REGULATION AND ECOLOGICAL ROLES OF PHENAZINE BIOSYNTHESIS IN THE BIOLOGICAL CONTROL STRAIN *PSEUDOMONAS CHLORORAPHIS* 30-84

A Dissertation

by

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ABSTRACT

Pseudomonas chlororaphis 30-84 is a plant growth-promoting rhizobacterium that was selected for its ability to control plant disease. Disease suppression by 30-84 is the result of production of phenazines, which both inhibit plant pathogens and facilitate rhizosphere competence. Many phenazine producing pseudomonads, such as 30-84, produce more than one phenazine derivative mediated by unique phenazine modification enzymes. To determine the role of different phenazine derivatives on key biocontrol properties, isogenic derivatives of 30-84 that produced different phenazines were constructed. The production of different phenazine derivatives significantly affected the specificity of fungal pathogen inhibition as well as biofilm eDNA release, which correlated with differences in biofilm formation.

Sequence analysis of the phenazine operon of 30-84 identified several DNA sequence features downstream of the phenazine operon promoter. These features potentially are capable of forming stem-loop structures, which lead to promoter attenuation. To analysis of unusual regulatory circuit in *phz* promoter, we generated mutants with a disruption of the stem-loop structures. Of significance, this disruption resulted in significantly higher phenazine production as well as increased expression of the *phzR/phzI* quorum-sensing system. These data suggest that this stem-loop region negatively regulates phenazine production, partially through *phzR/phzI.* In addition, this mutant had greater fungal inhibition capacity, biofilm formation and plant growth promotion, which are fundamental factors of biocontrol agents. Therefore, this work

provides potential applications for improved biological control by an enhanced phenazine strain.

Regulatory genes and systems including Gac/Rsm, RpeA/RpeB, RpoS, Pip and PhzR/PhzI tightly control phenazine production in 30-84. In this study, we identified a Tn5 generated mutant that disrupted the gene *miaA,* which encodes a tRNA modification enzyme. This mutant showed significantly decreased production of phenazines, exoprotease and quorum sensing signals. Moreover, transcript abundances of *phzR/phzI* as well as *pip*, and translational efficiency of RpeB and RpoS were significantly reduced in the *miaA* mutant. In addition, the *miaA* mutant showed reduced bacterial growth, survival rate in the soil, and antifungal activity. These results suggest MiaA influences the production of secondary metabolites and other traits involved niche adaptation in *P. chlororaphis* 30-84 via its tight control of the translation of key regulatory genes.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Plant disease is one of the most significant and chronic limitations to world-wide food production. Given projected population increases and uncertainty over climate, a critical need is to be able to feed the world in safe, environmentally-sustainable, dependable, cost-effective ways. Chemical pesticides remain the most widely-used methods to combat crop diseases, despite increasing concerns over their acute and cumulative effects on public health and the environment. Long-term strategies for sustainable pest management require that we develop alternatives to supplement and reduce our use of chemical pesticides. Biological control approaches that utilize beneficial plant-associated microorganisms for disease control are extremely promising. However, in order to improve availability, consistency, and performance of biological control we need a better understanding of the bacterial mechanisms that contribute to plant health and disease suppression.

To date, many types of biological control methods have been extensively studied and some are commercially available. However, many microorganisms that show a great potential in terms of efficacny as biological control agents unders laboratory or greenhouse-based conditions, and fail to perform consistently in field tests (Thomashow, 1996, Compant et al., 2005). For that reason, further understanding the mechanisms of

interaction with hosts and the development of effective application protocols are still area to be explored in order to realize the promise biological control has to offer.

My dissertation research is focused on *Pseudomonas chlororaphis* 30-84, a well-studied plant growth promoting rhizobacteria capable of suppressing plant diseases. Disease suppression by 30-84 results primarily from the production of secondary metabolites known as phenazines, which are required for pathogen inhibition and disease suppression. Phenazines also play critical roles in host-microbe interactions including rhizosphere competence and induction of plant disease resistance. Because of their interesting redox chemistry, antibiotic properties and ecological benefits to the producers, phenazines have been studied longer than most other secondary metabolites. Nevertheless, there are several fundamental questions that need to be resolved in order to gain a better understanding and improve their usage in commercial applications. I believe the results of my dissertation will guide the next step forward in the development of innovative and effective biological control methods using phenazine-producing strains for the management of soil-borne crop diseases.

Specific Objectives

My dissertation study aims to elucidate the ecological roles of different phenazine derivatives in terms of fitness benefits to the producers as well as gain a better understanding of the genetic regulation of the phenazine biosynthetic genes as a means of gaining insight into how producing cells control the expression of this important

secondary metabolite. *Pseudomonas chlororaphis* strain 30-84 serves as the biological system for my studies. There are three specific objectives:

Objective 1: **To understand the contribution of different phenazines structural derivatives to biological control efficacy.** I constructed isogenic derivatives of *P. chlororaphis* 30-84 that produced different phenazine structural derivatives. I found that these derivatives differed in their capacity to inhibit fungal plant pathogens, form biofilms, and release extracellular DNA, which are key characteristics to understanding biological control efficacy (Chapter II).

Objective 2: **To understand the role of an interesting feature in the promoter of the phenazine biosynthetic genes, which may determine the timing and quantity of phenazines produced.** I found unique sequence features in the promoter region of phenazine biosynthetic operon in *P. cholororaphis* 30-84. This region is potentially capable of forming stemloop structures and causing promoter attenuation. To delineate the function of this negative regulatory region, I generated a mutant strain with a disruption in a stemloop structure, and investigated the function and mechanism of this region. Additionally, using this mutant strain, I also studied the ecological benefits of enhanced phenazine production in terms of biological control capability (Chapter III).

Objective 3: To understand the functional role of tRNA modification enzyme in phenazine production and other ecological traits for biological control aspects. I identified a Tn5-plasposon generated mutant that disrupted a gene, *miaA,* which encodes a tRNA modification enzyme. The roles of MiaA were investigated, including how

MiaA fits into the current understanding of the regulatory network of genes that control phenazine production, as well as how MiaA influences ecological characteristics important for its capabilities as a biological control agent (Chapter IV).

Literature Review

Biological control is the method of applying live organisms to suppress and control plant disease. It is an approach that offers an alternative to the use of synthetic chemicals and thus is safer for human and environmental health and more sustainable. Fluorescent pseudomonads have been studied extensively for biological control applications because of their wide host range and effective antagonism of many soil-borne pathogens (Pierson & Pierson, 2010). The production of secondary metabolites is often crucial for effective disease suppression (Haas & Défago, 2005). The secondary metabolites known to be produced by biological control pseudomonads include 2,4-diacetylphloroglucinol, phenazines, pyoluteorin, pyrrolnitrin, and hydrogen cyanide, as well as exoenzymes including chitinase and exoproteases. Many excellent studies have demonstrated that one or more of these traits are essential for the control of plant pathogens in the rhizosphere (reviewed in Leisinger & Margraff, 1979, Budzikiewicz, 1993, Cook *et al.*, 1995, Raaijmakers *et al.*, 2002, Haas & Défago, 2005, Mercado-Blanco & Bakker, 2007, Lugtenberg & Kamilova, 2009). In addition to direct antagonism of the pathogen, some of these and other secondary metabolites have been shown to contribute to disease suppression via ecological competition with major and

minor pathogens, plant growth promotion, and induction of host resistance (Thomashow & Weller, 1996, Haas & Défago, 2005, Pal & Gardener, 2006, Weller, 2007).

In this study, I focused on a single fluorescent *Pseudomonas* biological control strain, *Pseudomonas chlororaphis* 30-84. *P. chlororaphis* 30-84 was originally selected as a biological control strain with the potential for the control of take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (*Ggt*) (Pierson & Thomashow, 1992). Significantly, it was isolated from a wheat-field rhizosphere in Kansas where natural take-all disease decline had been observed. Similar to other biological control pseudomonads, it possesses an arsenal of secondary metabolites that may contribute to its ability to inhibit fungal pathogens and suppress disease. These include the production of phenazines, pyrrolnitrin, hydrogen cyanide, and extracellular enzymes e.g. exoproteases, chitinases, gelatinases, and lipases (Chancey *et al.*, 1999, Loper *et al.*, 2012). Subsequent studies demonstrated that phenazines account for the majority of the strain's ability to inhibit to the target pathogen (Thomashow *et al.*, 1990). As described further in this chapter, phenazines also have been shown to contribute to other aspects of bacterial fitness and plant-microbe interactions.

Phenazines

Phenazines comprise a large group of heterocyclic nitrogen-containing secondary metabolites produced by variety of bacteria, including *Pseudomonas, Streptomyces, Burkholderia*, *Vibrio, Brevibacterium* and *Erwinia* (Turner & Messenger, 1986, Mavrodi *et al.*, 2006, Mentel *et al.*, 2009, Mavrodi *et al.*, 2010, Pierson & Pierson, 2010). Phenazine compounds produced by *Pseudomonas* species are biologically active

metabolites that function in microbial competitiveness (Mazzola *et al.*, 1992, Beifuss & Tietze, 2005, Pierson & Pierson, 2010), biofilm development (Maddula *et al.*, 2006, Maddula *et al.*, 2008, Ramos *et al.*, 2010, Wang *et al.*, 2016), electron shuttling (Price-Whelan *et al.*, 2007, Wang & Newman, 2008, Wang *et al.*, 2010), signal factors for gene expression (Dietrich *et al.*, 2006, Wang *et al.*, 2016) and the inhibition of soilborne plant pathogens (Thomashow *et al.*, 1993, Thomashow & Weller, 1996, Anjaiah *et al.*, 1998, Chin-A-Woeng *et al.*, 1998).

