapouge et al. 2008). Using *RNA Structure* (Reuter et al. 2010), we predicted that in *phz2*, the optimal secondary structure of the leader region spanning 46 nucleotides from +160 to +205 is likely to form a large stem-loop structure such that the SD sequence is trapped. The binding of RsmA to the target motif of the *phz2* transcript is likely to destroy the stem-loop structure, and might improve access of the 30S ribosomal subunit to SD sequence of the transcript, thus allowing translational initiation (Fig. 9.4). The possible RsmA-mediated activation of *phz2* expression via direct binding of its target was further demonstrated by substitutions of the predicted RABS and its flanking base-paired region in the 5′-non-coding stem-loop structure and by a series of experiments including gel shift analysis of the RsmA-*phz2* transcript in vitro and *phz* RNA-RsmA immunoprecipitation in vivo. All the data indicated that the different locations of the RABS on

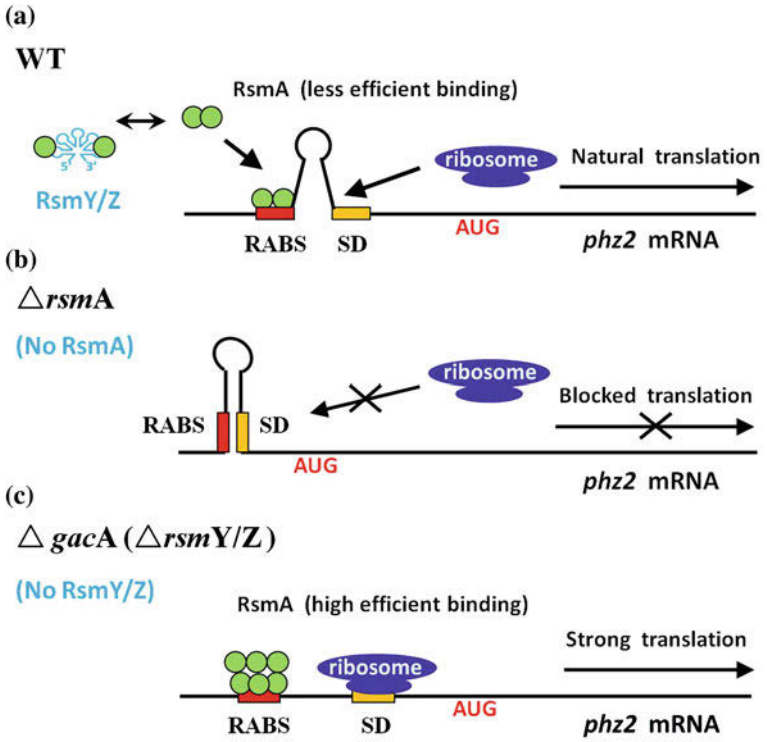

Fig. 9.4 The Gac/Rsm activation model of expression of the *phz2* gene in *P. aeruginosa* M18. **a** In the wild-type strain, RsmA is partially sequestered by the two sRNAs (RsmY and RsmZ), and the weak stem-loop structure favors inefficient RsmA post-translational activation of the *phz2* transcript. **b** In the *rsmA* mutant, the RABS and SD sequence are bound together to form a strong stem-loop structure resulting in translation inhibition. **c** In the *gacA* mutant, the stem-loop structure is completely prevented by direct binding of RsmA to the transcript and strong translation is triggered. Symbols: *green circles* indicate RsmA protein (Ren et al. 2013)

the 5′-untranslated region of the two *phz* RNAs led to the opposite RsmA-mediated activity, either activation or interference, with the two *phz* post-transcriptional events.

9.3.4 *Contribution of the Two* phz *Gene Clusters to PCA Production at Different Temperatures*

Temperatures of 28 °C, representing a soil environment, and 37 °C, representing the human body, were selected to investigate the contributions of each of the two *phz* gene clusters to PCA production in *P. aeruginosa* M18 (Ren et al. 2013). To eliminate interference from PCA modification by phzS and phzM and the influence

of RsmA on expression of the two *phz* gene clusters, the quadruple mutant strains M18ΔMSAP1 and M18ΔMSAP2 were used in these studies. The results indicated that the relative contributions of the two *phz* gene clusters to PCA production were reversed as the temperature changed from 28 to 37 °C. Thus, the *phz2* gene cluster made a major contribution to PCA production at 28 °C, whereas at 37 °C the *phz1* gene cluster made the major contribution (Table 9.1). Temperature-sensitive regulation of the two *phz* gene clusters occurred mainly at the post-transcriptional level as determined by measuring *β*-galactosidase activity of *phz′-′lacZ* transcriptional and translational fusions in the *rsmA*-inactivated strain M18ΔRA at both 28 and 37 °C. Expression of the two transcriptional fusions was not temperature-sensitive, but translation of *phz2* mRNA doubled at 28 °C compared with that at 37 °C and translation of *ph1* mRNA at 37 °C was 3-fold greater than that at 28 °C. These results suggest the involvement of unknown factor(s) independent of RsmA in regulating translation of the two *phz* gene clusters in response to temperature. Furthermore, as the temperature increased from 28 to 37 °C, the expression of the *phz1* gene cluster was up-regulated while that of the *phz2* cluster obviously was downregulated. The complex differential expression of the two *phz* clusters, with unique "functions" of each cluster, appears to provide a distinct advantage over a single cluster and would facilitate adaptation over a wide range of environments including not only variations in temperature, but also in oxygen availability in environmental and host-associated biofilms, as recently described by Recinos et al. (2012).

Table 9.1 Temperature-dependent contribution of two *phz* gene clusters to PCA production

Strain	PCA (μg/ml)[a]		Transcriptional fusion		Translational fusion[b]	
	28 °C	37 °C	28 °C	37 °C	28 °C	37 °C
M18ΔMSAP2	26.2 ± 6.0	79.2 ± 3.9				
M18ΔMSAP1	39.7 ± 3.4	15.9 ± 1.0				
M18ΔRA/pMP1C			6,028.4 ± 71.2	10,010.1 ± 412		
M18ΔRA/pMP2C			3,201.2 ± 209	3,075.3 ± 111.9		
M18ΔRA/pMP1L					141.7 ± 3.9	731.9 ± 42
M18ΔRA/pMP2L					273.2 ± 12	138.3 ± 18

[a] PCA production was measured in two quadruple mutant strains, M18ΔMSAP2, in which *phzM*, *phzS*, *phz2,* and *rsmA* were inactivated, and M18ΔMSAP1, in which *phzM*, *phzS*, *phz1,* and *rsmA* were inactivated, at temperatures of 28 and 37 °C

[b] The *β*-gal activity (Miller units) of the *phz* transcriptional fusions pMP1C and pMP2C and the translational fusions pMP1L and pMP2L was determined in the *rsmA* mutant M18ΔRA at 28 and 37 °C Each value is the mean ± standard deviation of triplicate cultures (Ren et al. 2013)

9.3.5 Temperature-Dependent Expression of *phzM* and its Regulatory Genes *ptsP* and *qscR*

PhzM, one of the enzymes involved in the conversion of PCA to PYO in *P. aeruginosa* PAO1 (Mavrodi et al. 2001; Parsons et al. 2007), is a predicted 36-kDa protein similar to enzymes involved in the methylation of polyketide antibiotics by *Streptomyces* spp. (Yang et al. 1999). The different amounts of PCA and PYO accumulated by strains M18 and PAO1 encouraged us to further investigate the expression and regulation of *phzM*. The differential production of PCA and PYO was found to be related to temperature-dependent and strain-specific expression of *phzM*, which was more highly expressed at 37 °C than at 28 °C in *P. aeruginosa* strains. 3-fold and 5-fold increased activity of *phzM*'-'*lacZ* translational fusions was detected in strains M18 and PAO1, respectively, at 37 °C compared with that at 28 °C. The temperature-dependent and strain-specific expression of *phzM* resulted in much greater PCA accumulation in strain M18 at 28 °C and more PYO in strain PAO1 at 37 °C (Huang et al. 2009). Much more PCA was produced by both wild-type M18 and the *phzM*::Km inactivation mutant M18 pM than by wild-type PAO1 or the *phzM*::Km inactivation mutant PAO1 pM, and PYO production was completely abolished in both *phzM* mutant strains M18pM and PAO1pM. The amount of PCA produced by both M18 and M18pM at 28 °C was almost 10-fold greater than that produced by PAO1 and PAO1pM. PYO was the predominant phenazine produced at 37 °C only in wild-type strain PAO1. The temperature-dependent expression of *phzM* in strains M18 and PAO1 occurred mostly at the transcriptional level, as we obtained nearly the same expression ratios of the *phzM*′-′*lacZ* translational and transcriptional fusion activities at the two temperatures, though the detailed mechanism(s) involved in the temperature-dependent and strain-specific expression of *phzM* remains unknown.

The effects of several global regulatory genes including *gacA*, *rsmA*, *rhlI*, *lasI*, *ptsP,* and *qscR* on *phzM* gene expression were also investigated. It was found that the regulators GacA and RsmA had no obvious effect on *phzM* gene expression, while LasI and RhlI had a positive effect and QscR and PtsP had a negative effect. Interestingly, we found that the expression of *lasI* and *ptsP* also occured in a temperature-dependent pattern and that the two regulatory genes exerted a strong regulatory effect on *phzM* expression.

The differential production of PCA and PYO by strains M18 and PAO1 may be a consequence of selective pressures imposed in different niches over a long evolutionary process. Expression and regulation of the two *phz* phenazine biosynthetic gene clusters as well as QS systems and other global regulators in *Pseudomonas* spp. strains are strongly controlled by environmental conditions (Pierson et al. 1994). The environment-dependent expression and differential regulation of the two *phz* gene clusters in *P. aeruginosa* seems to allow it to adapt to diverse environment conditions, such as variations in nutrition, pH value, cell density, etc. and especially temperature and oxygen, as mentioned in the sections

above. Additional environmental conditions and other regulators involved in the expression of the two *phz* gene clusters and the different amounts of PCA accumulated should be further investigated.

9.3.6 Small RNA Chaperone Hfq Regulates PCA Biosynthesis Through Direct and Indirect Pathways

In addition to two-component signal transduction and cell density-dependent quorum sensing, small noncoding RNAs (sRNA) comprise another important class of regulators utilized by bacteria to coordinate expression of secondary metabolite genes in response to environmental and metabolic stresses (Waters and Storz 2009; Sonnleitner et al. 2011). Hfq is a conserved RNA chaperone protein which originally was characterized as a host factor (HF-1) for phage Qβ RNA replication and subsequently was shown to be widely distributed in the bacterial kingdom, with multiple homologs in the annotated genomic data base (Brennan and Link 2007). The ubiquitous RNA chaperone Hfq is known for its role in global posttranscriptional regulation by binding AU-rich sequences of target mRNA and facilitating pairing between sRNAs and mRNAs, and for its involvement in regulating bacterial virulence and stress tolerance (Brennan and Link 2007).

The molecular mechanism adopted by the Hfq protein to control PCA biosynthesis was investigated in *P. aeruginosa* M18 (Wang et al. 2012). In culture, an *hfq* mutant of *P. aeruginosa* M18 exhibited an obvious, deep blue color in comparison to the parental strain. The blue-green pigment produced by *P. aeruginosa* is mainly composed of PYO. The possibility of elucidating the unique gene regulatory network controlling secondary metabolism in *P. aeruginosa* M18 prompted a further investigation of the Hfq-mediated global regulation of the biosynthesis of PYO and PCA. Hfq was shown to bind to the 5′-noncoding region of *qscR* and *phzM* transcripts and to repress post-transcriptional processing. The two Hfq targets shared similar secondary structure, with a short single-stranded AU-rich spacer (a potential Hfq-binding motif) linking two stem-loops in the 5′-noncoding region. Mutation of *hfq* resulted in overproduction of PYO and reduced PCA due to reduced Hfq interference with post-transcriptional processing of *phzM* mRNA and consequent promotion of the conversion of PCA to PYO.

The LuxR family transcriptional regulator QscR has been reported to negatively control *phz* expression (Chugani et al. 2001; Wang et al. 2008). It was also found that Hfq positively controlled the expression of the *phz2* cluster and enhanced PCA biosynthesis through both QscR-mediated transcriptional regulation at the promoter and the regulation of an unknown factor(s) at the operator. Taken together, these results suggest that Hfq, potentially in cooperation with unknown small noncoding RNAs (sRNAs), tightly controls phenazine biosynthesis through both direct posttranscriptional inhibition of *phzM* gene expression and indirect repression of *qscR* transcription.

Table 9.2 PCA production by wild-type *P. aeruginosa* strain M18 and its genetically modified derivatives

Strain	M18	M18G	M18GQ	M18GQ/phz	M18UMS/phz
Feature	Wild-type strain	GacA mutant	GacA, QscR mutant	GacA, QscR mutant harboring plasmid-borne *phz* cluster	Triple mutant in *phzM*, *phzS*, and the 5′-noncoding region of *phz1* cluster contained on a plasmid
PCA(g/L)[a]	0.1–0.2	0.5–1.0	2.0–3.0	3.0–4.0	5.0–6.0

[a] The ranges of PCA produced are the mean values from multiple cultures

9.4 Construction of a PCA-Overproducing Strain by Genetic Modification of *P. aeruginosa* M18

Based on the results obtained from the our studies of the regulation of expression of the two *phz* gene clusters in *P. aeruginosa* strain M18 and the finding that expression could be enhanced by supplementation with exogenous PCA or the introduction of additional *phz* gene clusters into strain M18, multiple genetically modified M18 derivatives have recently been developed using nonscar deletion strategies and augmentation with recombinant plasmids carrying *phz* gene clusters. Finally, the *phz* gene clusters have been highly expressed in these genetically modified strains in which elevated PCA production has been achieved (Table 9.2). The optimal medium components and fermentation condition also have been investigated using surface response methodology, and PCA production has successfully reached more than 5 g/l of culture after 72 h under cost-efficient production conditions (Li et al. 2010; Su et al. 2010; Zhou et al. 2010). A new antifungal pesticide, Shenqinmeisu, with PCA as a major component, was first registered in China in 2011 and is now widely applied in commercial farm fields to protect rice and vegetables against diseases caused by *Rhisoctonia solani*, *F. oxysporum* and other phytopathogens of agricultural and economical importance. Sales have reached 2.2 million US dollars during the last 2 years in China. We believe that this new pesticide will be applied over even larger scales in the future because of its high efficiency, low cost, and safety to growers and the environment compared with traditional chemically synthesized pesticides.

