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

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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 (Fitz-patrick 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
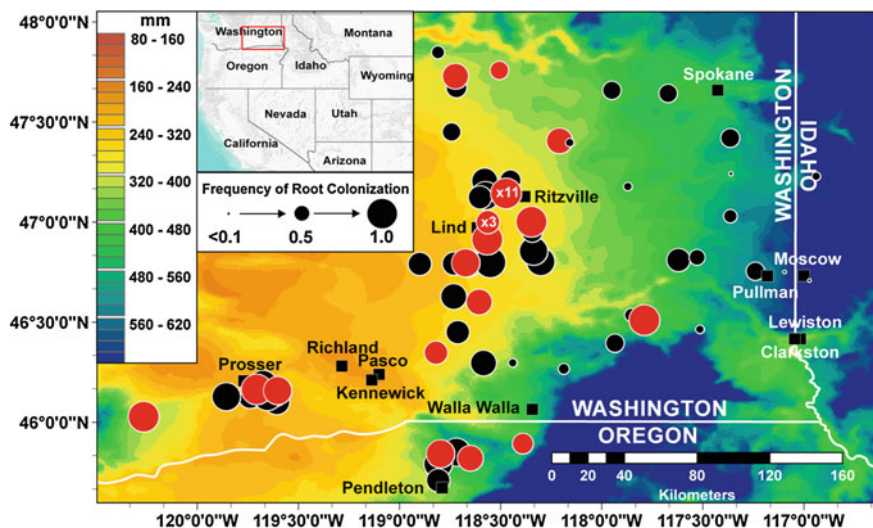
Fig. 10.1 Distribution of *phz*⁺ pseudomonads in wheat fields from Eastern Washington, Northeastern Oregon, and Western Idaho. Map of the surveyed area is overlaid with mean annual precipitation values for the years 1971 through 2000 (see inset for scale). The locations of sampling sites are indicated 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
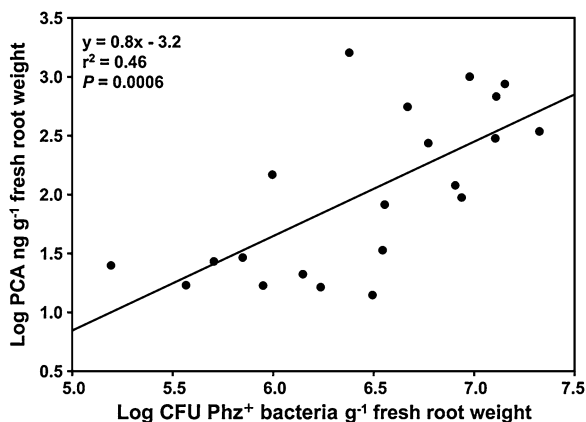
Fig. 10.2 Relationship between the accumulation of PCA in field-grown cereals and populations of indigenous *phz*[+] rhizobacteria. Amounts of PCA were determined by extracting the antibiotic from 15-g samples of wheat roots and performing a quantitation of PCA by HPLC-Q-TOF–MS/MS. In each location from 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
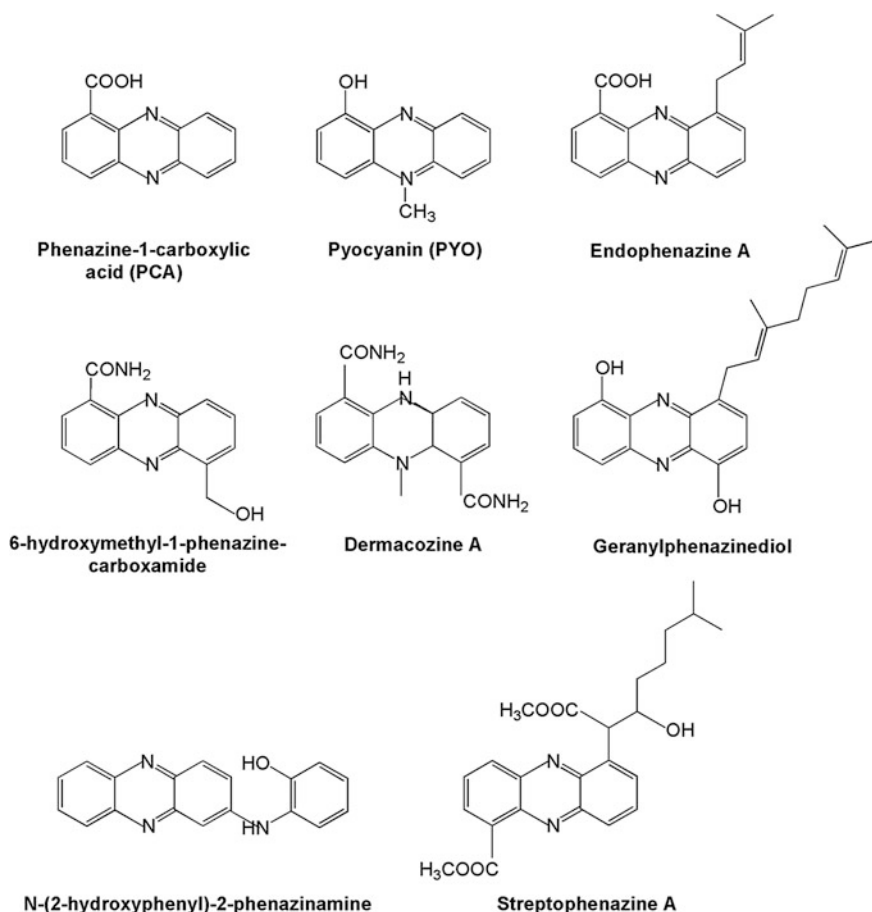
Fig. 10.3 Representative phenazine structural derivatives described in the text

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 temporally 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
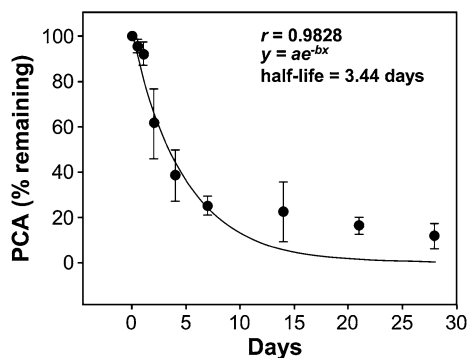

Fig. 10.4 Half-life of PCA in the rhizosphere. PCA (10 μg) in acetonitrile was spiked onto 10 g of wheat roots with rhizosphere soil collected from seedlings that had been grown for 3 weeks in a Shano sandy loam soil. The roots were incubated at room temperature in the dark and sampled in triplicate 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 $(5 \times 10^{11}$ 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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