Most phenazine producing *Pseudomonas* strains produce more than one phenazine derivative, however there are strains such as *P. fluorescens* 2-79 that produce only one phenazine, phenazine-1-carboxylic acid (PCA) (Mavrodi *et al.*, 2006, Pierson & Pierson, 2010). Production of phenazines requires a conserved seven-gene *phz* operon that encodes enzymes for synthesizing PCA, as the core phenazine. In *P. chlororaphis* 30-84 the genes in the biosynthetic operon are designated *phzXYFABCD* (according to the original nomenclature), which corresponds to *phzABCDEFG* (according to the *P. fluorescens* nomenclature) (Pierson *et al.*, 1995, Mavrodi *et al.*, 2006). The core phenazine PCA can be modified into other phenazine derivatives by specific phenazine modifying enzymes often referred as terminal modifying enzymes (Fig. 1.1). For example, *P. chororaphis* 30-84 produces a mixture of phenazine derivatives including PCA and a small amount (~10%) of 2-hydroxy-phenazine-carboxylic acid (2OHPCA) and 2-hydroxy-phenazine (2OHPZ) (Pierson & Thomashow, 1992, Pierson *et al.*, 1994). The terminal modifying gene *phzO*, which is located immediately downstream of the *phz* operon, encodes a bacterial aromatic monooxygenase (Delaney *et al.*, 2001). It is

responsible for the conversion of the small amount of PCA to 2OHPCA, and 2OHPZ is generated from 2OHPCA by spontaneous loss of the carboxylic acid (Pierson *et al.*, 1995, Delaney *et al.*, 2001) (Fig. 1-1). In addition to strain 30-84, another biocontrol strain of *P. chlororaphis* PCL1931 contains *phzH* instead of *phzO.* This terminal modifying gene encodes a putative transamidase which is responsible for the conversion of portion of PCA to phenazine-1-carboxamide (PCN) (Chin-A-Woeng *et al.*, 1998) (Fig. 1.1).

Bioinformatic and biochemical analysis of phenazine producing pseudomonads demonstrates that they contain a diversity of terminal modifying enzymes and many strains use multiple enzymes or combinations of these enzymes to produce multiple phenazine structural derivatives (Mavrodi *et al.*, 2006, Mavrodi *et al.*, 2010, Pierson & Pierson, 2010). For example, *P. aeruginosa* uses several different genes singly or in combination, including *phzH*, *phzM*, *phzS*, and *phzM/phzS* to produce phenazine-1-carboxamide (PCN), 5-methoxy-phenazine-1-carboxylic acid (5MPCA), 1-hydroxy-phenazine (1OHPZ), and pyocyanin (PYO),respectively (Mavrodi *et al.*, 2001, Chin-A-Woeng *et al.*, 2003, Mavrodi *et al.*, 2006, Pierson & Pierson, 2010) (Fig. 1.1). One focus of my work was to produce isogenic derivatives of 30-84 that produced different known phenazines examine the ecological consequences of altering phenazine production.

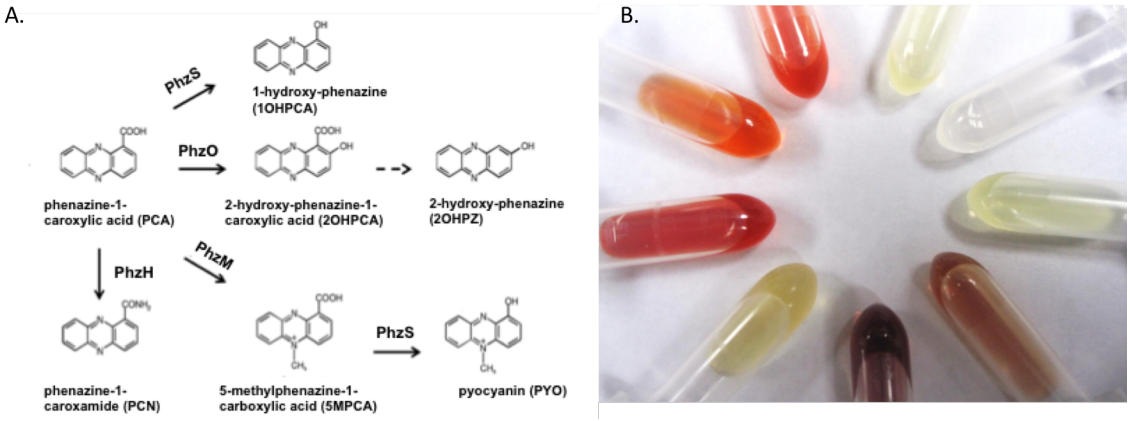


Figure 1. 1 Biosynthesis of phenazine derivatives and phenotypes.
A. Biosynthesis of PCA from chorismic acid via the *phz* operon and conversion to its phenazine derivatives by different terminal modifying enzymes. This figure modified from Chin-A-Woeng et al. (2003). B. Extracted phenazines from isogenic derivatives of strain 30-84 that produce different types of phenazines. Starting at 1 o'clock and going clockwise these derivatives are: 30-84PCA, 30-84ZN, 30-84H, 30-84S, 30-84M, 30-84MS, 30-84O*, 30-84RpeB, 30-84 wild type (See Chapter II).

Phenazines as antibiotic compounds for pathogen inhibition

Phenazines have broad spectrum antibiotic activity against a diversity of soil-borne plant pathogens (Fig. 1.2A and 1.2B). The mode of action is still not clear; however, several studies suggest that the antibiotic can diffuse into the cell membrane of pathogens. This results in the uncoupling of oxidative phosphorylation and the generation and accumulation of reactive oxygen species including superoxide and hydrogen peroxide, which are toxic to organisms (Hassett *et al.*, 1995, Britigan *et al.*, 1997, Mavrodi *et al.*, 2006, Pierson & Pierson, 2010).

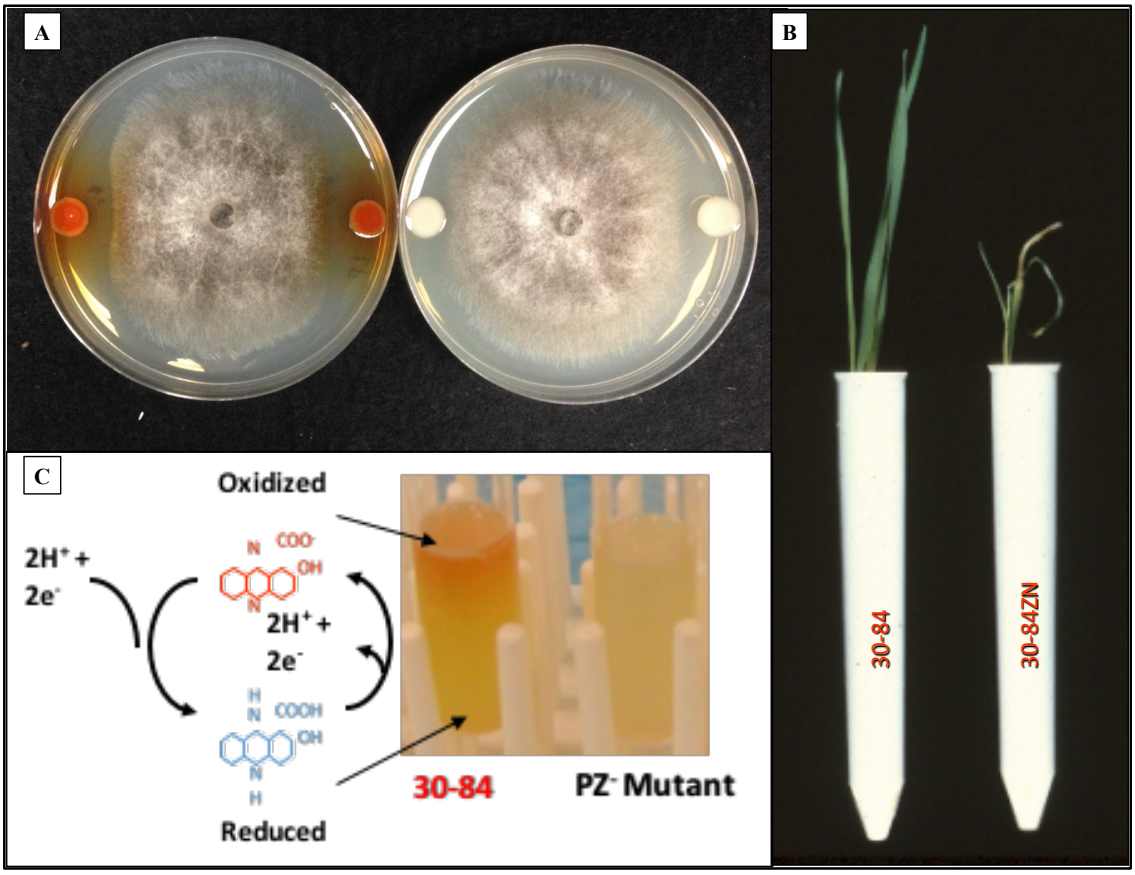


Figure 1. 2. Characteristics of *P. chlororaphis* 30-84.
A. In vitro fungal inhibition assay. Strain 30-84 and a phenazine null mutant (30-84ZN) were co-cultured with *Gaeumannomyces graminis* on PDA plate for 7 days. B. Protection of wheat from *G. graminis* var. *tritici* by phenazine producer 30-84. Phenazine is essential to suppress disease and phenazine null mutant failed to suppress the disease. Image source: Dr. L.S. Pierson III. C. Phenazines are redox-active molecules. Phenazines can reversibly undergo oxidation and reduction through transformation of 2 electrons and 2 protons. Image source: Dr. L.S. Pierson III