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Chapter 10
Phenazines in the Environment: Microbes, Habitats, and Ecological Relevance

Linda S. Thomashow

Abstract Phenazines, the pigmented, redox-active metabolites produced by certain fluorescent pseudomonads, streptomycetes, and members of a few other bacterial genera, have long been recognized for their broad-spectrum antibiotic activity. Much has been learned in recent years about the synthesis of these compounds and the diverse roles they play in the physiology of the microorganisms that produce them, but surprisingly little is known about their presence and turnover in natural ecosystems or their significance in microbial habitats. Phenazine producers are found throughout nature in association with plant and animal hosts and in terrestrial, freshwater, and marine habitats, and may reach sufficient numbers that the phenazines they produce can be extracted directly from environmental samples. This chapter focuses on recent reports that highlight the diversity of habitats from which phenazine producers have been recovered and the significance of the phenazines they produce in the ecosystems in which they reside.

10.1 Introduction

Long recognized as colorful "secondary metabolites" with broad-spectrum antibiotic activity, natural phenazines are now known to include more than 100 different structural derivatives. The simplest phenazines are produced by fluorescent *Pseudomonas* spp. that are typically associated with plant and animal hosts, and these compounds have been studied extensively as microbial fitness determinants (Mazzola et al. 1992; Lau et al. 2004b), for their roles in the biological control of plant pathogens (reviewed in Mavrodi et al. 2006; Pierson and Pierson 2006) their

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effects on plant hosts (Phillips et al. 2004; De Vleesschauwer and Höfte 2009), and as virulence factors in plant and animal disease (Rahme et al. 1995; Denning et al. 1998; Mahajan-Miklos et al. 1999; Lau et al. 2004a, b; Allen et al. 2005; Caldwell et al. 2009; Recinos et al. 2012). More recent studies have revealed that phenazines have an active role in the microbial community dynamics and physiology of the cells that produce them: they contribute to biofilm formation and architecture (Maddula et al. 2006, 2008; Dietrich et al. 2008; Ramos et al. 2010; Wang et al. 2011; Recinos et al. 2012); regulate patterns of gene expression (Dietrich et al. 2006); and function as electron shuttles that can facilitate iron acquisition (Wang et al. 2011), modulate redox homeostasis (Price-Whelan et al. 2007), and support survival under conditions of low oxygen availability (Wang et al. 2010). Synthesis of these compounds proceeds via a common genetic pathway, *phzABCDEFG,* responsible for the assembly of the core phenazine tricycle (Mentel et al. 2009; Mavrodi et al. 2008, 2010, 2012b; and see Chap. 1), with one or more accessory genes responsible for derivatization of the core structure either linked to the *phz* operon or located elsewhere in the genome. The structural modifications of the core tricycle directed by these accessory genes contribute to many of the biological functions of phenazines, thereby influencing the fitness and activities of the producing bacteria. Evidence of conservation of the core biosynthesis operon (Fitzpatrick 2009; Mavrodi et al. 2010; Schneemann et al. 2011) has opened the way to the use of molecular approaches to detect and monitor populations of phenazine producers in terrestrial habitats and to rapidly screen environmental samples for the presence of bacteria capable of producing phenazines of potential pharmaceutical value. This chapter focuses on recent reports that highlight the diversity of habitats from which phenazine producers have been recovered and the significance of the phenazines they produce in the ecosystems in which they reside.

10.2 Phenazines and Phenazine Producers in Terrestrial Habitats

The hundreds of reports of phenazine-producing strains of fluorescent pseudomonads that have been recovered from plant tissues or rhizosphere soil and studied as agents for the control of plant diseases (see Table 10.1 to examples) serve for document the worldwide distribution of these bacteria and their frequent association with plant hosts, but they provide little insight into the relative abundance and population biology of phenazine producers in nature. In a study aimed at understanding the microbial basis for the natural suppressiveness to Fusarium wilt caused by *Fusarium oxysporum* of a soil from the Châteaurenard region of France, Mazurier and colleagues (2009) determined the frequency, diversity, and biological control activity of pseudomonads producing phenazine antibiotics in a Châteaurenard soil with low iron availability, and in a nonsuppressive (conducive)

Table 10.1 Phenazine producers isolated worldwide for biological control activity

Strain	Origin	Species	Source or reference
2-79	Wheat rhizosphere, USA	*P. fluorescens*	Weller and Cook (1983)
NSK2	Hydroponic barley roots, Belgium	*P. aeruginosa*	Iswandi et al. (1987)
30-84	Wheat rhizosphere, USA	*P. chlororaphis*	Pierson and Thomashow (1992)
PGS12	Corn, Belgium	*P. chlororaphis*	Georgakopolous et al. (1994)
In-b-109 In-b-784	Rice, Philippines	*P. aeruginosa*	Rosales et al. (1995)
PCL1391	Tomato roots, Spain	*P. chlororaphis*	Chin-A-Woeng et al. (1998)
PNA1	Chickpea roots, India	*P. aeruginosa*	Anjaiah et al. (1998)
TX-1	Creeping bentgrass, USA	*P. aureofaciens*	Powell et al. (2000)
*phz*24	Tomato rhizosphere, France	*P. chlororaphis*	Schoonbeek et al. (2002)
GC-26	Grassland, Korea	*P. aeruginosa*	Lee et al. (2003)
Eh1087	Apple tree, New Zealand	*Pantoea agglomerans*	Giddens et al. (2003)
PA23	Soybean root tips, Canada	*P. chlororaphis*	Savchuk and Fernando (2004)
PUPa3	Rice rhizosphere, India	*P. aeruginosa*	Kumar et al. (2005)
M18	Sweet melon rhizosphere, China	*P. aeruginosa*	Hu et al. (2005)
UQ12 UN38	Soybean, Brazil	*P. fluorescens*	Botelho and Mendonça-Hagler (2006)
CMR5c CMR12a	Red cocoyam rhizosphere, Cameroon	*Pseudomonas* sp.	Perneel et al. (2007)
GP72	Green pepper rhizosphere, China	*P. chlororaphis*	Liu et al. (2007)
BP35	Black pepper shoot endosphere, India	*P. aeruginosa*	Aravind et al. (2009)
PB-St2	Sugarcane stem, Pakistan	*P. aurantiaca*	Mehnaz et al. (2009)
UPM P3	Oil palm, Malaysia	*P. aeruginosa*	Fariman and Meon (2009)
Pf1TZ	Almond rhizosphere, Tunisia; endophyte	*P. fluorescens*	Kilani-Feki et al. (2010)
RM-3	Maize rhizosphere, India	*P. aeruginosa*	Minaxi and Saxena (2010)
LBUM 223	Strawberry rhizosphere soil, Canada	*Pseudomonas* sp.	St.-Onge et al. (2011)
Psd	Black gram rhizosphere, India	*P. fluorescens*	Upadhyay and Srivastava (2011)
HC9-07 HC13-07 JC14-07	Wheat stem endosphere, China	*P. fluorescens*	Yang et al. (2011)
R2-7-07 R4-34-07 R11-23-07	Wheat rhizosphere soil, USA	*P. fluorescens*	Mavrodi et al. (2012a, b, c)
BS1393	Barley rhizosphere soil, Russia	*P. chlororaphis*	V. V. Kochetkov pers. comm. (2012)
SLPH10	Take-all decline soil, The Netherlands	*Pseudomonas* sp.	J. Raaijmakers pers. comm. (2012)

Carquefou soil of lower pH where iron is more biologically available. The results showed that pseudomonads carrying the phenazine biosynthesis gene *phzC* accounted for up to 13.9 % of the total rhizosphere population of culturable pseudomonads from the roots of flax and tomato grown in the wilt-suppressive Châteaurenard soil, but that such bacteria were not detectable in the rhizospheres of plants grown in the conducive Carquefou soil. The indigenous *phzC*$^+$ isolates produced phenazine compounds in vitro and comprised eleven distinct BOX-PCR genotypes, some of which clustered with the phenazine-1-carboxylic acid (PCA)-producing reference strain *Pseudomonas fluorescens* 2-79 and others with the phenazine-carboxamide (PCN) and hydroxyphenazine-producing *Pseudomonas chlororaphis* strains PCL1391 and PGS12. The suppressiveness of the Châteaurenard soil is thought to be due to fluorescent pseudomonads acting in synergy with nonpathogenic *F. oxysporum* to starve pathogenic fusaria of carbon and iron (Lemanceau et al. 1993), and when introduced in combination with the nonpathogenic strain *F. oxysporum* Fo47, isolates representative of each of five different indigenous genotypes of *phzC*$^+$ pseudomonads were effective in controlling Fusarium wilt of flax. No disease suppression was observed when the bacteria were introduced alone or upon introduction of a phenazine-nonproducing mutant strain (Mazurier et al. 2009). Collectively, these results provide evidence of a diverse population of phenazine producers in the Châteaurenard soil and indicate that phenazine production is integral to the ability of the bacteria to contribute to the soil's natural wilt suppressiveness.

10.2.1 Phenazines in Dryland Agriculture: A Case Study

Recent studies of the diversity and population structure of phenazine-producing bacteria in the USA have focused on strains recovered from wheat fields in the low precipitation zone of the Columbia Plateau of the inland Pacific Northwest, USA, a region that receives 150–350 mm of precipitation annually and has been farmed almost exclusively to cereals for over 125 years. In a recent PCR-based analysis of DNA extracted from the rhizosphere soil of crops grown in this area, Mavrodi and colleagues (2010) identified *phzF* gene sequences consistent with the presence of a diverse population of indigenous phenazine producers in eleven commercial farm fields. Follow-up surveys of 80 fields scattered over more than 22,000 km^2 (Fig. 10.1) revealed that most fields harbored populations of *phzF*$^+$ bacteria of up to log 7.1 cfu g^{-1} (fresh weight) on the roots of cereals and native plants and that root colonization by the bacteria was inversely correlated with annual precipitation or irrigation (Fig. 10.2; Mavrodi et al. 2012a, c). Analyses of the population structure and diversity of more than 400 *phzF*$^+$ isolates from across the region by BOX-PCR identified 31 distinct genotypes that formed four clusters (Parejko et al. 2012). All of the isolates exhibited high 16S rDNA sequence similarity to

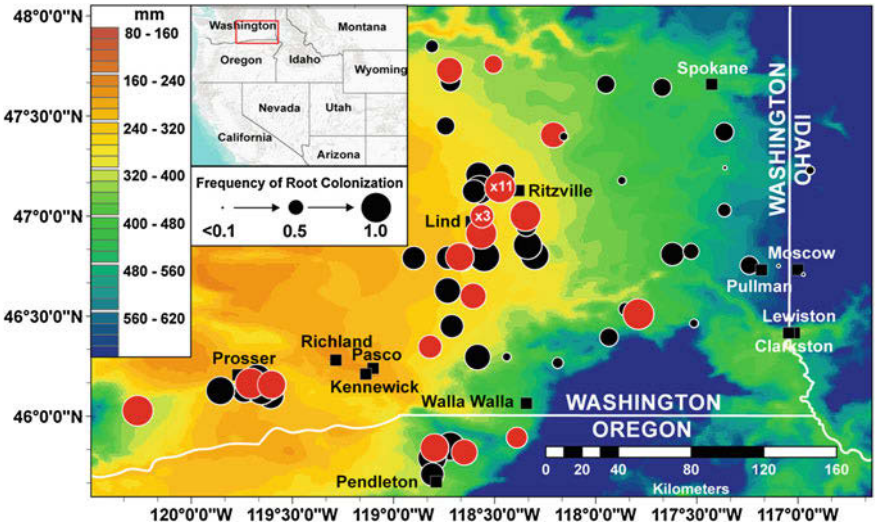

Fig. 10.1 Distribution of *phz*$^+$ pseudomonads in wheat fields from Eastern Washington, Northeastern Oregon, and Western Idaho. Map of the surveyed area is overlaid with mean annual precipitation values for the years 1971 through 2000 (see inset for scale). The locations of sampling sites are indicated by *circles* whose sizes are proportional to the frequency of rhizospheres colonized by *phzF*$^+$ *Pseudomonas* spp., as determined for 8–16 individual plants (see inset for scale). *Red circles* indicate sites from which samples were extracted for PCA. (Reproduced from Mavrodi et al. 2012a)

members of the *P. fluorescens* species complex including *Pseudomonas gessardii, Pseudomonas orientalis, Pseudomonas libanensis,* and *Pseudomonas synxantha* and, like the model strain *P. fluorescens* 2-79, representative isolates of each genotype produced PCA but not PCN or hydroxyphenazines in culture. PCA at concentrations of up to 1.6 μg (2.5 nmol) per gram (fresh weight) of root were also recovered from the roots and rhizosphere soil of wheat seedlings from 26 of 29 fields sampled directly for the antibiotic, and the amounts detected were correlated with the population size of *phzF*$^+$ pseudomonads on the roots (Fig. 10.2; Mavrodi et al. 2012a). These results are the first to demonstrate the accumulation of a natural antibiotic across a large terrestrial ecosystem and, taking into account the size of the phenazine-producing population and the patchy distribution of bacteria on roots, they provide evidence that PCA can accumulate in the environment in quantities sufficient not only for inter- and intraspecies signaling, but also for direct inhibition of sensitive organisms (Mavrodi et al. 2012a). More generally, the results of these studies indicate that indigenous phenazine-producing pseudomonads are enriched on the roots of crops grown under conditions of moisture deficit that are known to favor the formation of biofilms, complex microbial assemblages supportive of microbial survival in low-moisture habitats. Phenazines are known to

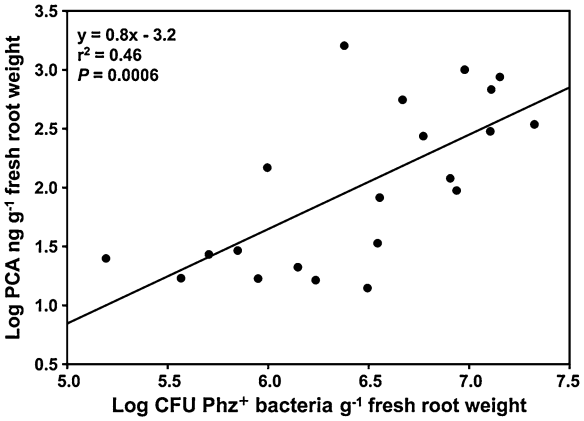

Fig. 10.2 Relationship between the accumulation of PCA in field-grown cereals and populations of indigenous *phz*$^+$ rhizobacteria. Amounts of PCA were determined by extracting the antibiotic from 15-g samples of wheat roots and performing a quantitation of PCA by HPLC-Q-TOF–MS/MS. In each location from which samples were extracted for PCA, the populations of *phz*$^+$ rhizobacteria were determined by analyzing 8–16 individual wheat rhizospheres. (Reproduced from Mavrodi et al. 2012a)

be involved in biofilm formation (Mavrodi et al. 2006, 2012c; Maddula et al. 2006, 2008; Ramos et al. 2010; Wang et al. 2011), and Mavrodi and colleagues (2012a, c) have speculated that phenazine producers are adapted for survival under the arid conditions prevalent on the Columbia Plateau due to their ability to resist desiccation via biofilm formation.

10.2.2 Phenazine Producers from the Guts of Arthropods

Like the fluorescent pseudomonads, actinomycetes are ubiquitous in terrestrial habitats. Soil and plant-associated isolates of these bacteria include such well-known phenazine producers as *Streptomyces antibioticus, Streptomyces griseoluteus, Streptomyces luteogriseus,* and *Streptomyces prunicolor* (Laursen and Nielsen 2004), but there have been only two descriptions to date of phenazines produced by microbial symbionts of arthropods. The first reported arthropod-associated phenazine producers were recovered from the guts of wood-lice, beetles, and millipedes collected at different locations in Europe. Somewhat surprisingly, four separate isolates from different arthropods were all identified as strains of *Streptomyces anulatus* by 16S rDNA analysis, and all were found to produce PCA as well as novel prenylated phenazine derivatives, endophenazines A-D (Fig. 10.3), which exhibited different degrees of antimicrobial activity against

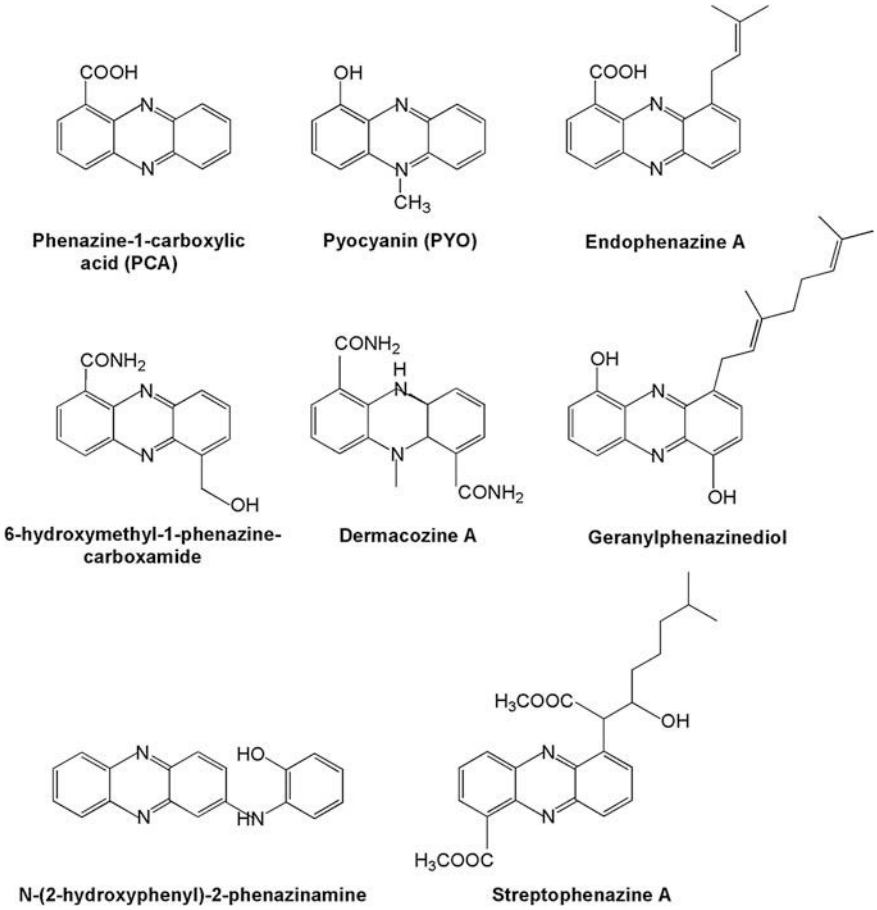

Fig. 10.3 Representative phenazine structural derivatives described in the text

Gram-positive bacteria and some filamentous fungi (Gebhardt et al. 2002). More recently, Patil and colleagues (2010) reported that over 70 % of sampled honeybees in a season carried culturable actinomycetes including *S. anulatus*. Another isolate, BE74, recovered from the guts of bees collected throughout the year was found to be closely related to *Nocardiopsis alba* by 16S rDNA analysis and to contain a putative phenazine biosynthesis locus detected by amplification with PCR primers based on an alignment of *phzD* sequences from *Nocardia dassonvillei, Streptomyces cinnamonensis,* and *Streptomyces anulatus*. Preliminary analysis by RT-PCR indicated that the gene was transcribed in cultures grown under a variety of conditions, suggesting that the strain might be capable of phenazine synthesis in various environments and leading the authors to speculate that "production of phenazine-like redox-active molecules by this isolate could

contribute to its ability to temporarily survive the anoxic or anaerobic conditions that may occur in honeybee guts" (Patil et al. 2010). An alternative hypothesis, derived from a recent model proposed by Scheuring and Yu (2012), is driven by a partner-choice mechanism in which the host fuels interference competition by providing abundant resources and the resulting competition favors the recruitment of antibiotic-producing bacteria. The proposed partner-choice mechanism is more effective when the antibiotic producer is vertically transmitted or has a high immigration rate, which might occur in a hive. Whether either or both explanations can account for the selection of phenazine producers in the guts of arthropods remains to be determined, but given the current interest in mining the environment for novel antibiotics and the availability of molecular tools to facilitate the screening of arthropod microbiomes, further investigation of these interesting associations would seem to be warranted.

10.2.3 Phenazine Turnover in the Environment

The ability to detect phenazines in environmental samples has been facilitated by the availability of efficient and sensitive methods of recovery and detection, but the dynamics of synthesis and decay in situ are still poorly understood. Mavrodi et al. (2012b) recently described a study in which the persistence of PCA that had been applied to the roots and rhizosphere soil of wheat at a concentration of 1 μg g^{-1} was monitored over 4 weeks by HPLC-coupled mass spectrometry. The half-life

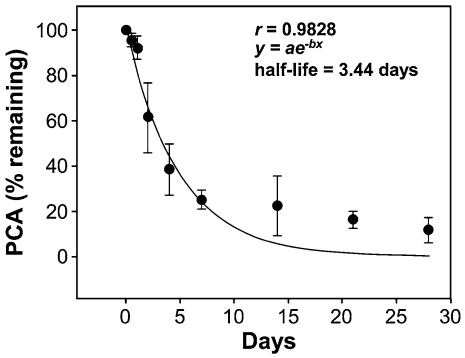

Fig. 10.4 Half-life of PCA in the rhizosphere. PCA (10 μg) in acetonitrile was spiked onto 10 g of wheat roots with rhizosphere soil collected from seedlings that had been grown for 3 weeks in a Shano sandy loam soil. The roots were incubated at room temperature in the dark and sampled in triplicate at 0 and 12 h, 1, 2, and 4 days, and 1, 2, 3, and 4 weeks. PCA was extracted from the roots essentially as described by Mavrodi and colleagues (2012a) and amounts recovered from the roots were quantified using a Q-TOF-2 mass spectrometer. The half-life of the antibiotic, 3.4 days, was calculated by the experimental decay model $y = ae^{-bx}$. Error bars correspond to standard deviations of the mean. (Reproduced from Mavrodi et al. 2012b)

under these conditions was 3.4 days (Fig. 10.4), suggesting that PCA is not long-lived in the rhizosphere and that synthesis must be sustained to maintain concentrations sufficient to inhibit fungal root pathogens. Similar studies in which PCA was applied to soil (0.3 μg g^{-1}), recovered by solid-phase extraction, and monitored by stacking capillary electrophoresis indicated that the compound could not be recovered from rhizosphere soil after 3 days, or from near-surface soil after 7 days (Guo et al. 2010). Whether the decay values in these studies are due to microbial degradation or irreversible binding to soil constituents remains to be determined, but phenazine-degrading bacteria can readily be recovered from soil. For example, the recently sequenced strain *Sphingomonas wittichii* DP58 from the rhizosphere of pimiento can reportedly use PCA as a sole source of carbon and nitrogen (Yang et al. 2007; Ma et al. 2012). The activity in soil of such bacteria probably helps to explain why in some cases phenazine producers that are highly antagonistic to fungal pathogens in vitro may fail to provide consistent biological control in the field.

10.3 Aquatic Habitats as a Source of Phenazine Producers

Because *Pseudomonas aeruginosa* is an important opportunistic pathogen of humans and other animals, studies of phenazine producing pseudomonads from aquatic habitats have focused almost exclusively on this species, and a recent report by Selezska and colleagues (2012) suggests that water, rather than soil, is its primary habitat. In a two-pronged study, these authors first conducted a comprehensive survey of the literature to test the idea that *P. aeruginosa* thrives equally well "close to everywhere" and concluded that the overwhelming majority of intentionally isolated strains were of aquatic origin, including both freshwater and marine sources, with water giving rise to larger numbers of isolates with greater isolation efficiency than soil (Selezska et al. 2012). These authors then determined the diversity and population structure of 381 strains obtained from among planktonic isolates collected from aquatic habitats along two river systems in Northern Germany extending over a distance of 150–200 km each. Genetic analysis, combined with ecological and phenotypic data, revealed the presence of six extended clonal complexes and suggested that water quality might be an important driver of the microevolutionary population dynamics of *P. aeruginosa* in the environment. Aquatic strains of *P. aeruginosa* have been collected worldwide, not only from water but also from deep-sea sediments and the surfaces of marine plants and animals. Like isolates of terrestrial origin, these strains produce a range of products in culture including PCA, 1-hydroxyphenazine, and the blue-colored compound pyocyanin (PYO; Fig. 10.3) first described by Fordos (1859), who isolated it from purulent wound dressings. Whether these compounds are produced in aqueous habitats or if they function in microbial interactions in the environment is unclear, but it is interesting to note that one marine strain of *P. aeruginosa*

produced PYO only when it was co-cultured with other isolates, identified as *Enterobacter* sp., from the same habitat (Angell et al. 2008).

More structurally complexes are the phenazines produced by marine isolates (mainly actinomycetes). There is considerable interest in these compounds and the bacteria that produce them because they are considered to be novel resources for drug discovery. Phenazine producers from marine habitats are typically detected first based on pigmentation or biological activity in culture, with subsequent purification by solvent partitioning and chromatography and structure determination by spectroscopy and NMR. Functional assessments can include determinations of antibacterial, antifungal, anticancer, and radical scavenging activity, activity as cancer preventive agents, and inhibition of enzymes such as acetylcholinesterase, or the chemoprevention targets quinone reductase 2, NF-κB, and inducible nitric oxide synthase (Cimmino et al. 2012, and see Chap. 13). Examples of novel phenazines isolated recently from marine bacteria include 6-hydroxymethyl-1-phenazine-carboxamide and 1,6-phenazine-dimethanol from the marine strain *Brevibacterium* sp. KMD, associated with a purple vase sponge (Choi et al. 2009); the dermacozines, a new family of compounds from deep-sea actinomycetes isolated from a Mariana Trench sediment (Abdel-Mageed et al. 2010); geranylphenazinediol from *Streptomyces* sp. strain LB173, isolated from ambient sediment of a brown alga growing in the Kiel Fjord (Ohlendorf et al. 2012); and N-(2-hydroxyphenyl)-2-phenazinamine, a novel anticancer and antifungal compound from *N. dassonvilii* isolated from arctic sediment (Gao et al. 2012). Structures of some of these phenazines are illustrated in Fig. 10.3. Whether they are produced in nature, and what their role there might be, remains to be determined. In one case, however, members of a class of novel compounds termed streptophenazines were produced in varying amounts by the sponge-derived *Streptomyces* sp. strain HB202 only when it was exposed to subinhibitory levels of tetracycline or bacitracin, suggesting a role in defense (Mitova et al. 2008).

10.4 Phenazines in the Clinical Environment

Despite widespread distribution in terrestrial and aquatic habitats, *P. aeruginosa* is most commonly described as an opportunistic pathogen of humans and other animals, especially patients with cystic fibrosis (CF) and other chronic pulmonary diseases. It has long been appreciated that phenazines produced by *P. aeruginosa* are present in high enough concentrations in the airways of such patients that they can contribute to the persistent inflammation and pathological changes seen in their bronchial tissues (Wilson et al. 1988; Denning et al. 1998; Lau et al. 2004a, b; Allen et al. 2005; Caldwell et al. 2009). However, remarkably little is known about how the presence of phenazines relates to the irreversible progression of pulmonary decline that culminates in respiratory failure, the primary cause of

mortality in patients with CF. Hunter and colleagues (2012) recently detected PYO and its precursor, PCA, at concentrations of up to 87 μM in the sputum of severely obstructed adult CF patients chronically infected with *P. aeruginosa* and showed that increased phenazine concentrations were significantly correlated with both the severity of lung function impairment and the rate of lung function decline. Patients whose sputum had the highest phenazine concentrations harbored isolates of *P. aeruginosa* that produced the least amount of PYO per cell when grown in culture, but these low levels were compensated by high cell densities of *P. aeruginosa* such that the collective population was sufficient to generate the PYO concentrations detected in sputum during late stages of infection. Moreover, as phenazine concentrations increased in more seriously affected patients, the overall diversity of the microbiota in the sputum declined as detected by 454 multiplex pyrosequencing (Hunter et al. 2012). Collectively, the results of these studies confirm previous reports about the quantities of phenazines present in the airways of CF patients and indicate that they are among the environmental variables that can be linked to CF lung function and the polymicrobial communities that are currently being recognized as an important part of the etiology of CF airway infection. The strong correlation between phenazine concentrations in sputum (but not in culture) and lung function decline observed by Hunter and colleagues (2012) led these authors to conclude that phenazines might have value as biomarkers in adult CF patients chronically infected with *P. aeruginosa.*

10.5 Phenazines Applied in Biological Control

At least one phenazine-producing strain, *P. aureofaciens* BS1393, is currently in use as a commercial biological control agent (V. V. Kotchetkov and D. V. Mavrodi, personal communication). Strain BS1393, isolated in 1991 from the rhizosphere of barley near Voronezh, Russia, is an active ingredient in the biopesticide "Pseudobacterin-2," which was developed at the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences. Liquid ($2 - 3 \times 10^{9-10}$ cfu/ml) and concentrated (5×10^{11} cfu/ml) formulations of "Pseudobacterin-2" have been shown to be harmless to insects, fish, animals, and humans and were officially licensed and approved in 1999 for use in plant disease control in the Russian Federation. "Pseudobacterin-2" is marketed for the control of a wide range of phytopathogenic bacteria, oomycetes, and fungi including *Phytopthora*, *Rhizoctonia,* and *Fusarium,* as well as for induction of resistance to plant diseases and increased crop yields in organic and conventional farming systems. Extensive trials in cereal, vegetable, and oilseed crops throughout Russia indicate that Pseudobacterin-2 reduced disease severity by 65–88 %, resulting in a 20–25 % yield increase in cereals, grapes, vegetables, and oilseed crops (V. V. Kotchetkov and D. V. Mavrodi, personal communication).