It also has been observed that phenazine derivatives generally differ in the specificity of their antagonism of different fungal species. Phenazines produced by *P. chlororaphis* 30-84 (PCA, 2OHPCA and 2OHPZ) and *P. fluorescens* 2-79 (producing PCA only) are able to suppress take-all disease. They specifically inhibit the pathogen *Gaeumannomyces graminis* var. *tritici* (*Ggt*), but are unable to inhibit *F. oxysporum* f.

sp. *radicis-lycopersici* (Chin-A-Woeng *et al.*, 2001a). The PCN producing strain *P. chlororaphis* PCL1391 has been shown to be most effective against *Fusarium oxysporum* f. sp. *radicis-lycopersici,* which is a causal agent of tomato foot and root rot. Also strain PCL1391 has been reported to inhibit *Rhizoctonia solani, Pythium ultimum,* and *Verticillium albo-atrum* (Chin-A-Woeng *et al.*, 1998). Mixtures of PCA and PCN produced by *P. aeruginosa* PNA1 inhibit *F. oxysporum* f. sp. *ciceris, Pythium splendens*, and *P. myriotylum* (Flaishman *et al.*, 1990, Anjaiah *et al.*, 1998). In addition, PYO producing *P. aeruginosa* LEC1 effectively inhibits *Septoria tritici* growth (Flaishman *et al.*, 1990). The underlying mechanism is unclear, but researchers believe that this specificity may be related to the chemical properties of each specific derivative, including their solubility and/or activity under different pH conditions (Chin-A-Woeng *et al.*, 2001b, Cezairliyan *et al.*, 2013).

Phenazines as signal molecules to modulate plant immunity

In addition to their chemical characteristic as antibiotics, phenazines are reported to activate plant defense pathways via induced systemic resistance (ISR). ISR is activated by colonization of roots with plant growth-promoting rhizobacteria (PGPR) and may confer resistance to diseases caused by plant pathogenic fungi, viruses and pathogenic bacteria. ISR is typically controlled by the jasmonic acid (JA) and ethylene (ET) dependent signaling pathways (Pieterse *et al.*, 2014). Root colonization of ISR-inducing bacteria prime plant defense systems, which leads to a faster and stronger response to pathogen attacks on above ground parts of plants. Several pseudomonads have been reported to be activating ISR inducers including *P. fluorescens* WCS417r, *P.*

fluorescens Pf29Arp, *P. fluorescens* CHA0, *P. fluorescens* 89B61, *P. putida* WCS358r, and *P. putida* KT2440 (Leeman *et al.*, 1996a, Audenaert *et al.*, 2002, Planchamp *et al.*, 2014). Recent unpublished studies in our lab confirm that strain 30-84 is also capable of inducing resistance to certain pathogens. Studies of *P. fluorescens* CHA0 and *P. aeruginosa* 7NSK2 induction of plant defense system in grapevine against *Botrytis cinerea* led authors to suggest that those strains triggered systemic resistance by an oxidative burst and phytoalexin accumulation in grape cells upon challenge with *B. cinerea* (Verhagen *et al.*, 2009). These results suggested that *Pseudomonas*-derived metabolites (e.g. pyochelin, pyoverdin, and salicylic acid) may serve to mediate oxidative burst and/or priming phytoalexin responses of plant cells to induce plant defense system.

There are a few reports that phenazine also serves as a signal molecule to activate ISR in plants. For example, the production of PYO by the rhizobacterium, *P. aerugonisa* 7NSK2 was shown to be involved in the activation of ISR against *Botrytis cinerea* in tomato and bean (Leeman *et al.*, 1996a, Audenaert *et al.*, 2002). *P. aeruginosa* 7NSK2 produces secondary metabolites including pyochelin (Pch), salicylic acid (SA), and PYO (Audenaert *et al.*, 2002). It was hypothesized that Pch and PYO act together to produce reactive oxygen species, which can lead to induced resistance (Audenaert *et al.*, 2002). Later De Vleesschauwer et al. (2006) reported that inoculation of *P. aeruginosa* 7NSK2 onto rice plants triggers systemic resistance to the fungal hemibiotrophic rice blast pathogen *Magnaporthe grisea*, however loss of PYO production resulted in the of loss resistance. This group also found that application of purified PYO to rice seedlings

resulted in increased H_2O_2 locally and systemically (De Vleesschauwer *et al.*, 2006). It is believed that phenazine, which is a redox active molecule, directly stimulates resistance in plant tissue by an oxidative burst (Spencer *et al.*, 2003). These results suggest that PYO has a critical role in activating ISR against leaf fungal pathogens.

Roles of phenazine in rhizosphere competence and root colonization

Phenazines are known to play roles in many aspects of host-microbe interactions, including rhizosphere competence, and root colonization (Mazzola *et al.*, 1992, Maddula *et al.*, 2006). Previous work showed that as compared to wild types, populations of phenazine deficient mutants of 30-84 and *P. fluorescens* strain 2-79 inoculated into soil colonized wheat roots equally effective. However, their numbers decreased more rapidly during multiple plant-harvest cycles compared to wild-type in the presence of other microorganisms in natural soil (Mazzola *et al.*, 1992). By comparison, no significant differences between wild-type and the phenazine deficient mutant in colonization were observed over repeated plant-harvest cycles in sterile soil (Mazzola *et al.*, 1992). These results indicated that phenazine production by 30-84 has a major role in its rhizosphere colonization and persistence in the natural soil where it must compete with the indigenous microorganisms.

The importance of phenazine producers in agricultural soils was also recently demonstrated by a survey of the diversity and population of fluorescent pseudomonads in the inland Pacific Northwest of the USA (Mavrodi *et al.*, 2011, Mavrodi *et al.*, 2012, Parejko *et al.*, 2012). This study showed that phenazine producing *Pseudomonas* populations dominate in the rhizosphere of winter wheat under dryland production,

where there is less than 165mm of annual precipitation (Mavrodi *et al.*, 2011). A follow-up study also reported that high accumulation of PCA was observed under arid conditions, and that the population of phenazine producing rhizobacteria was negatively affected by irrigation (Mavrodi *et al.*, 2012). These studies suggested that phenazine producers thrived under water stress conditions, possibly due to biofilm formation preventing desiccation under dry conditions (Mavrodi *et al.*, 2013).

Redox properties of phenazines

Phenazines are known as redox compounds that able to accept and donate two electrons, in a stepwise manner, according to the redox properties of nearby compounds (Fig. 1.2C) (Price-Whelan *et al.*, 2006). Phenazines facilitate carrying electrons from intracellular reductants to extracellular oxidants, and may serve as electron shuttles, to generate energy in biofilms (Abken *et al.*, 1998, Hernandez *et al.*, 2004). Under biofilm conditions, cells adjacent to the surface attached are more oxygen limited than cells at the liquid interface of the biofilm due to the depletion of oxygen in the biofilm. It is hypothesized the phenazine redox cycling facilitates energy production via cellular respiration by accepting electrons for the re-oxidation of accumulating NADH, resulting in the maintenance of redox homeostasis and essential metabolism (Price-Whelan *et al.*, 2006).

Influence of phenazines on bacterial biofilm formation

Biofilm formation is one of the most important characteristics for survival and persistence of rhizosphere bacteria (Pierson & Pierson, 2010). Quorum sensing plays a major role in phenazine production as well as critical role in biofilm development (De

Kievit *et al.*, 2001, Maddula *et al.*, 2006). A study conducted by Maddula et al. (2006) demonstrated that phenazine production in strain 30-84 is essential for the formation of biofilms. In this study, biofilm production by wild type 30-84 and a phenazine deficient mutant were compared using microscopic analysis of biofilm formation in specialized chambered microscope flow cell slides. The authors demonstrated that the phenazine deficient mutant of 30-84 was unable to form biofilms, but that this defect could be complemented via exogenously supplied phenazines into the growth media.

Subsequently, Maddula et al. (2008) reported that altering the ratio of phenazines produced by strain 30-84 affected biofilm density and structure. Using flow cells, they compared the development and architecture of biofilms produced by 30-84 wild-type and strains with altered ratios of phenazine production (e.g. 2-OH-PCA overproducer and PCA only producer). The 2-OH-PCA overproducer adhered more quickly to the chamber surface, formed thicker but flat biofilm structure than wild-type and had a lower dispersal rate. However, the PCA only producer had a thicker (4-fold) and mushroom- shaped biofilm compared to the wild-type and the 2OHPCA overproducer (Maddula *et al.*, 2008). Similarly the importance of phenazine for biofilm morphology was shown using *P. aeruginosa* strain PA14 (Ramos et al., 2010). The biofilm produced by the phenazine null-mutant of strain PA14 was reduced in total biomass, thickness and number of micro-colonies, but higher in surface-to-volume ratio than observed for biofilms of wild type. These results suggest that phenazines play important roles in biofilm formation, and that different phenazine derivatives influence the development and architecture of the biofilm.

The presence of extracellular DNA (eDNA) in bacterial biofilms has been observed for nearly a decade (Allesen-Holm *et al.*, 2006). Recent studies have taken a closer look at the importance of eDNA for the structural integrity of the biofilm formation (Das *et al.*, 2013b). The origin of eDNA has been hypothesized to derive from chromosomal DNA resulting from cell lysis, which may be mediated by the generation of ROS (Wei & Ma, 2013, Okshevsky & Meyer, 2015). Recently, Wang et al. (2016) demonstrated that release of eDNA by 2OHPCA producers of *P. chlororaphis* 30-84 was greater than by strains that produce only PCA or produce no phenazines and that this eDNA resulted in the production of greater structured biofilm matrix. A gene cluster encoding a bacteriophage-derived pyocin and its lysis cassette was upregulated in 2-OH-PCA producing derivatives. The holin encoded in this gene cluster was found to contribute to the release of eDNA in 30-84 biofilm matrices, demonstrating that the influence of 2OHPCA on eDNA production is due in part to cell autolysis as a result of pyocin production and release. Similarly, phenazine production (e.g. PYO) by *P. aeruginosa* is responsible for promotion of eDNA release via generation of H_2O_2 whereas phenazine-deficient strains demonstrate lower amounts of cell lysis and eDNA release (Das & Manefield, 2012).