10.5.1 Biological Control and Safety

Given the frequency with which phenazine-producing strains of *P. aeruginosa* have been isolated as potential biological control agents (Table 10.1) and considering the importance of this species as an opportunistic pathogen of humans, it is not surprising that the need to address the safety of such environmental strains as an alternative to the use of synthetic chemical pesticides has been recognized in recent years (Handelsman 2002; Berg et al. 2005). There are still relatively few studies, however, in which plant-associated strains of *P. aeruginosa* have been characterized for traits associated with pathogenesis in animals. Working with the rice rhizosphere strain *P. aeruginosa* PUP3a, Steindler and colleagues (2009) evaluated two quorum sensing systems, LasI/R and RhlI/R, which in clinical strains have a key role in the expression of many virulence factors including phenazines. Both systems were involved in the regulation of plant growth-promoting traits including antifungal activity in vitro and colonization of the rice rhizosphere. PUPa3 was also pathogenic in the infection model animals *Galeria melonella* and *Caenorhabditis elegans* and in both cases pathogenesis was significantly attenuated only when both systems were inactivated, implying that both are independently involved in virulence factor regulation (Steindler et al. 2009).

In a more comprehensive study, Wu and colleagues (2011) compared the complete genome sequence of *P. aeruginosa* M18, an effective biocontrol agent isolated in 1996 from the rhizosphere of sweet melon, to genomic sequences of four *P. aeruginosa* strains of clinical origin. Unlike clinical isolates that typically produce mainly PYO in culture, M18 produces mainly PCA, a PYO precursor, as its major biocontrol compound. The core genome of M18 is largely similar to those of four strains of clinical origin; they all contain two copies of the seven-gene phenazine core biosynthesis operon (see Chaps. 1 and 4), and most genomic differences are confined to accessory regions that account for less than 10 % of the ORFs present. Strain M18 was more susceptible than the virulent strain LESB58 to several antimicrobial agents applied against *P. aeruginosa* in clinical settings and was more readily cleared than LESB58 from the lungs in the acute mouse infection model (Wu et al. 2011). Comparative transcriptome analyses of M18 and PAO1 grown at 28 °C ("rhizosphere temperature") and 37 °C ("human temperature") revealed that M18-induced more genes at 37 °C than at 28 °C and conversely, that more genes were induced in PAO1 at 28 °C than at 37 °C, consistent with gene mobilization in both strains to adapt to stresses associated with temperature-shifted growth (Wu et al. 2012). The results of these analyses provide insight into possible therapeutic strategies in the event of human infection by strain M18, but its similarity to virulent strains and its ability to grow at 37 °C are strong deterrents to its use as a biocontrol agent in the field. Instead, through genetic modification of the strain and optimization of the culture medium, yields of PCA as high as 5 g L^{-1} (20 mM) have been achieved and the phenazine itself is now registered and

marketed as the pesticide Shenquinmycin in China (He and Xu 2011; and see Chap. 7).

The issue of environmental safety also has been addressed directly by Kumar and colleagues (2012), who assessed a number of phenotypic and genetic traits of *P. aeruginosa* BP35, an endophyte from the stem of black pepper with biocontrol potential against *Phytophthora capsici,* a serious constraint to black pepper production in India. Strain BP35 colonized the stems, leaves, and roots of black pepper and tomato, inhibited hyphal growth of *P. capsici,* and produced both phenazines and rhamnolipids effective against oomycete pathogens. Genotyping by analysis of the *recN* gene showed that BP35 clustered most closely to *P. aeruginosa* M18 (described above); analysis by the eBURST algorithm, which examines relationships within clonal complexes, showed that it was a single-locus variant of a clinical isolate from the Netherlands; and multilocus sequence typing with seven conserved housekeeping genes indicated that BP35 was novel among a global collection of 1210 *P. aeruginosa* isolates (Kumar et al. 2012). However, like other isolates of *P. aeruginosa*, BP35 grew at 25°–41 °C, was resistant to numerous antibiotics, exhibited moderate to strong cytotoxicity on mammalian cells in culture, and was pathogenic in an acute mouse airway infection model. These results led Kumar and colleagues (2012) to conclude that BA35 is as virulent as clinical strains of *P. aeruginosa,* and to abandon consideration of the strain for biological control of Phytophthora rot of black pepper and other plant diseases. They indicate further that "It is in the interest of public safety as well as the continuance of sustainable crop protection technologies to develop an unambiguous strain selection policy with due importance to biosafety."

10.6 Concluding Remarks

Of the many insights gained over the past decade into phenazines and the bacteria that produce them, two that are relevant to understanding the role of these compounds in the environment are first, that their biological activity depends largely on their electrochemical properties, and second, that the pathway for phenazine biosynthesis is conserved in nature. Together, these advances provide a foundation for future efforts to decipher the relevance of phenazines in the microbial habitats in which they are produced.

It is now clear that at least among fluorescent *Pseudomonas* spp., electron shuttling contributes not only to the pathogenic and biocontrol potentials of the producing strains, but also to their cellular physiology and ecological fitness. These biological functions are modulated by the physical and chemical properties of individual phenazines, which in turn vary according to the nature and position of the substituents on the heterocyclic core. Evolutionary pressure on bacteria may have selected for different phenazines to serve different roles in the ecosystems in which the bacteria reside, as suggested by Pierson and Pierson (2010), but it is reasonable to speculate that at least some functions of the more highly derivatized phenazines produced by actinomycetes will be carried out by electron shuttling.

Conservation of the phenazine core biosynthesis pathway provides a means not only to detect and track populations of phenazine-producing bacteria in situ, but also to monitor pathway expression under environmental conditions. Phenazine biosynthesis is metabolically costly, and the capacity to produce "expensive" metabolites is more likely to be maintained if the metabolites serve multiple functions (Wink 2009). Whether functional diversity is achieved by individual compounds, by varying the ratio of compounds in precursor-product relationships, or via families of structurally related compounds, it is remarkable that the core biosynthesis genes are conserved among isolates from habitats as diverse as the guts of arthropods and the depths of the sea.

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Chapter 11
Phenazine as an Anticancer Agent

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Abstract Since 1859, when Fordos reported the isolation of the blue pigment 5-N-methylphenazine-1-one, named pyocyanin, more than 100 different natural phenazines have been isolated and more of 6,000 compounds with a phenazine-based skeleton have been synthesised. The biological activities of these compounds including antimicrobial, antimalarial and antiparasitic activities have been reported, although since 1959, phenazines have been associated with anticancer activity and several publication and patents are available thus far. This chapter critically discusses the structural features of both natural and synthetic phenazines in relation to their in vitro, in vivo and available clinical anticancer activity along with a focus on the mode of action.

11.1 Introduction

Phenazines are a large group of natural and synthesised nitrogen-containing heterocyclic compounds. Natural phenazines are derived from the shikimic acid biosynthetic pathway, which is involved in the production of numerous metabolites necessary for bacteria primary growth, including aromatic amino acids and *para*-aminobenzoic acid (Laursen and Nielsen 2004; Budzikiewicz 1993; Mavrodi et al. 1998, 2010; Pierson and Pierson 2010). In particular, shikimic acid is converted to chorismic acid, which represents a key step to obtain the basic phenazine aromatic structure such as phenazine-1-carboxylic acid and phenazine-1,6-dicarboxylic acid (**1** and **2**, Fig. 11.1), which in turn are precursors for more complex

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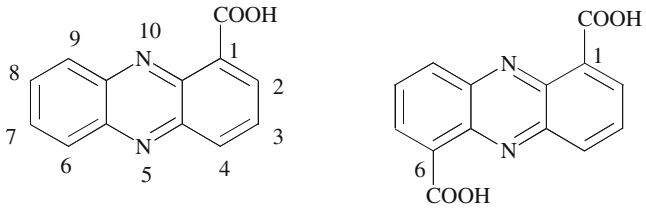


1 Phenazine-1-carboxylic acid **2** Phenazine-1,6-dicarboxylic acid

Fig. 11.1 Chemical structure of phenazine-1-carboxylic acid and phenazine-1,6-carboxylic acid (**1** and **2**)

phenazines (Laursen and Nielsen 2004; Budzikiewicz 1993; Mavrodi et al. 1998, 2010; Pierson and Pierson 2010). It is evident that bacteria produce a wide variety of phenazines and multiple phenazine derivatives. Bioinformatics comparisons of the phenazine biosynthetic genes among several bacteria demonstrate a high degree of conservation of five genes (Mavrodi et al. 2006; Mentel et al. 2009; Gross and Loper 2009). These five genes are considered as the 'core' biosynthetic genes because each of them is required for the synthesis of the basic tricyclic phenazine structure. Recent evidences suggest that these core biosynthetic genes moved among diverse bacterial genera via horizontal transmission (Mavrodi et al. 2010). In most phenazine-producing bacteria, the core biosynthetic genes are flanked by one or more accessory genes that encode different terminal-modifying enzymes that result in the production of additional phenazine derivatives (Pierson and Pierson 2010).

Natural phenazines are mainly isolated as secondary metabolites from *Pseudomonas*. However, phenazines are also produced by a wide variety of Eubacteria including both Gram-negative and Gram-positive species. Phenazine producers include *Nocardia*, *Sorangium*, *Brevibacterium*, *Burkholderia*, *Erwinia*, *Pantoea*, *Vibrio*, *Pelangiobacter* genera (Mavrodi et al. 2006, 2010; Mentel et al. 2009) and also members of actinomycetes, especially *Streptomyces* (Turner and Messenger 1986). Additionally, *Methanosarcina*, a member of the *Archea*, was shown to produce a phenazine derivative (Abken et al. 1998). New phenazine producers continue to be identified such as *Brevibacterium* sp. KMD 003 isolated from a marine purple sponge (Choi et al. 2009; Pierson and Pierson 2010).

The Gram-negative pathogen *Pseudomonas* was known as the first and for several years also considered as the only bacterial genera to produce phenazine pigments. The blue pyocyanin and the green chlororaphine, both produced by *Pseudomonas aeruginosa*, were isolated in the late nineteenth century; this was followed by isolation of the purple iodinin from *Pseudomonas aureofaciens* in 1938 (Turner and Messenger 1986). Phenazines isolated from *Pseudomonas* strains are mostly simple compounds showing a hydroxyl and/or a carboxyl group as substituent at C-9, and are often C2-symmetric compounds. **1** and **2** are believed to be metabolic precursors for other phenazines. These simple phenazines display

antibiotic activity, while anticancer activity is not reported in the literature, at least to the best of our knowledge. Genetic approaches are currently developed to identify novel phenazine-producing bacteria (Schneemann et al. 2011).

Most of the described effects of phenazines during pathogenesis and competition are attributed primarily to their ability to generate reactive oxygen species (ROS) in other organisms and tissues (Pierson and Pierson 2010). In some cases, this may result in host beneficial effect, such as the inhibition of pathogenic organism development (Pierson and Pierson 2010). In other cases, bacterial virulence is enhanced by phenazine production as it interferes with normal host cell functions (Pierson and Pierson 2010). These dual outcomes are due ultimately to the ability of phenazines to accept or donate electrons because of their aromatic structure. Whether they accept or donate electrons is dependent on their redox potential relative to that of other electron transfer compounds in the cell or in the environment (Pierson and Pierson 2010). Early studies on cellular respiratory chains demonstrated the ability of phenazines to uncouple oxidative phosphorylation by shunting electrons from the endogenous pathway in mammalian cells (Pierson and Pierson 2010).

Phenazines are known to be associated with anticancer activities since 1959 and recent reviews discuss the formation of ROS and oxidative stress induced by phenazines (Laursen and Nielsen 2004; Mavrodi et al. 2006; Pierson and Pierson 2010). The generation of these ROS contributes to the virulence of the infection and may contribute to successful host invasion and disease due to their negative effects on a range of host cell functions, including respiratory, ciliary beating, epidermal cell growth, calcium homeostasis, prostaglandin release, neutrophil apoptosis, interleukin-2-release, immunoglobulin G secretion and a protease-antiprotease activity (Laursen and Nielsen 2004; Pierson and Pierson 2010). Cells that are actively respiring, such as tumour cells, appear to be more susceptible to respiratory interference and ROS generation caused by phenazine compounds (Laursen and Nielsen 2004; Pierson and Pierson 2010). Additionally, phenazines known to interfere with topoisomerase I and II activities in eukaryotic cells have been identified and cancer cells, having high levels of both topoisomerases, are more susceptible to this interference (Laursen and Nielsen 2004; Pierson and Pierson 2010). For example, active proliferation of human lymphocytes is inhibited by pyocyanin (Sorensen et al. 1983).

Searching of new biological sources of natural phenazines versus the syntheses of novel phenazine derivatives is an ongoing area of research aimed to obtain novel anticancer compounds (Pierson and Pierson 2010). Indeed, considering the promising biological properties of natural phenazines, several compounds that contain phenazine as a typical moiety have been prepared to increase and/or modulate their activity as well as to decrease their toxicity (Cimmino et al. 2012).

This chapter describes the major structural differences of natural versus synthetic phenazines when exerting anticancer activity. Furthermore, an in depth understanding of their mode of action including cell proliferation inhibition versus

induction of cell death due to interaction with polynucleotides, inhibition of topoisomerases, radical scavenging and charge transfer, etc. was also reported for some phenazine derivatives.

11.2 Natural Phenazines

11.2.1 Functionalised Phenazine

Several simple phenazines described above are also produced by the Gram-positive actinomycete, *Streptomyces*, but it has the ability to produce also more highly substituted phenazine including aldehydes, thioesters, ethers and amides, which besides being antibiotic exhibit radical scavenging and antitumor activity (Laursen and Nielsen 2004).