Phenazine regulation

One focus of my thesis research was on gaining a better understanding of the genetic regulation of the phenazine biosynthetic genes as a means of gaining insight into how producing cells control the expression of this important secondary metabolite. In strain 30-84, phenazine biosynthesis is tightly regulated by a complex network of

regulatory systems (Fig. 1-3). These regulatory systems include transcriptional regulation via the PhzI/PhzR quorum sensing system. Other regulators include the Gac/Rsm system that controls phenazine production post-transcriptionally via the control of small non-coding RNA (*rsmZ*) and the translational repressor RsmE (Wang *et al.*, 2013). Additionally the RpeA/RpeB two component regulatory system, Pip the 'phenazine inducing protein', and RpoS the stationary sigma factor have been shown to contribute phenazine gene regulation (Wang *et al.*, 2012a). Strain 30-84 also has a secondary quorum sensing system, CsaI/CsaR, but it has been shown to only have a minor effect on phenazine production (Zhang & Pierson, 2001).

Phenazine production is regulated by quorum sensing system

Quorum sensing is a well described regulator of bacterial gene expression. Bacteria produce small signal molecules that are thought to diffuse freely through bacterial cell membranes (Kaplan & Greenberg, 1985). At high signal densities or under diffusion limited conditions, the signal molecules accumulated within their cells. In liquid culture, signal accumulation is typically proportional to cell density, hence the term "quorum sensing". However under other conditions such as might be experienced in nature, signal accumulation may depend more on limitation to signal diffusion than population density. A typical quorum sensing system contains of two genes: one encoding an AHL synthase and one encoding a transcriptional regulatory genes (Pierson *et al.*, 1998, Miller & Bassler, 2001, Waters & Bassler, 2005). Quorum sensing was originally described in *Vibrio fisheri* (Nealson & Hastings, 1979), where *luxI* encoded

AHL synthase and *luxR* encoded the transcriptional regulator (Eberhard *et al.*, 1981, Engebrecht *et al.*, 1983, Engebrecht & Silverman, 1984).

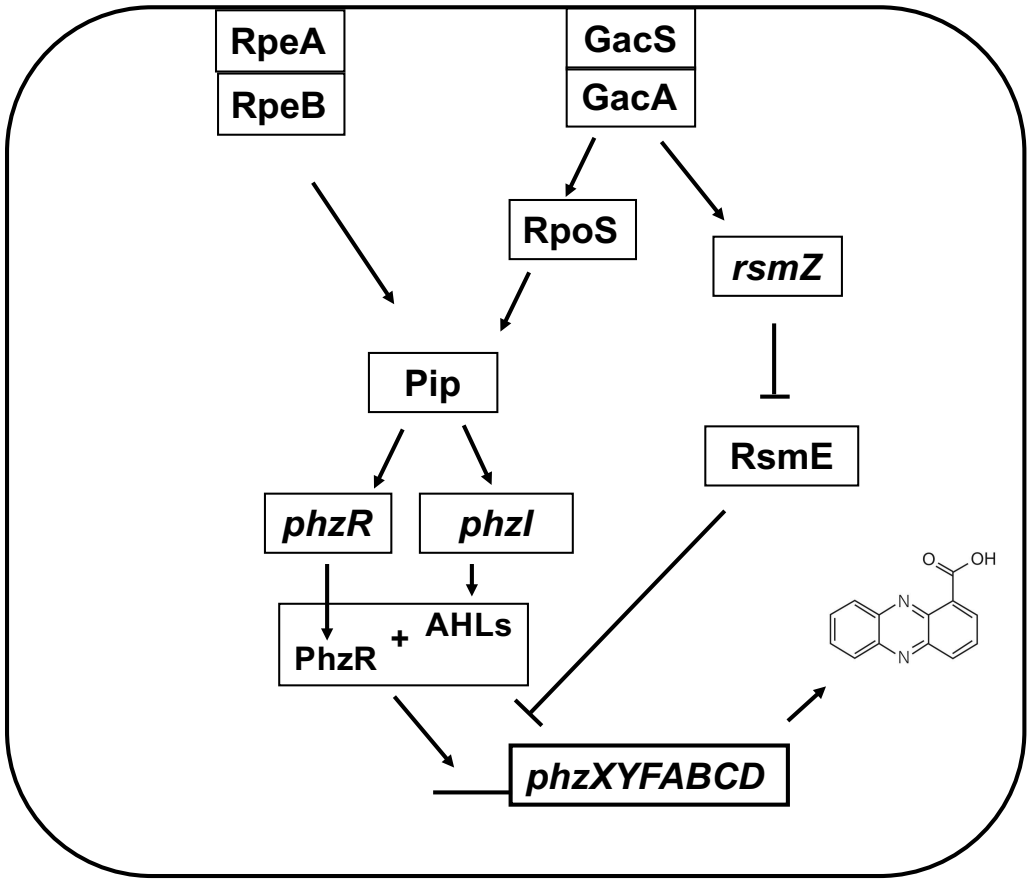


Figure 1. 3. Simplified model for phenazine biosynthesis in *P. chlororaphis* 30-84.
RpeA and GacS are transmembrane sensor proteins. RpeA is proposed to have a net phosphatase activity resulting in the dephosphorylation of RpeB. RpeB is possibly controlled by RpeA or other phosphor-donors, and phosphorylated RpeB promotes the transcriptional expression of *pip* resulting in the positive regulation of phenazine production through the PhzR/PhzI quorum sensing system. GacS is autophosphorylated and transfers its phosphate to GacA. Subsequently phosphorylated GacA promotes the expression of *rpoS,* and *rpoS* stimulates *pip* expression. GacA also positively regulates transcription of small non-coding RNAs (e.g. *rsmZ*), and these small non-coding RNAs titrate repressor protein RsmE to enhance the expression of *phz* genes. This figure is modified from Wang *et al.* (2012, 2013).

The PhzR/PhzI quorum sensing system controls expression of phenazine biosynthetic operon in *P. chlororaphis* 30-84 (Wood & Pierson, 1996), The gene *phzI,* a homolog of *luxI,* encodes the AHL synthase and *phzR,* a homolog of *luxR,* encodes a DNA binding transcriptional regulatory protein whose activity is controlled by AHL signals. The PhzR/PhzI quorum sensing system is highly conserved among other phenazine-producing *Pseudomonas* strains including *P. chlororaphis* strains 30-84, PA23 and PCL1391, *P. fluorescens* 2-79, and *Pseudomonas* sp. CMR12a (Pierson *et al.*, 1994, Wood & Pierson, 1996, Chin-A-Woeng *et al.*, 2001b, Khan *et al.*, 2005, De Maeyer *et al.*, 2011, Selin *et al.*, 2012). These genes are typically located directly upstream of the phenazine biosynthetic operon. According to the model proposed for other quorum sensing systems, when AHL signals reach a threshold density, the signal molecules bind and activate PhzR. Once PhzR is activated, it recruits RNA polymerase and increases its binding affinity to the "*phz* box" sequence located in the promoters of phenazine biosynthetic (*phz)* operon and the *phzI* gene*,* to initiate transcription of both (Fig. 1-2) (Wood & Pierson, 1996). Originally it was reported that *phzI* of strain 30-84 and PCL1391 was responsible for the synthesis of the AHL signal molecule of hexanoyl-homoerin lactone (HHL) (Wood & Pierson, 1996, Chin-A-Woeng *et al.*, 2001b), but later Khan et al., (2007) identified *N*-(3-hydroxyhexanoyl)-L-homoserine lactone as the predominant AHL signal produced by *phzI* in strain 30-84, PCL1391 and *P. fluorescens* 2-79. Strain 30-84 contains a second quorum sensing system, CsaI/CsaR, which is similar to PhzI/PhzR. It appears to play a minor role in phenazine production, but

contributes to exoprotease production and cell aggregation under laboratory conditions (Zhang & Pierson, 2001).

Phenazine regulated by two-component regulatory systems

In bacteria, two-component regulatory systems (TCS) placed on the top of signaling hierarchies play important roles in the coordinate regulate cellular pathways potentially by facilitating sensing and response to environmental conditions (Parkinson & Kofoid, 1992). Typically, TCS consist of cognate pair of proteins, transmembrane sensor kinase and cytoplasmic regulatory response protein. In pseudomonads, many important traits involved in virulence, survival, motility, biofilm formation, phase variation and production of metabolites are regulated by TCS system, GacS/GacA (Parkinson & Kofoid, 1992, Chancey *et al.*, 2002, Heeb *et al.*, 2002, Chatterjee *et al.*, 2003, De Souza *et al.*, 2003, van den Broek *et al.*, 2005, Driscoll *et al.*, 2011). Mutations in gacS or gacA are common resulting in phase variants with distinctly different patterns of gene expression and distinctly different phenotypes.