The hexasubstituted phenazine PD 116,152 (**3**, Fig. 11.2), the methyl ester of 6-formyl-4,7,9-trihydroxy-8-methyl-1-phenazinecarboxylate isolated from *Streptomyces sp.*, showed IC_{50} values of 0.52 and 0.71 μg/mL, respectively, on mouse L1210 lymphocytic leukaemia and human colon adenocarcinoma (HTC-8) cells; moderate in vivo antitumor activity was observed as 49 % of increase in survival

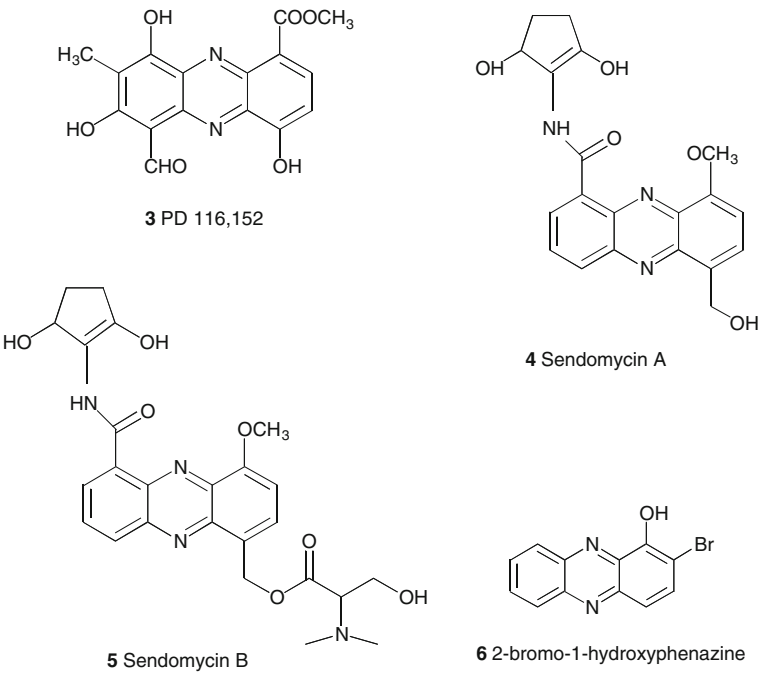


Fig. 11.2 Chemical structure of functionalized phenazine derivatives (**3**–**6**)

of P388 lymphocytic leukaemia-bearing mice treated with 20 mg/kg(i.p. administration) (Smitka et al. 1986; Tunac et al. 1986).

The enol amide sendomycin A and its *N*, *N*-dimethylserine ester, sendomycin B (also known as senacarcin A or DC-59A) (**4** and **5**, Fig. 11.2), with unknown stereochemistry, were isolated from *S. endus* subsp. *aureus* DO-59. Sendomycin A displays in vivo anticancer activity in the mouse sarcoma 180 model with 67 % reduction in murine sarcoma growth at 9.4 mg/kg every day. Toxicity was evidenced at 25 mg/kg (Nakano et al. 1982). No anticancer activity has been reported for the congener sendomycin B.

Recently, Conda-Sheridan and coworkers (Conda-Sheridan et al. 2010) have isolated 2-bromo-1-hydroxyphenazine (**6**, Fig. 11.2) from a marine *Streptomyces* species (strain CNS284) and demonstrated anti NF-KappaB activity. The transcription factor nuclear factor kappa-light chain of activated B cells (NF-KappaB) and, the inducible nitric oxide synthase (iNOS), are potential chemopreventive target along with Phase I (aromatase, quinone reductase 2 (QR2) and Phase II (QR1, glutathione-S-transferase (GST) enzymes that detoxify carcinogens (Conda-Sheridan et al. 2010).

11.2.2 Terpenoid Phenazines

Several phenazines from *Streptomyces* contain isoprenylated C- or N-side chains as common structural feature. Benthocyanin B (**7**, Fig. 11.3), the *N*-monoterpenoid phenazine fused with a phenyl-substituted-γ-lactone, which has a geranyl substituent at N-5, and its congener benthocyanin C (**8**, Fig. 11.3), were isolated from *S. prunicolor*. They appeared to be new free radical scavengers that inhibit lipid peroxidation induced by free radicals in rat liver microsomes with activities that are 30–70 times stronger when compared to vitamin E (Shin-ya et al. 1993; Laursen and Nielsen 2004). Benthocyanin B (**7**) and C (**8**) were also reported to inhibit rat erythrocyte hemolysis with IC_{50} values of 0.56 and 1.30 μg/mL, respectively (Shin-ya et al. 1993; Laursen and Nielsen 2004), although no anticancer activity has yet been reported thus far.

Phenazinomycin (**9**, Fig. 11.3) another *N*-isoprenylated compound isolated from *Streptomyces* sp. WK-2057 (Funayama et al. 1989) is the first phenazinone alkaloid including a sesquiterpene moiety in its structure. The compound exhibited antitumor activity against experimental murine tumours both in vitro with IC_{50} values of 25 μg/mL in normal mouse P388 leukaemia cells and of 3.1 μg/mL in adriamycin-resistant P388 leukaemia cells (Funayama et al. 1989) and in vivo with 40 % of increase in the survival of sarcoma 180-bearing mice when treated at 22 mg/kg day (Funayama et al. 1989; Omura et al. 1989).

WS-9659A (**10**, lavanducyanin, Fig. 11.3) and its chlorinated derivative WS-9659B (**11**, Fig. 11.3), isolated from *Streptomyces* sp. CL190 and 9659, are two other related *N*-substituted monoterpene analogues, showing antitumor activity against mouse leukaemia cells with IC_{50} values of 0.09 and 0.10 μg/mL

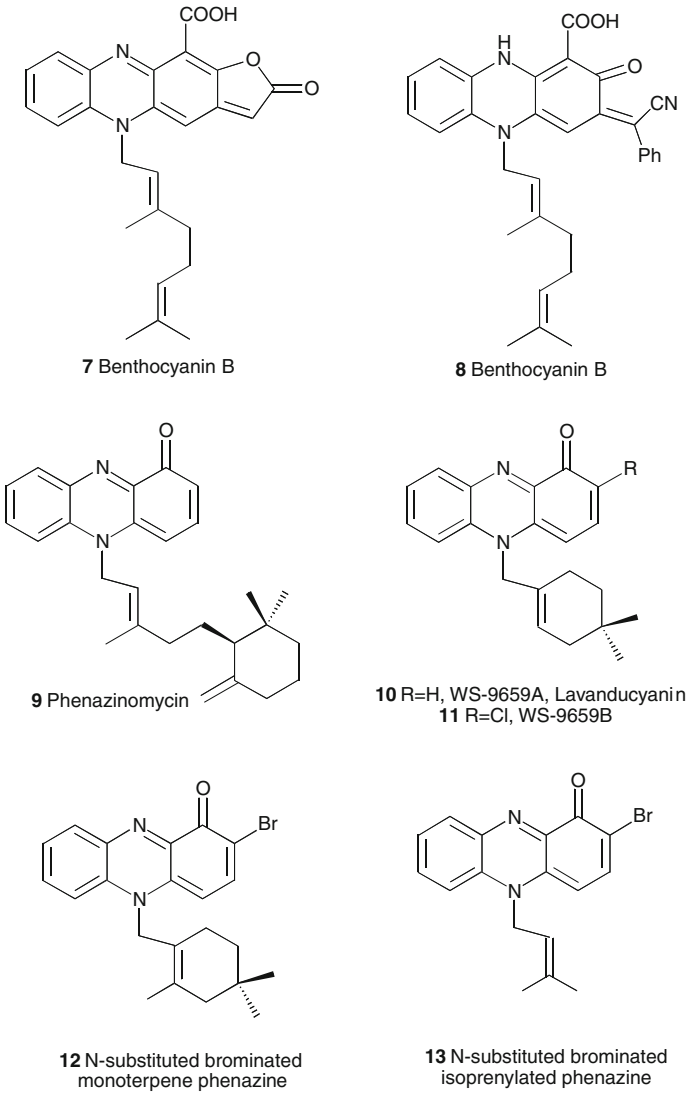


Fig. 11.3 Chemical structure of terpenoid phenazines (**7**–**13**)

respectively in mouse P388 and L1210 leukaemia cells (Imai et al. 1989). At low concentrations (0.0001–0.06 μg/mL), lavanducyanin stimulates the growth of normal cells (Imai et al. 1993; Mastumoto and Seto 1991).

WS-9659A (**10**) and WS-9659B (**11**) are also competitive inhibitors of testosterone 5-alpha-reductase in rat, dog and human prostate tissues (Nakayama et al. 1989a, Nakayama et al. 1989b). Inhibition of the enzyme testosterone 5α-reductase represents a new pharmacological approach toward the treatment of

benign prostate hyperplasia which appears to be mediated by 5α-dihydrotestosterone. WS-9659A appeared to be the most potent with IC_{50} values of 0.5–0.8 μg/mL. In the in vivo evaluation experiment, WS-9659A was administered subcutaneously in immature castrated rats causing a mild decrease in the weight of the ventral prostate induced by testosterone propionate, but did not decrease the weight of the seminal vesicles compared with the vehicle-treated controls (Nakayama et al. 1989b; Laursen and Nielsen 2004).

Recently, from the marine-derived *Streptomyces* sp. (strain CNS284) were isolated together to lavanducyanin (**10**), two new 2-brominated, terpenoid phenazines, as the *N*-substituted brominated monoterpene phenazine (**12**, Fig. 11.3) and the *N*-substituted isoprenylated phenazine (**13**, Fig. 11.3).

In mammalian cell culture studies, compounds **10**, **12** and **13** inhibited TNF-α-induced NFκB activity (IC_{50} values of 4.1, 24.2 and 16.3 μM, respectively) and LPS-induced nitric oxide production (IC_{50} values of >48.6, 15.1 and 8.0 μM, respectively). PGE2 production was blocked with greater efficacy (IC_{50} values of 7.5, 0.89 and 0.63 μM, respectively), possibly due to inhibition of cyclooxygenases in addition to the expression of COX-2. Treatment of cultured HL-60 cells led to dose-dependent accumulation in the subG1 compartment of the cell cycle, as a result of apoptosis (Kondratyuk et al. 2012).

11.2.3 Saphenic Acid and Analogs

The 6-(1-hydroxyethyl) phenazine-1-carboxylic acid named saphenic acid (**14**, Fig. 11.4), was isolated from *S. antibioticus* together with several its analogs having a modified chemical structures. The hydroxyacetic ester of saphenic acid DC-86-M (**15**, Fig. 11.4), was isolated from the soil habitant *S. luteogriseus* DO-86 (Takahashi et al. 1986). The LD_{50} value against experimental murine sarcoma 180 tumours was below 25 mg/kg when administrated i.p (Takahashi et al. 1986; Laursen and Nielsen 2004). It showed 64 % reduction in murine sarcoma 180 growth at 20 mg/kg. A dose of 40 mg/kg is toxic (Takahashi et al. 1986).

The 3-hydroxy-2-methoxypropionic acid ester analogue DOB-41 (**16**, Fig. 11.4), isolated from *Streptomyces* sp., showed moderate effect on the longevity of mice with implanted murine lymphatic P338 leukaemia cells (Shoji et al. 1988; Laursen and Nieslon 2004) with 53 % of increase in survival of sarcoma 180-bearing mice at 10 mg/kg. However, it exhibited a weak effect with only 29 and 13 % survival increases in the mouse L1210 leukaemia and B16 melanoma models, respectively. A dose of 20 mg/kg is toxic (Shoji et al. 1988).

(-)-Saphenamycin (**17**, Fig. 11.4), the (2-hydroxy-5-methyl)-benzoyl ester of saphenic acid isolated from *S. canaries* and *S. antibioticus*, showed an IC_{50} values of 0.15 μg/mL in L5178Y and of 0.6 μg/mL in L1210 mouse leukaemia cell lines (Kitahara et al. 1982) and IC_{50} of 0.6 μg/mL in CCRF/CEM T-cell leukaemia cells (Geiger et al. 1988; Laursen and Nielsen 2004). Moreover, saphenamycin displays a modest life-prolonging effect on mice with leukaemia cell implants. Indeed,

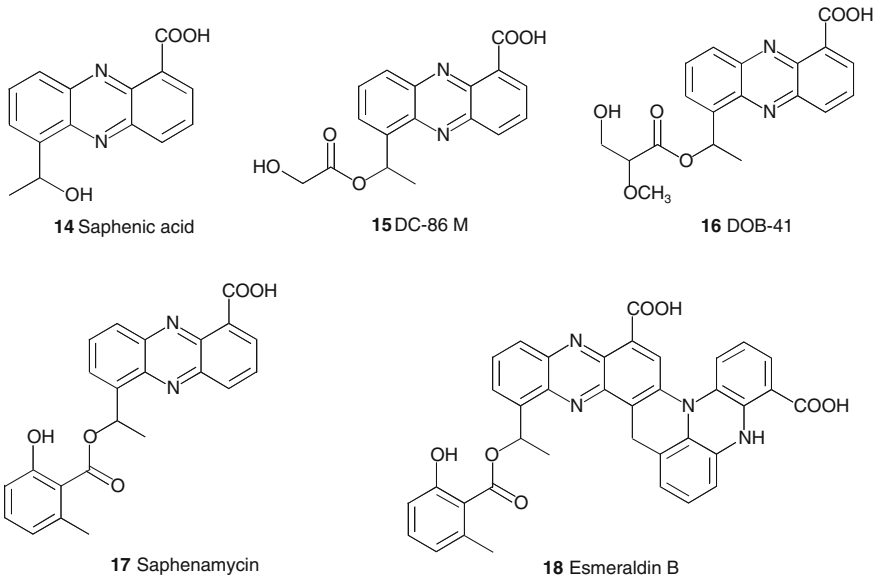


Fig. 11.4 Chemical structure of saphenic acid and analogs (**14–18**)

250 μg/mL mouse/day × 10 days slightly ($p > 0.05$) prolonged by 19 and 20 % the survival of mice intraperitoneally implanted with the L1210 leukaemia and the Ehrlich ascite carcinoma, respectively (Kitahara et al. 1982; Cimmino et al. 2012)

Bahnmüller et al. (1988) have hemisynthetised 9 esters from racemic saphenic acid and they observed that the IC_{50} concentrations in the CCRF/CEM tumour cell line were 18 μg/mL for the *p*-bromobenzoate, 4 μg/mL for the salicylate and 7.4 μg/mL for the acetate esters, but much higher for the remaining ones (Bahnmüller et al. 1988; Cimmino et al. 2012).

The dimeric phenazine derivative esmeraldine B (**18**, Fig. 11.4), which is isolated from *S. antibioticus* (Keller-Schierlein et al.1988), seems to be derived from saphenamycin joined to 6-methylen phenazine-1-carboxylic acid. Its anticancer activity, at least in vitro, with an IC_{50} value of 0.4 μg/mL against eukaryotic tumour cell lines, is similar to those reported for the other phenazines derived from saphenic acid (Geiger et al. 1988; Laursen and Nielsen 2004; Cimmino et al. 2012).