The GacS/GacA two-component global regulatory system (TCS) is essential for biosynthesis of bacterial secondary metabolites including phenazines, AHL signals, exoprotease, lipase, gelatinase and HCN in 30-84 (Chancey *et al.*, 1999, Chancey *et al.*, 2002, Wang *et al.*, 2013) and other *Pseudomonas* species (Heeb *et al.*, 2002, van den Broek *et al.*, 2005, Selin *et al.*, 2014). Mutation in Gac system in strain 30-84 results in loss of ability to form biofilm, pathogen inhibition, and rhizosphere competence (Chancey *et al.*, 1999, Driscoll *et al.*, 2011). GacS is a transmembrane histidine kinase (HK) sensor protein and GacA is cytoplasmic response regulatory (RR) protein. The

mechanism of GacS/GacA regulatory system has been suggested that the HK sensor GacS perceives external stimuli through its N-terminal periplasmic domain. This induces a conformational change that traverses the cell membrane, and then transfers the phosphate group from the histidine residue of the GacS to a conserved aspartate residue on the GacA. When the GacA is phosphorylated by GacS, it increases binding affinity to specific promoter region to regulate downstream of gene expressions (Chancey *et al.*, 1999, Chancey *et al.*, 2002, Wang *et al.*, 2013). Mutations in either GacS, the sensor kinase, or GacA, the response regulator, result in the loss of phenazine and other secondary metabolite production. Recent transcriptomic analysis demonstrates that the expression of phenazine biosynthetic genes as well as phenazine regulatory genes including *phzI/phzR, iopA/iopB, rpoS,* and *pip* are regulated by Gac system (Wang *et al.*, 2013).

The Gac system also positively controls the expression of several non-coding RNAs, which are part of a downstream regulatory system called Rsm (regulator of secondary metabolism). In *Pseudomonas* species, Gac/Rsm system comprised of three small non-coding RNAs (e.g. *rsmX, rsmY* and *rsmZ*) and two RNA binding repressor protein RsmA and RsmE (Heeb *et al.*, 2002, Kay *et al.*, 2005, Kay *et al.*, 2006, Lapouge *et al.*, 2008, Wang *et al.*, 2013). The function of RsmA/E proteins are post-transcriptional repressors by binding to a specific sequence motif (e.g. –GGA- or ribosome binding site) in the target mRNA and blocking translation. Once Gac is activated, it initiates transcription of small non-coding RNAs of *rsmX, rsmY,* and *rsmZ,* and multiple copies of those RNAs binds to RsmA/E to titrate the translational

repression (Haas & Keel, 2003, Haas & Défago, 2005, Kay *et al.*, 2005, Wang *et al.*, 2013). In strain 30-84, Gac system controls the expression of *rsmZ*, and in turns activates the expression of *phz* genes by titrating the translational repressor protein, RsmE (Fig. 1-2) (Wang *et al.*, 2013).

Strain 30-84 has another TCS system, RpeA/RpeB (repressor of phenazine expression) that regulates phenazine production (Fig. 1-2). RpeA is a putative sensor kinase and negatively regulates phenazine production (Whistler & Pierson, 2003). Compared to the wild-type, *rpeA* mutants have excessive phenazine production in rich media as well as minimal media. However, recent study indicates that cognate response regulator RpeB positively modulates phenazine production by controlling the phenazine regulatory genes, *pip* and *phzR* (Wang et al., 2012a). Phenazine production by an *rpeB* mutant is significantly decreased along with expression of phenazine biosynthetic operon and *pip* and *phzR* (Fig. 1-2) (Wang *et al.*, 2012a). These studies suggest that GacA/GacS mainly controls *phz* expression via post-transcriptional mechanisms, whereas RpeA/RpeB control phenazine expression via control of other regulators (e.g. *pip*) in a hierarchical manner, possibly in response to nutrient stress (Wang *et al.*, 2012a).

Phenazine regulation influenced by environmental conditions

The regulation of phenazine biosynthesis is clearly influenced by intracellular and/or extracellular environmental conditions (Chin-A-Woeng *et al.*, 2001b). Growth and amount of phenazine produced by *P. chlororaphis* PCL1391 was greatly affected by various environmental factors such as pH, temperature, nutritional sources, and

environmental stresses (van Rij *et al.*, 2004, van Rij *et al.*, 2005, Mavrodi *et al.*, 2006). For instance in *P. chlororaphis* PCL1391, PCN production is significantly decreased with decreasing pH (from 7 to 6) or temperature (from 21 to 16 ºC) (van Rij *et al.*, 2004). Glucose when supplied as a carbon source, results in more PCN production compared to other carbon sources such as fructose, glycerol, or sucrose (van Rij *et al.*, 2004). In addition to the environmental and nutritional factors, stress conditions such as excess salt or oxygen limitation dramatically affect phenazine production (van Rij *et al.*, 2004).

Furthermore some strains that produce multiple phenazine derivatives tend to produce certain phenazines under specific environmental and cultural conditions (Kanner *et al.*, 1978). For example, in minimal nutrient medium, *P. aeruginosa* tended to produce more PYO rather than PCN when grown at 37 ºC with shaking, however, more PCN was produced when it was growing at 28 ºC without shaking (Kanner *et al.*, 1978). Huang *et al*., (2009) reported that *P. aeruginosa* strain M18 also produces dramatically different amounts of the two phenazines derivatives, PCA and PYO depending on culture conditions. The predominant phenazine produced by strain M18 at 28 ºC is PCA, whereas the levels of PYO increases at 37 ºC due to the temperature dependent expression of *phzM* (Huang *et al.*, 2009). The strain M18 was isolated from the sweet melon rhizosphere, because of its capability to protect plants against various phytopathogens via the production of PCA (Hu *et al.*, 2005). In both organisms, the phenazine responsible for the most significant effect is produced at the temperature where they are most likely to be effective, e.g. PYO in causing disease in humans with

body temperature of 37 ºC and PCA at more typical environmental temperatures. The results suggested that production of different phenazine derivatives were evolved for its fitness under specific environments. All together the results suggested that regulation of phenazine production is habitat-specific.

CHAPTER II

ROLES OF DIFFERENT PHENAZINES PLAY IN THE BACTERIAL FITNESS OF THE BIOLOGICAL CONTROL STRAIN *PSEUDOMONAS CHLORORAPHIS* 30-84

Summary

Pseudomonas chlororaphis 30-84 is a biological control agent selected for its ability to suppress diseases caused by fungal pathogens. Phenazine production is required for fungal pathogen inhibition and wheat rhizosphere competence. *P. chlororaphis* 30-84 produces three distinct phenazines: phenazine-1-carboxylic acid (PCA), 2-hydroxy-phenazine-1-carboxylic acid (2OHPCA) and a small amount of 2-hydroxy-phenazine (2OHPHZ). The two, 2-hydroxy derivatives are produced from PCA via the activity of a monooxygenase, a modifying enzyme encoded by the gene *phzO*. In addition to the biosynthetic genes responsible for the production of PCA, many *Pseudomonas* strains possess one or more modifying genes such as *phzH*, *phzM*, or *phzS*, which encode enzymes that act independently or together to convert PCA into other phenazine derivatives. In order to understand the fitness effects of producing different phenazines, we constructed isogenic derivatives of strain 30-84 that produced phenazines made by other pseudomonads. This was accomplished by introducing the phenazine modifying genes of other organisms *in trans* into strain *Pseudomonas chlororaphis* 30-84PCA, a mutant that produces only PCA (*phzO*::Tn5). The present study demonstrated that altering the type of phenazines produced by *P. chlororaphis* 30-84 affected the specificity of fungal pathogen inhibition. Altered phenazine-producing

strains also differed in their ability to promote eDNA release, which resulted in different levels of biofilm formation. However, data indicated that all derivatives were equally important for persistence in the wheat rhizosphere under the conditions tested. This work expands the current understanding in roles of phenazine derivatives in terms of biological control application.

Introduction

Most plant-beneficial pseudomonads that are capable of suppressing plant disease are able to produce a variety of secondary metabolites (Haas & Défago, 2005). Phenazines, one of the well-studied secondary metabolites produced by these bacteria, are diffusible, heterocyclic compounds that play important roles in inhibiting plant pathogens of agricultural crops (Pierson & Thomashow, 1992, Mavrodi *et al.*, 2006). Distinct *Pseudomonas* strains often produce one or more phenazines derived from the substitution of functional groups (e.g. carboxy- and hydroxy-) on the ring structure of the core phenazine, phenazine-1-carboxylic acid (PCA) produced by most phenazine-producing pseudomonads. PCA synthesis is mediated by a highly conserved phenazine biosynthetic operon (*phzXYFABCD* in *P. chlororaphis* 30-84) (Pierson *et al.*, 1995, Mavrodi *et al.*, 2001). In most cases, the phenazine biosynthetic operon is flanked by one or more accessory genes that encode modifying enzymes, responsible for converting PCA to the different phenazine derivatives by methylation, transamidation, hydroxylation or decarboxylation (Chin-A-Woeng *et al.*, 2001b, Delaney *et al.*, 2001, Mavrodi *et al.*, 2001, Mavrodi *et al.*, 2006, Pierson & Pierson, 2010). For example, *P.*

chlororaphis 30-84 contains a modifying gene *phzO,* which encodes a monooxygenase responsible for the conversion of some PCA to 2OHPCA (Delaney et al., 2001). Similarly, the opportunistic human pathogen *P. aeruginosa* PAO1 contains specific phenazine modifying genes, including *phzH, phzM,* and *phzS*, which convert PCA to four additional phenazine derivatives (Mavrodi *et al.*, 2001). These include: phenazine-1-carboxamide (PCN) (via the activity of *phzH*), 5-methyl-phenazine-1-carboxylic acid (5MPCA) (via the activity of *phzM*), 1-hydroxy-phenazine (1OHPZ) (via the activity of *phzS*), and pyocyanin (PYO) (via the activity of both *phzM* and *phzS*) (Mavrodi *et al.*, 2001, Mavrodi *et al.*, 2006, Pierson & Pierson, 2010). However, the ecological function and individual physiological contribution of different phenazines to the fitness of the producer has yet to be elucidated.

As a group, phenazine compounds are electro-chemically active metabolites, which mechanistically are believed to contribute to bacterial fitness in part via antagonistic effects on competitors through the generation of reactive oxygen species (ROS) including superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Mavrodi *et al.*, 2001, Price-Whelan *et al.*, 2006, Pierson & Pierson, 2010). Phenazines, which diffuse out of the producing-cells, may become incorporated into the membranes of other organisms resulting in the uncoupling of oxidative phosphorylation, the generation of ROS, and ultimately interference in electron flow and enzyme functions related to cellular respiration (Baron *et al.*, 1989, Gu & Imlay, 2011). Similarly, phenazines are associated with virulence in humans when they produce changes in the redox balance of host cells

causing cell injury to the host cells or imbalanced oxidative stress responses (Price-Whelan *et al.*, 2006, Pierson & Pierson, 2010).

Additionally, the redox properties and reactivities of phenazines are believed to contribute to host fitness via their conditioning of the biofilm environment. Evidence suggests that phenazines contribute to electron shuttling and iron acquisition within biofilms and even to the establishment and architecture of biofilms (Maddula *et al.*, 2006, Price-Whelan *et al.*, 2007, Maddula *et al.*, 2008, Wang *et al.*, 2011, Glasser *et al.*, 2014). Under biofilm conditions, where oxygen diffusion is limited, basal cells experience more oxygen-limited conditions than the cells in the outer layers of the biofilm. Price-Whelan et al. (2006) proposed that PYO produced by *P. aeruginosa* may extend energy production via cellular respiration, by serving as electron shuttles from intracellular reductants (i.e. NAD(P)H) to extracellular oxidants (i.e. molecular oxygen). Consistent with this hypothesis is the observation that levels of reduced NAD are greater in a PYO deficient mutant of *P. aeruginosa* under oxygen limited conditions and that reduced NAD levels decrease when exogenous PYO is added (Price-Whelan *et al.*, 2007). Additionally, the redox activities of PCA (produced by *P. aeruginosa*) and PCN (produced by *P. chlororaphis* PCL1391) have been shown to contribute to the reduction of ferric iron (Fe^{3+}) to the more bioavailable, soluble form ferrous iron (Fe^{2+}), resulting in the promotion of biofilm development and enhanced microbial survival under anaerobic conditions (Hernandez *et al.*, 2004, Wang *et al.*, 2011). The essential role of phenazines for biofilm formation by *P. chlororaphis* 30-84 was demonstrated in a study in which a phenazine-defective mutant, 30-84ZN (*phzB::lacZ*), was significantly

impaired in cell attachment/biofilm formation, compared to wild type (Maddula *et al.*, 2006). Subsequently, the same phenazine mutant was found to be impaired in biofilm matrix production (Wang *et al.*, 2016). Modifying the type of phenazines produced also altered *P. chlororaphis* 30-84 biofilm development and architecture (Maddula *et al.*, 2008). In that study, altered phenazine-producing derivatives were constructed that produced only PCA (30-84PCA, Table 2.1) via the disruption of *phzO* or produced more 2OHPCA (30-84O*) due to the introduction of extra copies of *phzO in trans*. Compared to the wild-type, overproduction of 2OHPCA resulted in enhanced initial attachment, altered the three-dimensional biofilm structure, and reduced dispersal from mature biofilms, whereas production of only PCA resulted in thicker biofilms with greater bio-volume.

In natural environments, surface attached aggregates typically become encapsulated in a self-produced matrix of exopolymeric substance (EPS) (Parsek & Singh, 2003). This EPS matrix protects bacterial cells from physical and mechanical stresses (i.e. desiccation, antibiotics, host immune system), having a major role in their survival and persistence (Okshevsky & Meyer, 2015). The mature biofilm matrix is composed primarily of polysaccharides, secondary metabolites, proteins, lipids and nucleic acids (such as extracellular DNA) (reviewed in Flemming & Wingender, 2010, Mann & Wozniak, 2012, Das *et al.*, 2013b, Wei & Ma, 2013). Additionally, extracellular DNA (eDNA) has been found to be a ubiquitous component of the biofilm matrix, with hypothesized roles relating to initial bacterial adhesion and surface aggregation (Das *et al.*, 2010), biofilm development (Gloag *et al.*, 2013), promoting bacterial binding

affinities with polymers and metabolites (Das *et al.*, 2013b), and the development of cation gradients important for protection against antibiotics (Mulcahy *et al.*, 2008). In *P. chlororaphis* 30-84, a relationship between eDNA release and phenazine production recently was reported (Wang *et al.*, 2016). Using the altered phenazine producers 30-84PCA and 30-84O*, it was observed that the amount of eDNA produced was greater in strains that produced 2OHPCA (e.g. 30-84O* and wild type) compared to the strain that produced only PCA (30-84PCA) or no phenazines (30-84ZN). Moreover, structured biofilm matrix production depended on eDNA production and was abolished if eDNA was degraded by DNase I (Wang *et al.*, 2016). That study reported that eDNA production is correlated with the generation of ROS, which may promote eDNA release via several mechanisms including bacteriocin activity. The generation of hydrogen peroxide concomitant with PYO production by *P. aeruginosa* also has been linked to the promotion of eDNA release (Das & Manefield, 2012).

Despite the myriad of studies documenting the importance of phenazines, the specific roles of different phenazine derivatives are still not clear. The previous studies using the altered phenazine producers of *P. chlororaphis* 30-84 suggested that each phenazine has a distinct function in biofilm formation and other biological control capabilities including their pathogen inhibition (Maddula *et al.*, 2008, Wang *et al.*, 2016). In the present study, I created additional isogenic strains of altered phenazine producers that produce PCN, 1OHPZ, 5MPCA, and PYO in addition to the core phenazine, PCA. The new altered phenazine producers, together with the previously characterized strains of 30-84 wild-type, 30-84ZN, and 30-84PCA were used to study

the effect of altering the type of phenazines produced on characteristics important for biological control including pathogen inhibition, biofilm formation, and wheat root colonization.

Results and Discussion

Functional analyses of altered phenazine producers

The types of phenazines produced by each of the newly constructed altered phenazine producers were first characterized by colormetric observation and semi-qPCR. Each of the different phenazines has a distinct color under neutral pH. Thus successful expression of the modifying genes was first indicated by production of a diffusible compound with the characteristic color of the target phenazine. Compared to the bright orange phenazine produced by wild-type when grown to stationary phase in PPMD medium, 30-84H (PCN producer) produced a greenish tan phenazine, 30-84M (5-meth-PCA producer) produced a dark red phenazine, 30-84S (1OHPHZ producer) produced a tan brown phenazine, and 30-84MS (PYO producer) produced a dark green phenazine (Table 2.1, Fig. 2.1A and 2.1B). Similar results were reported by Mavrodi *et al.* (2001). Additionally, a consistent phenotype can be observed in strains of 30-84ZN (non-phenazine producer, white), 30-84PCA (PCA only producer, bright yellow), and 30-84O* (2OHPCA overproducer, dark orange) as described previously (Maddula *et al.*, 2008) (Table 2.1, Fig. 2.1A and 2.1B).

Table 2. 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
***Pseudomonas* strains**		
30-84	PCA^+, 2-OH-PCA^+, and 2-OH-PHZ+, Rif^R	Pierson and Thomashow (1995)
30-84 PCA	PCA^+, 2-OH-PCA^-, Rif^R, Km^R, *phzO*::Tn5	Maddula et al., (2008)
30-84ZN	Phz^- *phzB*::*lacZ* genomic fusion Rif^R	Wood et al., (1997)
30-84H	PCA^+, PCN^+, containing plasmid pGT2-*phzH*, Km^R, Rif^R, Gm^R	This study
30-84S	PCA^+, $1OHPZ^+$, containing plasmid pGT2-*phzS*, Km^R, Rif^R, Gm^R	This study
30-84M	PCA^+, $5MPCA^+$, containing plasmid pGT2-*phzM*, Km^R, Rif^R, Gm^R	This study
30-84MS	PCA^+, PYO^+, containing plasmid pGT2-*phzMS*, Km^R, Rif^R, Gm^R	This study
ATCC 17411	PCA^+, PCN^+	Mavrodi et al., (2001)
P. fluorescens 2-79	PCA^+, Rif^R	Weller (1983)
***E. coli* strains**		
DH5α	F^- *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*argF-lacZYA*)*I169* ϕ80*lacZ*ΔM15 λ^-	GIBCO-BRL
HB101	F^- *hsdS20*(r_B^- m_B^-) *supE44 recA1 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-5* λ^-	GIBCO-BRL
Plasmid		
pCR2.1-TOPO	Ap^R,Km^R, T7 promoters, f1 *ori, lacZα*	Invitrogen Corp.
TOPO-*phzH*	pCR2.1-TOPO carrying a 1.9-kb DNA fragment containing *phzH* gene	This study
TOPO-*phzS*	pCR2.1-TOPO carrying a 1.2-kb DNA fragment containing *phzS* gene	This study
TOPO-*phzM*	pCR2.1-TOPO carrying a 1.2-kb DNA fragment containing *phzM* gene	This study
TOPO-*phzM_BB*	pCR2.1-TOPO carrying a 1.2-kb DNA fragment containing *phzM* gene	This study
pGT2	pProbe-GT', pVS1 replicon, p15a origin of replication, gfp transcriptional fusion; Gm^R	Miller et al., (2000)
pGT2-PsP	pGT2 carrying a 127-bp DNA fragment containing truncated promoter sequence of *phzX* gene	This study
pKT2-PsP	pKT2 carrying a 127-bp DNA fragment containing truncated promoter sequence of *phzX* gene	Yu et al., unpublished
pGT2-*phzH*	pGT2-PsP carrying a 1.9-kb DNA fragment containing *phzH* gene	This study
pGT2-*phzS*	pGT2-PsP carrying a 1.2-kb DNA fragment containing *phzS* gene	This study
pGT2-*phzM*	pGT2-PsP carrying a 1.2-kb DNA fragment containing *phzM* gene	This study
pGT2-*phzMS*	pGT2-PsP carrying a 2.4-kb DNA fragment containing *phzM* and *phzS* gene together	This study

[a] Km^R, Ap^R, Gm^R and Rif^R = kanamycin, ampicillin, gentamycin and rifampin resistance, respectively.