11.2.4 Griseoluteic Acid and Analogs

No anticancer activity was reported for griseoluteic acid (**19**, Fig. 11.5) which represents the main modified phenazine of another subgroup of this family of bioactive bacterial metabolite (Wang et al. 2011). The 3-hydroxy-1-l-valyl ester of

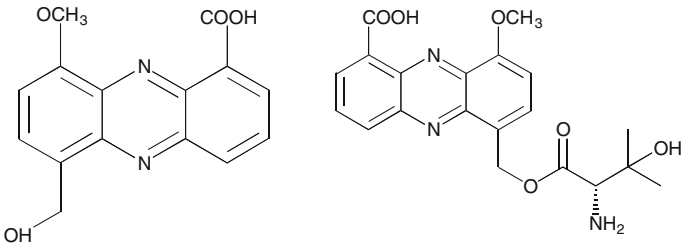


Fig. 11.5 Chemical structure of grisoluteic acid and analogs (**19** and **20**)

griseoluteic acid isolated from the halophile Gram-negative marine bacterium *Pelagiobacter variabilis* collected from a microalga and named pelagiomicin A (also known as LL-14l352alpha; **20**, Fig. 11.5) displays significant in vitro anticancer activity, with IC_{50} values of 0.04 μg/mL in HeLa cancer cell, 0.2 μg/mL in BALB3T3 fibroblasts and 0.07 μg/mL in H-ras transformed BALB3T3/H-ras fibroblasts (Imamura et al. 1997). Instead it appears to be associated with weak if any anticancer activity in vivo in the murine P388 leukaemia model. **20** were also isolated from another unidentified halophile marine bacterium living near a tunicate in the Pacific Ocean and in this case IC_{50} values of 0.2 μg/mL in A2780S ovarian, of 0.5 μg/mL in SW620 colon cancer cells, and of 0.7 μg/mL in CCRF-CEM T-cell leukaemia (Singh et al. 1997), were reported. Normal endothelial cells seem less sensitive with IC_{50} value of 2.7 μg/mL (Singh et al. 1997) but the lack of in vivo activity and the poor toxicological profile observed for **20**, would most likely limit the therapeutic usefulness (Singh et al. 1997). However, pelagiomicin A (LL-14I325alpha) is about five times more cytotoxic in vitro towards cancer cells than LL-14I325beta and it inhibits DNA, RNA and protein syntheses within 10 min when assayed at 0.1, 0.8 and 2.7 μg/mL, respectively (Singh et al. 1997).

11.2.5 Other Bioactive Phenazines

From the culture broth of a *Bacillus* sp. collected from a Pacific deep sea sediment samples (depth 5,059 m), a new phenazine (**21**, Fig. 11.6), which possess a [4.2.2] ring structure, with a semiquinone moiety joined to N^5, N^{10} of phenazine tricyclic ring, was isolated. The compounds were tested only at concentration of 50 μM, due to the low amount available, against P388 and K562 cells. At this concentration **21** was cytotoxic to P388 cells, inhibiting their proliferation by 78.3 % (Li et al. 2007).

Recently, three new phenazines were isolated from *Streptomyces* sp. IFM 1204 and named izumiphenazines A-C (**22**–**24**, Fig. 11.6). **22** is the first example of a phenazine dimer connected via a tetrahydrofuran ring. Izumiphenazines A–C were

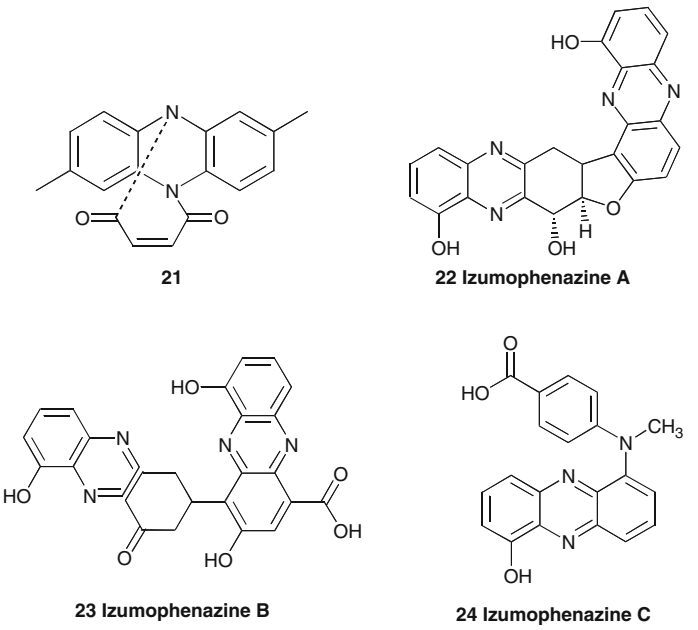


Fig. 11.6 Chemical structure of other bioactive phenazines (**21**–**24**)

evaluated for their activity in overcoming TRIAL (TNF-related apoptosis-inducing ligand) resistance in human gastric adenocarcinoma cells. The results showed that these compounds had a synergistic effect in combination with TRIAL against AGS cells, thereby suggesting their possible use in combination with TRIAL against human gastric adenocarcinoma (Abdelfattah et al. 2010).

11.3 Synthetic Phenazines

11.3.1 Phenazine-1-Carboxamide and Analogs

Inspired from the acridine-4-carboxamide (DACA, **25**, Fig. 11.7), which was reported in the late 1980s as a potent dual topoisomerase I/II inhibitor and anti-tumor agent and that was taken through to phase II clinical trials (Atwell et al. 1987; Antonini et al. 2000) until it was abandoned in 2000 due to its fast metabolism and low human responses (Gamage et al. 2002; Laursen and Nielsen 2004), a series of synthetic monosubstituted phenazine DACA analogues were explored. The attention was initially drawn to the N-[2-(dimethylamino) ethyl] phenazine-1-carboxamide (**26**, Fig. 11.7), which showed to have moderate in vivo activity against both the P388 leukaemia and the Lewis lung (LL) carcinoma, even

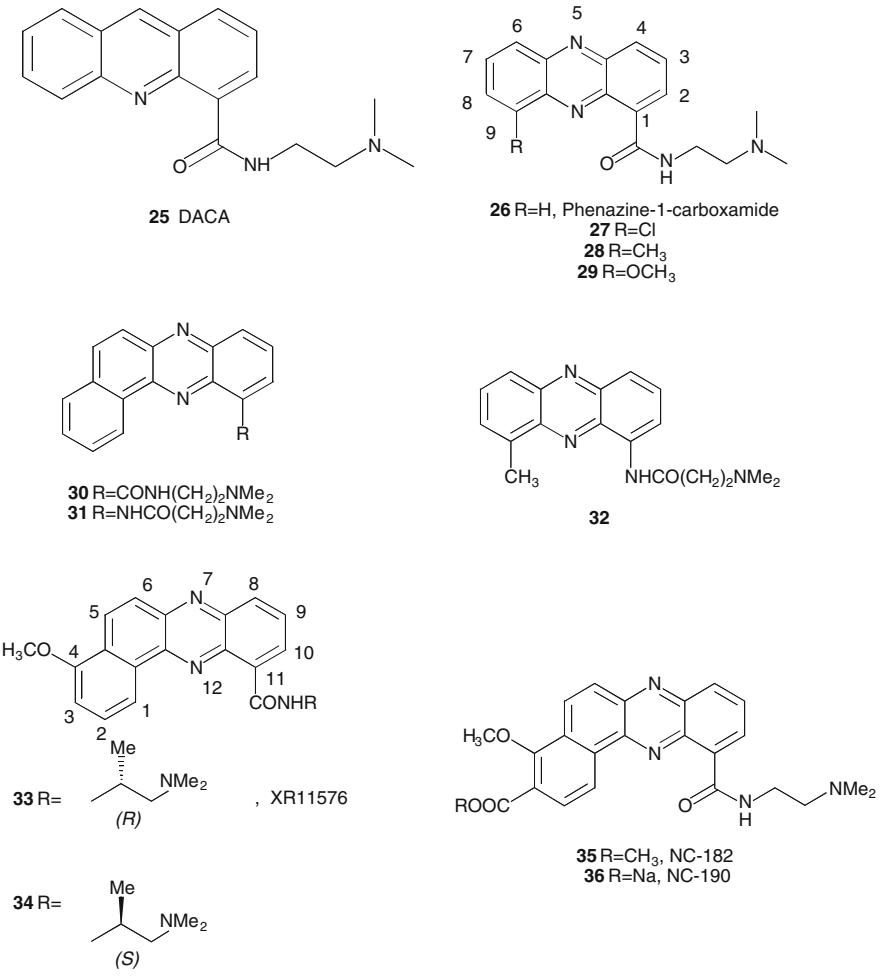


Fig. 11.7 Chemical structure of DACA, phenazine-1-carboxamide and analogs (**25**, **26**–**36**)

though the IC_{50} values against L1210 leukaemia cells of 1,400 nM, was much less cytotoxic compared to that of the parent DACA of 105 nM (Rewcastle et al. 1987). The SAR of a series of phenazine-1-carboxamide derivatives with varying positions (2, 3, 4, 6, 7, 8 and 9), as well as the electronic and lipophilic character of some substituents (Cl, CH_3, OCH_3), was explored. The data thus obtained indicating that moving the amide side chain from the position 1, as well as varying the length of the amide side chain, results in a loss or decrease of activity. A set of derivatives bearing CH_3, Cl, OCH_3 at every available phenazine position, showed the importance of substituent's at 9-position (Rewcastle et al. 1987; Laursen and Nielsen 2004). In fact derivatives having 9-Cl, 9-CH_3 and 9-OCH_3 (**27**–**29**, Fig. 11.7) showed in vivo activity higher of **26** with a IC_{50} values on L1210

leukaemia cells of 11, 42 and 48 nM respectively. 8, 9-benzofused phenazine analogue (**30**), were 10-fold more potent that the 3, 4- and 6, 7-benzofused analogues with an IC_{50} values of 33 nM (Rewcastle et al. 1987; Laursen and Nielsen 2004).

Successively, Gamage et al. (2006) compared the potency of phenazine carboxamides for the variation of the substituents on C-9 and the effects of the variations in the C-1 side chain on a panel of tumour cell lines in culture. Hydrophobic and small 9-substituents as CH_3-, OCH_3- and 8,9-benzo- (**28**–**30**, Fig. 11.7), improved the potency in respect to **26**, in murine P388 leukaemia cell line (P388) with IC_{50} values of 18, 63 and 37 nM, respectively; in H69 parental human cell lung carcinoma cell line (H69) with IC_{50} values of 200, 245 and 68 nM, respectively; and LX4 (P-glycoprotein overexpressing H69) cell lines with IC_{50} values of 290, 820 and 130 nM, respectively. Substitution with N-(2-methoxy) and N-(2-hydroxyphenyl) had only slight effect. There were generally a little difference in IC_{50}s in the parent and P-glycoprotein expressing lines, suggesting that most of the compounds are not affected by the presence of this efflux pump (Gamage et al. 2006).

Regards the effect of variations in the C-1 side chain, only the reversed amides (**31** and **32**, Fig. 11.7), showed significant potency and even these were 30- to 50-fold less effective than the parent compounds **30** and **28,** respectively (Gamage et al. 2006). All of the compounds with other side chains, including those with O, NH and CH_2NH linkers, were essentially inactive. This was consistent with the structural model of how the tricyclic carboxamides bind to DNA, with the carboxamides CO and NH moieties making specific binding contributions (Gamage et al. 2006).

Vicher et al. (2002) reported a series of new substituted 8,9-benzo[*a*]-phenazine carboxamide systems which were evaluated against the H69 parental human small cell lung carcinoma cell line (H69P) and H69/LX4 resistant cell lines which overexpressed P-glycoprotein. Selected analogues were also evaluated against the COR-L23 parental human non small cell lung carcinoma (L23/P) cell line and the COR-L23/R resistant (L23/R) cell line which overexpressed multidrug resistance protein. The introduction of chirality into the carboxamide side chain of these novel benzophenazine carboxamides has resulted in the discovery of a potent enantiospecific series of cytotoxic agents, exemplified by 4-methoxy-benzo[*a*]phenazine-11-carboxylic acid (2-(dimethylamino)-1-(*R*)-methyl-ethyl)-amide, named XR11576 (**33**, Fig. 11.7) (Vicker et al. 2002). **33** showed IC_{50} values against H69/P and H69/LX4 of 23 and 29 nM, respectively, while its enantiomer (**34**, Fig. 11.7), showed IC_{50} values of 30 and 44 nM, respectively, revealing some chirality dependence. XR11576 (**33**) showed also cytotoxicity on L23/P and L23/R with IC_{50} values of 10.1 and 12.1 nM, respectively, while the IC_{50} values of its enantiomer **34** (Fig. 11.7) was only 96 and 121 nM, respectively.

Furthermore, the *R*-enantiomer **33** demonstrated in vivo activity and was taken into preclinical development showing more than 70 % oral bioavailability in female mice after intravenous administration (Vicher et al. 2002; Laursen and Nielsen 2004; deJonge et al. 2004). Mistry et al. (2002) showed that XR11576 (**33**)

mediated both topoisomerase I and topoisomerase II associated complexes, although it showed different cleavage patterns from the topoisomerase inhibitors camptotechin and etoposide. XR11576 demonstrated potent cytotoxic activity against a variety of human and murine tumour cell lines (IC_{50} 6–47 nM) (Mistry et al. 2002). **33** was also unaffected by the presence of overexpresssed P-glycoprotein or MRP, or atypical drug resistance due to topoisomerase II down regulation. XR11576 exhibited a similar pharmacokinetic profile in mice and rats after either i.v. or p.o. administration. In vivo XR11576 showed marked efficacy against a number of tumours including sensitive (H69/P) and multidrug-resistant (H69/LX4) small cell lung cancer and the relatively refractory MC26 and HT29 colon carcinomas following i.v. and p.o. administration (Mistry et al. 2002). Ex vivo tumours have been shown to be more sensitive to **33** than other topoisomerase inhibitors (DiNicolantonio et al. 2004). Lewis et al. (2007) set out further study using human tumour cell lines, PEO1 ovarian cancer, MDA-MB-231 breast cancer and variants with acquired resistance to VP-16 and XR11576, showing **33** to induce DNA protein cross link following long exposure. In addition, an XR11576-resistant cell line showed resistance to campothecin and etoposide, suggesting a role for topoisomerase in the cytotoxic mechanism of action of these compounds (Lewis et al. 2007; Jobson et al. 2009). Further investigation on the role of topoisomerase inhibition in the mechanism of action of **33**, demonstrated that exposure of **33** results in accumulation of topoisomerase I- and II-DNA complexes after long periods, which coincides with the induction of gamma-H2AX foci as a marker for DNA damage (Jobson et al. 2009). Although **33** reached Phase I clinical trial in 2004, (de Jonge et al. 2004) it was not further pursued because some dimeric phenazine (as XR5944.14 for example) displayed higher activity and therapeutic ratio as detailed below.