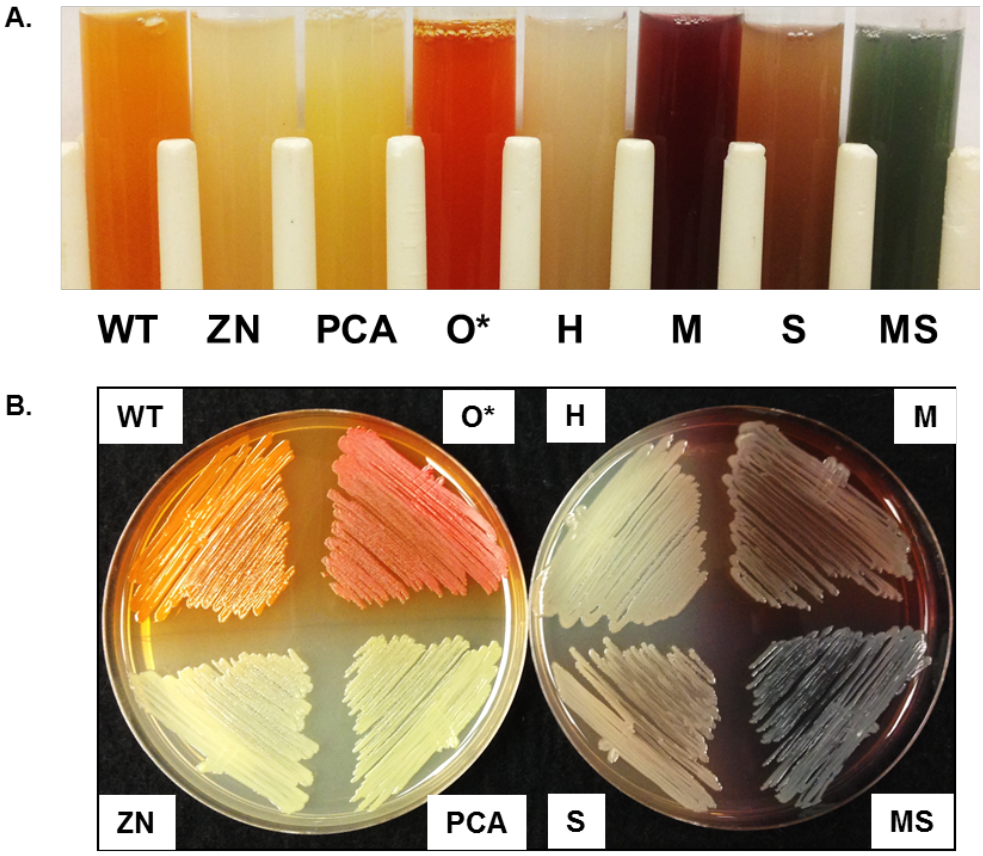


Figure 2. 1 Production of phenazines by *P. chlororaphis* 30-84 wild-type and altered phenazine-producing derivatives on liquid and solid media.
Strains shown include: *P. chlororaphis* 30-84 wild-type, 30-84ZN (phenazine non-producer, *phzB*::*lacZ*), 30-84PCA (PCA only producer, *phzO*::Tn5), 30-84O* (2OHPCA overproducer), 30-84H (PCA and PCN producer), 30-84M (PCA and 5MPCA producer), 30-84S (PCA and 1OHPZ), and 30-84MS (PCA and PYO producer). The wild type, 30-84ZN, 30-84PCA, and 30-84O* contain the empty vector pGT2pPsp. A. Phenazine production in liquid media. Cultures from individual single colonies were grown in 5 ml PPMD medium for 48 hr at 28 ºC with rapid shaking. B. Phenazine production in solid media. Individual colonies were picked from each plate, and streaked on PPMD agar plate. These plates were incubated at 28 ºC for 36 hr without shaking.

To verify the successful expression of the terminal modifying genes *in trans*, RNA was extracted from triplicate samples of each altered phenazine producer. The cDNA was synthesized by reverse transcription with random hexamers. Semi-qPCR was performed using specific primers for the genes *phzH, phzS* and *phzM* (Table 2.2). The gene *rpoD* was used as the reference gene. As shown in Fig. 2.2, expression of *phzH* can

be detected for the 30-84H samples, whereas expression of *phzS* or *phzM* can be detected only in the 30-84MS and the 30-84S or 30-84M samples. The results indicated that each of introduced phenazine modifying gene was successfully expressed *in trans* supporting the colorimetric observations that PCA was being converted to the other phenazine derivatives.

Table 2. 2 Oligonucleotides used for gene cloning and semi-qPCR

Oligonucleotides	Sequences (5'-3')[a]
phzH1	CGCACGGATCCTTTCAGCATGTTC (*Bam*HI)
phzH2	CGCACAAGCTTCGTCACGCTCA (*Hin*dIII)
phzM1	CGCACGGATCCTTTCGGTACGCAGGAAAAG (*Bam*HI)
phzM2	CGCACAAGCTTGTTGAAAGTTCCGATTCA (*Hin*dIII)
phzM3	CGCACGGATCCGTTGAAAGTTCCGATTCA (*Bam*HI)
phzS1	CGCACGGATCCAAAAGGAAGCACC (*Bam*HI)
phzS2	CGCACAAGCTTCTAGCGTGGCCGTT (*Hin*dIII)
phzHRT1	AATGCCGAACTGGTCTGCGAA
phzHRT2	ATGTCGCCGAAGGTGAAAGGTA
phzMRT1	AATGGAAGTCCCGTTGCGTCT
phzMRT2	TGTCGATCCCGCTCTCGAT
phzSRT1	TTCCTCGACGGCAAGACCAT
phzSRT2	CGAGATCGGATAGGCGACCA
RpoD-F1	ACGTCCTGAGCGGTTACATC
RpoD-R1	CTTTCGGCTTCTTCTTCGTC

[a] Underlined nucleotides are restriction sites added and the restriction enzymes are indicated at the end of primers

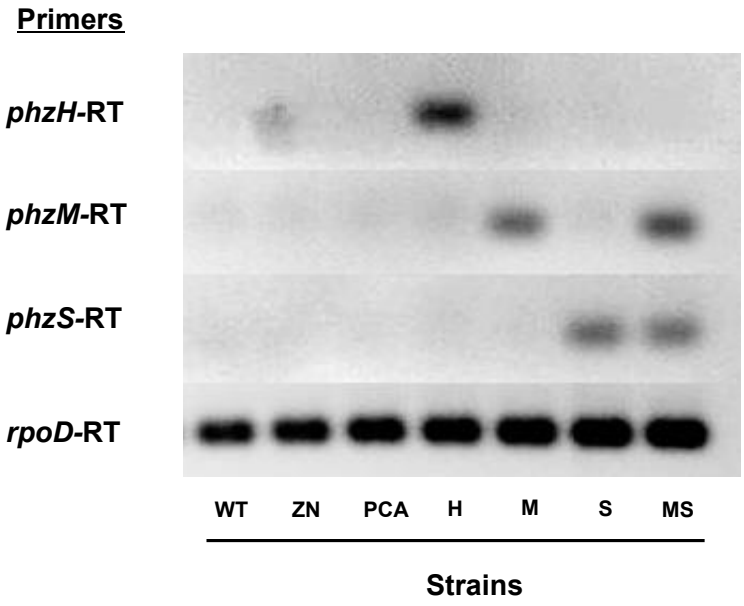


Figure 2. 2 Expression of phenazine modifying genes in the *P. chlororaphis* 30-84 wild-type and the altered phenazine-producing derivatives.
Total RNA was extracted from overnight cultures in AB minimal media supplemented 2% casamino acid at OD_{620}=1.2 and cDNA was synthesized. Semi quantitative PCR analysis was carried out to verify the expression of each of the introduced modifying genes using *rpoD* as the reference gene. The gene specific primer sets listed in the figure were used to check the expression of each gene. The assay was repeated twice, and one representative experiment is presented.