Substitution with a 4a,12b-benzofused system on the phenazine core resulted in the development of antitumor agents such as NC-182 and NC-190 (**35** and **36**, Fig. 11.7) (Tarui et al. 1994; Yamagishi et al. 1996). NC-182 promotes the unwinding of Z-form DNA to B-form and displays potent DNA intercalation activity with nearly the same binding ability as daunomycin, while having no base specificity (Tarui et al. 1994). The mode of interaction of NC-182 with DNA depends on the concentration of the drug, where the intercalative and electrostatic bindings are dominant at low and high concentrations of the drug respectively (Tarui et al. 1994). The absence of DNA binding specificity of NC-182 led to the development of NC-190, which is a topoisomerase II inhibitor that inhibits in fact the DNA strand-passing activity of DNA topoisomerase II (Yamagishi et al. 1996). NC-190 induces growth inhibition, protein-linked DNA breaks and DNA fragmentation in cultured HL-60 leukemic cells in a dose-dependent manner with activities comparable to those of etoposide (Yamagishi et al. 1996). It has then been demonstrated that in addition of being a topoisomerase II inhibitor, NC-190 displays tumour growth inhibition through the fact that it inhibits the expression of mRNA of thymidine kinase, while it does not directly inhibits the activity of thymidine kinase (Samata et al. 2002; Cimmino et al. 2012).

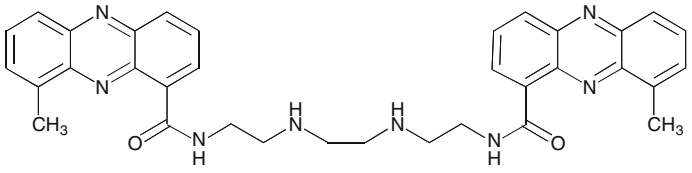
NC-190 has been assayed on a large number of tumour models and displayed significant antitumor activity in vivo in each model tested. ILS value, which represents the average increase in life span of treated animals over control groups of tumor-bearing untreated animals when the drug is given at an optimal dose, were of 200 % in P388 mouse leukaemia (at 50 mg/kg; days 1–5), of 280 % in L1210 mouse leukaemia (100 mg/kg, days 1–5), of 156 % in B16 mouse melanoma (50 mg/kg, days 1–5, of 98 % in mice with M5076 reticulum cell sarcoma (25 mg/kg, days 1, 5, 9 and 13) of >300 % in mice with sarcoma 180 (50 mg/kg, days 3–10, of 12 % in rats with Yoshida sarcoma (12.5 mg/kg, days 3–10 and of 161 % in rats with Yoshida ascite hepatoma AH130 (6.3 mg/kg, days 3–10). Values of ILS greater than 20 % (P388) or greater than 40 % (other tumour models) are considered statistically significant (Nakaike et al. 1989; Cimmino et al. 2012)

When vincristine (VCR)-resistant P388 leukaemia-bearing mice were treated with an optimal dose of NC-190, four of six mice were cured, whereas VCR contributed marginal therapeutic benefits only (Tsuruo et al. 1990). NC-190 also showed chemotherapeutic effect against adriamycin-resistant P388 leukaemia-bearing mice and was active against various multidrug-resistant human and murine cancer models in vitro (Tsuruo et al. 1990).

NC-190 entered Phase II clinical trials in oncology in Japan in the early 1990s but it was then abandoned because it displayed toxicities including bone marrow suppression, transient mild to moderate liver enzyme elevation, alopecia and mild gastrointestinal toxicities, while tumour responses were only occasionally encountered (Furue 1993; Cimmino et al. 2012).

11.3.2 Dimeric Phenazines

Dimeric analogues of phenazine-1-carboxamides were designed as topoisomerase I/II-targeted anticancer agents by Denny and coworkers (Garg et al. 2000; Spicer et al. 2000, 2002; Gamage et al. 2001). Studies focused on evaluation of the role of chromophore substituent's and showed that the same structure–activity relationship earlier derived for the corresponding monomers (Rewcastle et al. 1987) could be applied to the dimeric compounds (Garg et al. 2000; Spicer et al. 2000, 2002; Gamage et al. 2001). As for the monomeric analogues, 9-substitution with a small lipophylic group (CH_3 or Cl), led to a significant improvement of topoisomerase inhibition, up to 60-fold, also when compared to the corresponding dimeric bis (acridine-4-carboxamides) (bis-DACA analogues) (Spicer et al. 2000). The 9-methyl compound poisoned topoisomerase I and II at drug concentrations of 0.25 and 0.5 μM, respectively, and it inhibited the catalytic activity of these enzymes at concentrations of 1 and 5 μM, respectively (Spicer et al. 2000). The 9-methyl compound was also significantly more active as an anticancer agent in the murine colon 38 model when compared to the clinical dual topoisomerase I/II poison DACA (total dose 90 versus 400 mg/kg) (Spicer et al. 2000). Following the



37 XR5944

Fig. 11.8 Chemical structure of dimeric phenazine XR5944 (**37**)

identification of 9-methylphenazine as the optimal chromophore in terms of in vitro cytotoxicity (Spicer et al. 2000), a series of dimeric phenazine-9-carboxamides joined by a variety of dicationic $(CH_2)_nNR(CH_2)_mNR(CH_2)_n$ linkers were prepared by reaction of 9-methylphenazine-1-carboxylic acid imidazolide with the appropriate polyamines, changing the length, rigidity and charge density on the chain (Gamage et al. 2001). The novel dimeric phenazine, with $(CH_2)_2NH(CH_2)_2NH(CH_2)_2$ linker, named XR5944 (also know as MLN944) (**37**, Fig. 11.8), demonstrated potent cytotoxicity, inversely proportional to linker length (Gamage et al. 2001), against human and murine tumour cell lines in vitro and in vivo (Gamage et al. 2001; Stewart et al. 2001). IC_{50} value of 21 nM in murine P388 leukaemia, 3 nM in murin Lewis lung carcinoma and 0.2 nM in human Jurkat leukaemia cells were reported (Gamage et al. 2001). Additional in vitro anticancer activity has been reported by Stewart et al. (2001) showed inhibition of both topoisomerase I and II in vitro. In a range of cancer cell lines, XR5944 displays IC_{50} concentration ranging between 0.04 and 0.4 nM and was significantly more potent than TAS-103, originally proposed as a joint topoisomerase I and II inhibitor, as well as agents specific for topoisomerase I and II as topotecan, doxorubicin and etoposside (Stewart et al. 2001; Cimmino et al. 2012). In addition, **37** was unaffected by atypical drug resistance and retained significant activity in cells overexpressing P-glycoprotein or multidrug resistance-associated protein (Stewart et al. 2001). The first in vivo experiments carried out with XR5944 (**37**), demonstrated antitumor efficacy in murine colon 38 syngeneic and HT29 human colon tumour xenografts with a growth delay of 7 days at 20 mg/kg and 2.4 days at 13.3 mg/kg respectively (Gamage et al. 2001). Additional in vivo anticancer activity against human carcinoma xenograft models (H69 small cell lung cancer and H29 colon) has been reported by Stewart et al. (2001). In the HT29 human colon cancer model, which is relatively unresponsive to chemotherapy, XR5944 at 15 mg/kg induced tumour regression in the majority of animals, whereas TAS-103, doses at its maximum tolerated dose of 45 mg/kg, only induced a delay of tumour growth compared with control animals. In the HT29 model, chronic low doses of 31 (5 or 10 mg/kg) induced complete tumour regression in the majority of animals, while chronic treatments with topotecan at 20 mg/kg and etoposide at 30 mg/kg only slowed the tumour growth rate (Stewart et al. 2001; Cimmino et al. 2012).

Furthermore, an ATP-tumour chemosensitivity assay has been used to assess the ex vivo sensitivity of 90 solid tumours and the median IC_{90} and IC_{50} values for XR5944 in the 90 tumour-derived cell lines were 68 and 26 nM, respectively (Di Nicolantonio et al. 2004), demonstrating that ex vivo tumours were more sensitive (40- to 300-fold) to **37** than other topoisomerase inhibitors. In particular, breast and gynecologic malignancies were most sensitive, while gastrointestinal tumours showed the greater resistance (Di Nicolantonio et al. 2004).

The anticancer activity of XR5944 was also investigated in combination with 5-fluorouracil (5-FU) or irinotecan in human colon carcinoma cell lines (in vitro) and xenografts (in vivo) (Harris et al. 2005) and antagonism was observed in vitro following exposure of HT29 colon cancer cells simultaneously to XR5944 and 5-FU or SN38 (the active metabolite of irinotecan), while sequential exposure of either combination in either order demonstrated at least an additive response (Harris et al. 2005; Cimmino et al. 2012). In vivo anticancer activity in HT29 xenografts in immunocompromized mice was enhanced by sequential administration of 5-FU (65 mg/kg) or irinotecan (35 mg/kg) 48 h before XR5944 (5, 10 or 15 mg/kg) compared to single agent treatment at the same or higher doses (Harris et al. 2005; Cimmino et al. 2012).

XR5944 (MLN944, **37**) was thus reported originally as a dual topoisomerase I/II poison (Gamage et al. 2001), but the sustained potency of this compound in mammalian cells with reduced levels of both topoisomerases suggested other mechanisms of anticancer effects for this drug (Sappal et al. 2004). It seems in fact that the primary mechanism of action of XR5944 likely involves DNA binding and intercalation, but does not appear to involve topoisomerase inhibition (Sappal et al. 2004). It has then been demonstrated that XR5944 displays its potent cytotoxic effects through inhibition of transcription of all RNA polymerases (Byers et al. 2005).

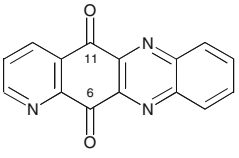
XR5944 (MLN944) was co-developed by Millenium Pharmaceuticals and Xenova Ltd. and has entered phase I clinical trials in 2003 as a therapeutic agent for solid tumours (Byers et al. 2005; Verborg et al. 2007). However, the lack of correlation between toxicity and PK values made difficult to recommend of dose for further study in Phase II trials (Verbog et al. 2007) and this compound does not seem to have entered Phase II clinical trials in oncology, at least to the best of our knowledge. However, this compound could still have a future in clinical development because XR5944 is capable of specifically inhibiting the binding of oestrogen receptor (ER) to its consensus DNA sequence and its subsequent activity (Punchihewa et al. 2007). These novel findings may allow the development of less toxic XR5944 analogues capable of overcoming resistance to current antiestrogens.

More recently, Jobson et al. (2009), further investigated the role of topoisomerase inhibition in the mechanism of action of **37**. As for X11576 (**33**, Fig. 11.7), the study demonstrated that exposure of **37** results in accumulation of topoisomerase I- and II-DNA complexes after long periods of exposure, which coincides with the induction of γ-H2AX foci as a marker for DNA damage. Exposure of X11576 and XR5944 in DNA-repair deficient cell lines indicated that these two

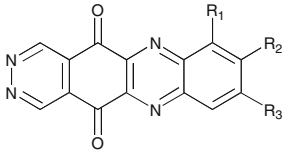
drugs possess slightly different mechanism of action which may be related to their other proposed cellular effects (Jobson et al. 2009).

11.3.3 Phenazine-6,11-Dione and Analogs

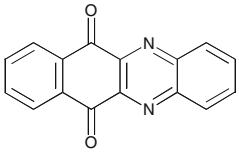
The structure of phenazinediones has a planar tetracyclic ring and *p*-conjugated ketone groups containing a nitrogen atom which enables hydrogen bonding with DNA, according to Moore and Pindur's thesis (Moore et al. 1989; Pindur et al. 1993). Lee and coworkers, focused on the synthesis and cytotoxic evaluation of nitrogen containing heterocyclic quinones such as 6,11-dihydrobenzo[2,3-*b*]phenazine-6,11-dione and their analogs (benzophenazinediones) (Kim et al. 2003), 6,11-dihydro-pyrido[2,3-*b*]phenazine-6,11-dione and their analogs (pyridophenazinediones) (Kim et al. 2003; Lee et al. 2004) and 6,11-dihydro-pyridazo[2,3-*b*]phenazine-6,11-dione (also known as 6,11-dihydro-quinoxalino[2,3-*b*]phtalazine-6,11-dione) and their analogs (pyridazophenazine diones) (Lee et al. 2004, 2007). 6,11-dihydro-pyrido[2,3-*b*]phenazine-6,11-dione (**38**, Fig. 11.9) exhibited a much greater cytotoxic activity upon the growth of all the human tumour cell lines tested than those of doxorubicin with ED_{50} values of 0.007–0.041 μg/mL against human lung (A549), human ovarian (SK-OV-3), human melanoma (SK-MEL-2), human CNC (XF 498) and human colon (HCT 15) cell lines (Lee et al. 2004). Several derivatives were prepared but no one exhibited better cytotoxicity respect to the parent compounds although the ED_{50} values of 0.007–0.061 μg/mL against SK-OV-3 tumour cell line were approximately 3–22-fold lower that that of doxorubicin



38
6,11-dihydro-pyrido[2,3-*b*]phenazine-6,11-dione



39 $R_1=R_2=R_3=H$, 6,11-dihydro-pyridazo[2,3-*b*]phenazine-6,11-dione
40 $R_1=CH_2CH_3$, $R_2=R_3=H$
41 $R_1=R_2=CH_3$, $R_3=H$
42 $R_1=R_3=H$, $R_2=N(CH_3)_2$
43 $R_1=OCH_2CH_3$, $R_2=R_3=H$
44 $R_1=R_3=OCH_3$, $R_2=H$



45 6,11-dihydro-benzo[*b*]phenazine-6,11-dione

Fig. 11.9 Chemical structure of phenazine-6,11-dione and analogs (**38**–**45**)

(ED_{50} = 0.160 μg/mL) (Lee et al. 2004). In general pyridophenazinediones possessing three nitrogen atom shown much better cytotoxicity than benzophenazinediones possessing two nitrogen atoms (Kim et al. 2003) according to the results of Johnson (Shaikh et al. 1986), which reported that the number of nitrogen atoms are important for cytotoxicity. 6, 11-dihydro-pyridazo[2,3-*b*]phenazine-6, 11-dione (**39**, Fig. 11.9) tetracyclic heteroquinone with four nitrogen atoms exhibited potent cytotoxic activity against all the tumour cell lines tested, and in particular its cytotoxic effect against HCT 15 (ED_{50} 0.004 μg/mL) was 25 times greater than that of doxorubicin (ED_{50} 0.093 μg/mL) (Lee et al. 2004).

However, the concept that there is an increasing cytotoxic effect with more nitrogen atoms (Shaikh et al. 1986) was invalidated by the experimental data they obtained. In fact it was hard to determine which one was more potent between **38** and **39**. In general, **38** with three nitrogen atoms, exhibited potent activities against SK-OV-3 and XF 498 tumour cell lines, while **39**, with four nitrogen atoms, showed especially cytotoxicities against SK-MEL-2 and HCT 15 tumour cell lines (Lee et al. 2004).

The same authors patented the preparation of **39** and some derivatives (Kim et al. 2005). Successively, several derivatives of **39** were prepared (Lee et al. 2007), some of them (**40**–**43**, Fig. 11.9) showed high cytotoxicity against all cancer cell lines tested. In particular, the cytotoxicity of **40** and **41** with IC_{50} values of 0.010-0.034 μM and 0.010-0.019 μM respectively, against A549, SK-OV-3, SK-MEL-2, XF 498 and HCT 15, was 10 times higher than that of doxorubicin (Lee et al. 2007). Compounds **42** (IC_{50} 0.024-0.088 μM), **43** (IC_{50} 0.015-0.053 μM) and **44** (IC_{50} 0.026-0.070 μM) were 2–9 times more cytotoxic than doxorubicin. However, none of them displayed significantly higher in vitro anticancer activity when compared to **38** (Lee et al. 2007).

Khalifa and coworkers (2008), reported the selective cytotoxicity of 6,11-dihydrobenzo[b]phenazine-6,11-dione (**45**, Fig. 11.9) against human lung carcinoma cell line (H460), with IC_{50} values of 16.25 μM, 1.3 times higher than that of doxorubicin, in contrast with the cytotoxic activity reported by Lee and coworkers for some benzophenazinedione derivatives (Kim et al. 2003), in which the IC_{50} values against A 549, SK-OV-3, SK-MEL-2, XF 498 and HCT 15 were much higher than that of doxorubicin (Kim et al. 2003).

11.3.4 Phenazine-5,10-Dioxide and Analogs

Phenazine-5,10-dioxides are reported as prodrugs for antitumor therapy that undergo hypoxic-selective bioreduction to form cytotoxic species (Cerecetto et al. 2005a; Lavaggi et al. 2008; Pachon et al. 2008). The majority of the cells in solid tumours are not dividing rapidly and hypoxia appears to be their common and distinct property that promotes an important mechanism for the specification activation of antitumoral action, namely bioreduction. Neo-angiogenesis fails to be successful in rapidly growing cancer clones within a given solid tumour, leading

therefore to hypoxic areas until the complete neo-angiogenesis process is accomplished, otherwise all the cancer cells die and the hypoxic area becomes necrotic (Hanahan and Weinberg 2011). In other words, the imperfect neovascularization seen in fast growing tumour areas results in limited and inefficient blood vessel networks in these tumour areas that becomes hypoxic, thus resistant to conventional radiotherapy and chemotherapy (Cerecetto et al. 2005b; Cerecetto et al. 2006; Lavaggi et al. 2010; Hanahan and Weinberg 2011). Hypoxic cancer cells could thus be selectively targeted over normoxic normal cells, or even normoxic cancer cells that are less biologically aggressive than hypoxic cancer cells (Hanahan and Weinberg 2011; Porporato et al. 2011), through bioreduction (Pachon et al. 2008), which is irreversible under hypoxic conditions (Cerecetto et al. 2005a; Pachon et al. 2008). It was hypothesised that hybrid compounds possessing an *N*-oxide and a π DNA-stacking moiety would be a new generation of bioreductive compounds (Cerecetto et al. 2005a). These could damage hypoxic cells by generating HO, and after bioreduction, damage the hypoxic cells by direct DNA interaction or DNA related biomolecules. (Cerecetto et al. 2005a; Lavaggi et al. 2010). Several Phenazine-5,10 dioxide derivatives, structurally related to other bioreductive agents were synthesised and selected for their DNA-π-stacking structure that potentially interact with DNA after the corresponding bioreduction in hypoxia conditions (Cerecetto et al. 2005; Lavaggi et al. 2008; 2010). 7(8)-bromo-2-hydroxyphenazine-5,10-dioxide (**46**, Fig. 11.10) showed selective toxicity toward hypoxic cells and behave as hypoxic trigger cytotoxin. **46** displayed a good hypoxia selective cytotoxicity at 20 μM on V-79 cells (Cerecetto et al. 2005a). 7(8)-methyl-2-aminophenazine-5,10 dioxide (**47**, Fig. 11.10) and 7(8)-methoxy-2-aminophenazine-5,10 dioxide (**48**, Fig. 11.10), displayed excellent selective cytotoxic properties, although presented less cytotoxic effect at 20 μM (Cerecetto et al. 2005a).

In all cases the products **46**–**48** were characterized and evaluated as a non-separable mixture of 7- and 8- isomers which is the result of the well known tautomerism of benzofuroxan reactant at room temperature (Boulton et al. 1967; 1970; Cerecetto et al. 2005b), used in this work for the preparation of phenazine dioxide derivatives (Cerecetto et al. 2005a).

In fact, **46**–**48** were obtained by a heterocycle expansion process by reaction of the corresponding benzofuroxan with different phenol derivatives, producing the

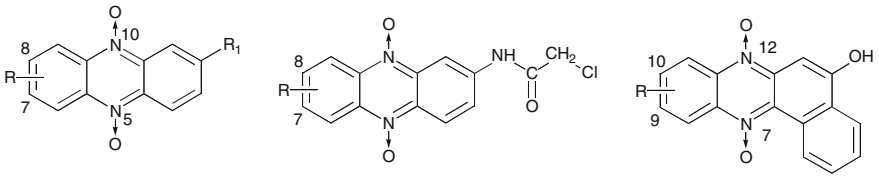


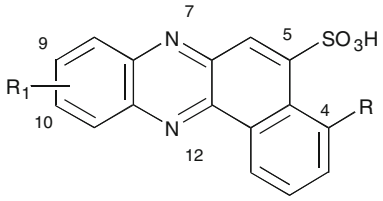
Fig. 11.10 Chemical structure of phenazine-5,10-dioxide and analogs (**46**–**52**)

corresponding 7- and 8-substituted-2-aminophenazine-5,10-dioxides (**47** and **48**), and 7- and 8-bromo-2-hydroxyphenazine-5, 10-dioxide (**46**) (Cerecetto et al. 2005b). However, previous results for quinoxaline dioxide derivatives demonstrated that both positional isomers have the same selective hypoxic cytotoxicity against V79 cells (Monge et al. 1995). The selective anaerobic-reduction of **46**–**48**, and its relation to bioreductive activity were proved using enzymatic mammals system, in particular rat liver microsomal and cytosol fractions (Lavaggi et al. 2008). The study showed that both compounds are bioreduced selectively under anaerobic conditions to phenazine monoxide and phenazine that concomitantly could produce cellular damage under low-oxygen concentrations. In particular, the 2-amino derivatives **47** and **48**, were found to yield the monoxidized and completely reduced products while the 2-hydroxy derivative **46** were found to produce only the complete reduced product (Lavaggi et al. 2008). Some enzymes responsible for the bioreduction were also identified (Lavaggi et al. 2008).

Besides these study the same authors reported the synthesis and antitumoral effects of some phenazine-5,10 dioxide displayed in vitro aerobic-antitumor activity against Caco-2-cells (Pachon et al. 2008).

2-chloroacetylamino-7(8)-nitrophenazine-N^5,N^{10}-dioxide (**49**, Fig. 11.10), containing an electrophile chloroacetamide and a very reactive group (nitro moiety), showed the higher antiproliferative potency in term of MTT assay with IC_{50} values of 1.7 μg/mL. When the nitro group was substituted by a less reactive dioxolyl group as in 2-chloroacetylamino-7(8)-(1,3-dioxol-2-yl)phenazine-N^5, N^{10}-dioxide (**50**, Fig. 11.10), IC_{50} values was only 2-fold higher. Interestingly, **49** showed an arrest in the G2/M phase at 24 and 48 h, which led cells to apoptosis at 24 h and to necrosis at 48 h. The fact that **49** is the most effective in arresting the cell cycle and in inducing apoptosis and necrosis could be due to the presence of two electrophilic group compared to **50** with an electrophile (chloroacetamide) and a nucleophile group (1,3-dioxol-2-yl) (Pachon et al. 2008). Also in this case, the products, were characterized and evaluated as a nonseparable mixture of 7- and 8-isomers for the known effect of benzofuroxanan (Boulton et al. 1967, 1970; Cerecetto et al. 2005b).

Successively, a new scaffold, the benzo[*a*]phenazine-7,12-dioxide system was investigated for the reason that none of the phenazine-5,10-dioxide derivatives reported previously, was able to interact with DNA. The expanded chromophoric system of the new scaffold, could potentially improve DNA-π-stacking properties, maintaining the bioreductive capacities through *N*-oxide pharmacophore (Lavaggi et al. 2010). The results demonstrated the potential biological properties of some of the derivatives, structural hits for further chemical modification to become into therapeutic for solid tumours (Lavaggi et al. 2010). In particular compounds **51** and **52** with cytotoxicity against V79 cells in both conditions (hypoxia and normoxia) were also cytotoxic against Caco-2 tumoral cells in aerobiosis (Lavaggi et al. 2010). Again, the compounds were characterized and evaluated as a nonseparable mixture of 9- and 10-isomers.



53 R=H, R_1=H, benzo[*a*]phenazine-5-sulfonic acid
54 R=H, R_1=10-CON$(CH_3)_2$
55 R=H, R_1=9-NH_2
56 R=4-NH_2, R_1=H
57 R=H, R_1=10-COOH
58 R=4-NO_2, R_1=10-COOH

Fig. 11.11 Chemical structure of benzophenazine-5-sulfonic acid and analogs (**53**–**58**)

11.3.5 Benzophenazine-5-Sulfonic Acid and Analogs

Benzo[*a*]phenazine-5-sulfonic acid (**53**, Fig. 11.11) was selected as a basic DNA intercalating polycyclic pharmacophore for the presence of flat aromatic ring system capable of insertion in between DNA base pairs. Furthermore, presence of multiple nitrogen atoms imparts greater degree of planarity to the nucleus reducing carcinogenicity and toxicity problems associated with non nitrogen polycyclic system (Moorthy et al. 2009). Benzo[*a*]phenazine-5-sulfonic acid had an IC_{50} values against HL-60 tumour cell lines of 91 μM. Several its derivatives were synthesised and some of them (**54**–**58**, Fig. 11.11) showed improved cytotoxic potency. Compound **54**, a 10-dimethylcarboxamido derivative of benzo[*a*]phenazine-5-sulfonic acid, was found to be the most active in the series with IC_{50} values of 19 μM. The presence of an 9-amino group as in **55** resulted in an IC_{50} values of 31 μM, while 4-amino group as in **56** gave an IC_{50} of 85 μM. Compound **57** with free carboxylic group at C-10 in benzo[*a*]phenazine-5-sulfonic acid ring, had an IC_{50} of 69 μM with increased in potency with a 4-nitro group at C-4 as in **58** (IC_{50} 59 μM). The results of thermal denaturation study suggest that **54** exhibit DNA binding affinity. It is also worthmentioning that **54** showed significant DNA intercalating ability which is evident from large transition in thermal melting points of the DNA (Moorthy et al. 2009).

11.4 Future Perspectives: Phenazine as an Anticancer Agent

With more than 6,000 phenazine-containing structures reported in the literature, both natural and synthetic, the potential for accessing high chemical diversity with this structural scaffold is immense. However, the utilisation of planar phenazine-based molecules for the development of novel anticancer agents is indeed a highly

challenging endeavour due to the inherent potential of these compounds for non-selective DNA binding leading to general toxicity. Recent research has demonstrated that while this may indeed be the case with small intercalative phenazines, the derivatization of the phenazine core with diverse pendant functionality may lead to selective agents targeting not only DNA and DNA-enzyme complexes but also many unrelated cellular macromolecules important for cancer progression.

Modulation of molecular targets that control cancer development and progression is one approach for chemoprevention (Kondryatuk et al. 2012). Chemoprevention aims to prevent, delay and ultimately reverse cancer development. For example, transcriptional regulation of NF-κB has been intensely studied. The transcription factor nuclear factor kappa-light chain of activated B cells (NF-κB) and, the inducible nitric oxide synthase (iNOS), are potential chemopreventive target along with Phase I (aromatase, quinone reductase 2 (QR2) and Phase II (QR1, glutathione-S-transferase (GST) enzymes that detoxify carcinogens) (Conda-Sheridan et al. 2010).

Major cellular targets for NF-κB are chemokines, immune receptors, adhesion molecules, stress response genes, regulators of apoptosis, transcription factors, growth factors, enzymes, and cell cycle regulators (Aggarval et al. 2006; Kondryatuk et al. 2012). These include the anti-apoptosis genes bcl-2 and bcl-xl, cylcooxygenase (COX)-2, matrix metalloproteinase-9 (MMP-9), genes encoding adhesion molecules, chemokines, inflammatory cytokines, and cell cycle-regulatory genes (Luqman et al. 2010; Kondryatuk et al. 2012). In addition, the NFκB pathway is central in iNOS induction. Activity of iNOS is tightly regulated and includes activation of IκB and mitogen-activated protein kinases (MAPKs) kinases (Gonda et al. 2009; Kondryatuk et al. 2012). In principle, agents that can suppress NF-κB activation and iNOS have the potential of suppressing carcinogenesis (Inoue et al. 2007; Konryatuk et al. 2012). Recent study have showed that 2-bromo-1-hydroxyphenazine (Conda-Sheridan et al. 2010) and 2-bromo-N-substituted monoterpene phenazine and 2-bromo-N-substituted isoprenylated phenazine (Kondryatuk et al. 2012), as well as several phenazine derivatives, displayed IC_{50} value versus QR1 induction and QR2 inhibition in the nanomolar range, suggesting some of them as cancer chemopreventive agents (Conda-Sheridan et al. 2010; Cimmino et al. 2012).

Furthermore, recent patents have considered extensive studies on the antitumour effect of phenazine derivatives and as result have found that some compounds are effective chemotherapeutic agents, in particular against cancerous pancreatic cells (MiaPaca cells). Accordingly, it can constitute new target for the development of novel therapeutic strategies against cancers or diseases linked to uncontrolled and/or abnormal growth of cells, and notably may serve as lead compound for a new generation of anticancer agents. (Camplo et al. 2011 patent wo2011/117830). However, highly potent compounds can be found by exploring dimeric phenazines or those containing pendant proton table functionality, such as tertiary amines. Finally, the prodrug approach, such as the use of hypoxic-selective *N*-oxides is expected to be another important research direction aimed to diminish the general toxicity associated with phenazines (Cimmino et al. 2012).

11.5 Conclusions

This chapter reports natural and synthetic bioactive phenazine belonging to different subgroups with a detailed description of their anticancer activity. The relationship between the structure and the activity is discussed as well as the results obtained by some in vitro, in vivo and available clinical experiments.

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