Purification and structural identification of phenazine derivatives

To verify the structural identification of the phenazines produced by each phenazine-producing derivative, total phenazines were extracted from late stationary phase cultures grown in PPMD medium using C_{18} reverse-phase SPE columns. Subsequently, purified phenazines were separated by HPLC-MS according to monoisotopic mass and extracted ion chromatograms are reported (Fig. 2.3). In total phenazine extracts from 30-84PCA, one peak was detected at RT=15.5 min in *m/z*= 224-225 range, which corresponded to PCA (monoisotopic mass, 224.05 g/mol, respectively) (Fig. 2.3A). The identity of PCA was confirmed by comparison to extracts from strain *P.*

fluorescens 2-79, which produces on PCA (data not shown) (Weller, 1983, Mavrodi *et al.*, 2001). Phenazines extracted from 30-84 wild-type demonstrated the characteristic 3 major peaks detected at RT=13.8 min, 15.5 min, and 16.2 min in *m/z* = 196-197, 224 - 225, and 240-241 ranges, respectively. These correspond to 2OHPHZ (196.06 g/mol), PCA (224.05 g/mol) and 2OHPCA (240.05 g/mol) (Fig. 2.3B). In addition to the PCA peak, phenazines extracted from 30-84H contained a peak at RT= 14.1 min in *m/z* = 223 – 224 (Fig. 2.3C) corresponding to PCN (223.07 g/mol). The identity of PCN was confirmed by comparison to phenazines extracted from *P. chlororaphis* ATCC 17411, which produces PCN (data not shown) (Mavrodi *et al.*, 2001). In addition to PCA, 30-84M should produce 5MPCA. However, only one clear peak was detected within the PCA range; numerous ambiguous noisy peaks also were detected (Fig. 2.3D). Similar results were reported previously (Mavrodi *et al.*, 2001, Bellin *et al.*, 2014). According to Mavrodi *et al*. (2001), no 5MPCA could be detected by C_{18}-reverse phase HPLC extraction of phenazines produced by either the *phzS* mutant of *P. aeruginosa* PAO1 or an *E. coli* strain expressing both *phzM* and one of the phenazine operons from *P. aeruginosa in trans*. They speculated that because 5MPCA is extremely hydrophilic, it could not be extracted with the methods used (Mavrodi *et al.*, 2001). For this reason, production of 5MPCA of strain 30-84M was confirmed only by colorimetric observations accompanied by semi-qPCR analysis. In addition to PCA, the phenazines extracted from 30-84S contained a peak corresponding to 1OHPHZ (196.06 g/mol) detected at RT = 15.53 in the range of *m/z* = 197 – 198, respectively (Fig. 2.3E). Finally, in the extracts from 30-84MS, in addition to a peak corresponding to PCA, a

peak corresponding to PYO (210.07 g/mol) peak was detected at RT = 3.7 min in the range of *m/z* = 211 – 212, respectively (Fig. 2.3F). The identity of PYO was confirmed by comparison to purified PYO (Sigma-Aldrich). Taken togher, the results of all of analyses indicated that the introduced genes were successfully expressed and the enzymes produced were functionally capable of converting some of the PCA to the appropriate phenazine derivatives.

Phenazines have specificity to inhibit the growth of plant pathogenic fungi

Phenazine production in *P. chlororaphis* 30-84 is responsible for the majority of its ability to inhibit the growth of pathogens (Pierson & Thomashow, 1992). To determine the antifungal capability of the modified phenazine producers to inhibit a diversity of plant pathogenic fungi, *in vitro* pathogen inhibition assays were performed. Only minor (less than 1 mm) or no inhibition was observed for the phenazine non-producing strain 30-84ZN, and the bacterial colony typically was surrounded by fungal mycelium at the end of the assays. In nearly every case the production of PCA resulted in significantly more pathogen inhibition than observed for 30-84ZN (Table 2.3). Significantly, the production of at least one additional phenazine by the altered phenazine-producing derivative generally resulted in greater fungal inhibition than observed for 30-84PCA (e.g. for 11 out of 12 fungi), suggesting a benefit to converting some PCA to another type of phenazine (Table 2.3).

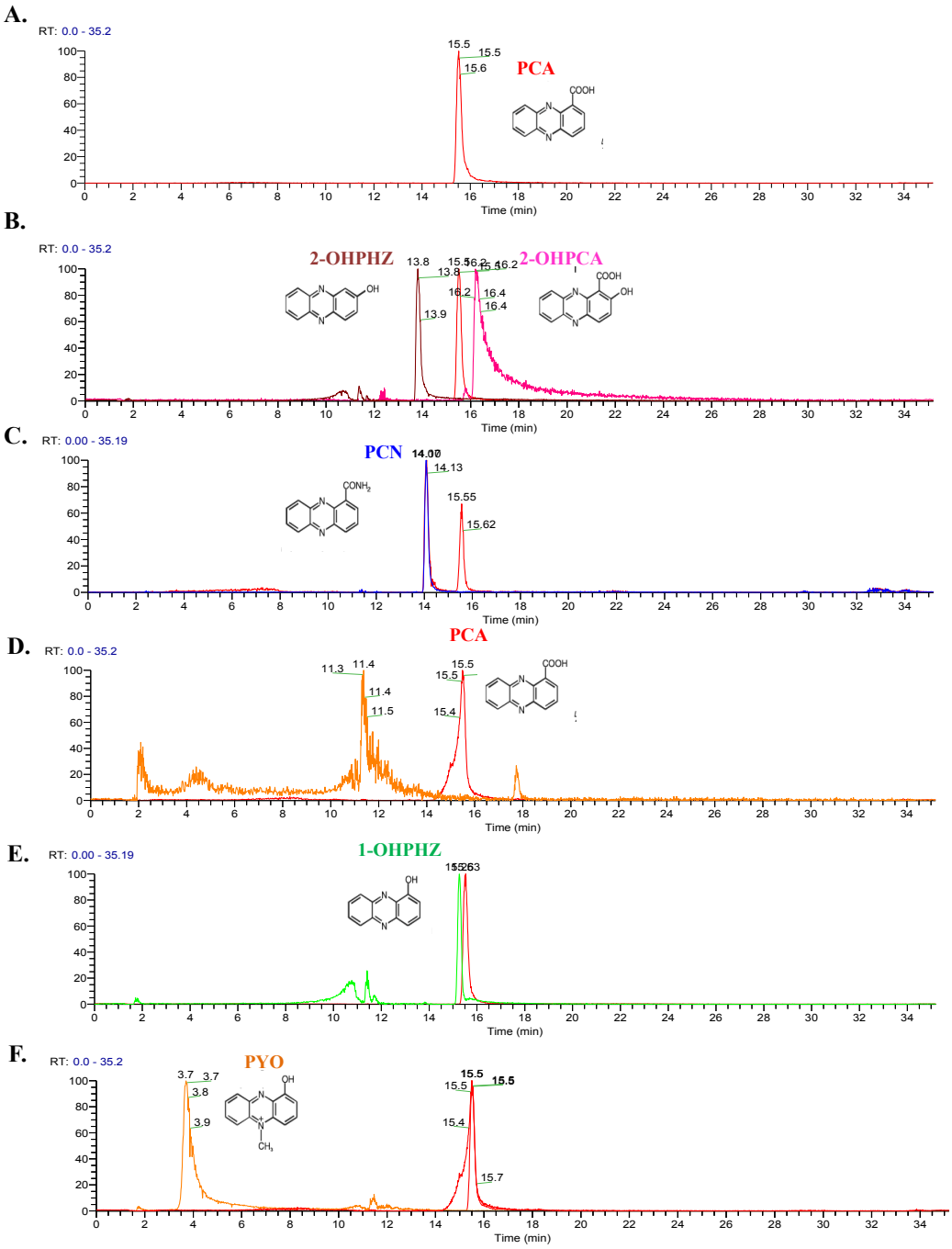


Figure 2. 3 HPLC-MS analyses of phenazines extracted from *P. chlororaphis* 30-84 wild type and each of the altered phenazine-producing derivatives

(A) *P. chlororaphis* 30-84PCA, (B) *P. chlororaphis* 30-84 wild-type, (C) 30-84H, (D) 30-84M, (E) 30-84S, (F) 30-84MS. Data indicate PCA is produced by all derivatives and that 2OHPCA, 2OHPZ, PCA, PCN, 1OHPCA, and PYO were produced by *P. chlororaphis* 30-84 wild-type, 30-84H, 30-84M, 30-84S, and 30-84MS, respectively. One microliter sample was used for the HPLC-MS separation and the identities of peaks were confirmed by monoisotopic mass analysis accompanied by comparisons to available positive control strains and compounds (i.e. PCA only producer *P. fluorescens* 2-79, PCN producer *P. chlororaphis* ATCC17411, and purified PYO (Sigma-Aldrich)). The assay was repeated three times.

However, the altered phenazine-producing strains differed in the spectrum of pathogens inhibited. For example, *Pseudomonas chlororaphis* wild-type (PCA, 2OHPCA and 2OHPZ producer) and 30-84MS (PCA and PYO producer) typically had greater fungal growth inhibition capabilities and a broader spectrum of activity than the other strains tested. Significantly, the production of at least one additional phenazine by the altered phenazine-producing derivative generally resulted in greater fungal inhibition than observed for 30-84PCA (e.g. for 11 out of 12 fungi), suggesting a benefit to converting some PCA to another type of phenazine (Table 2.3). However, the altered phenazine-producing strains differed in the spectrum of pathogens inhibited. For example, *Pseudomonas chlororaphis* wild-type (PCA, 2OHPCA and 2OHPZ producer) and 30-84MS (PCA and PYO producer) typically had greater fungal growth inhibition capabilities and a broader spectrum of activity than the other strains tested. Interestingly, only 2OHPCA producing *P. chlororaphis* 30-84 wild-type effectively inhibited the growth of *G. graminis*. Given that the isogenic derivatives differed only in the type of phenazines produced, these data demonstrate that phenazines are somewhat specific in their abilities to inhibit the growth of different plant pathogenic fungi.

Table 2. 3 *in vitro* Fungal inhibition assays.

	Inhibition zone (mm)[a]						
	ZN	PCA	WT	H	M	S	MS
Cochliobolus heterotrophus	0.8 ±0.71	1.25 ±0.71	4.25 ±0.75*	1.25 ±0.75	4.75 ±0.85*	3.75 1.44*	5.75 ±0.75*
Gaeumannomyces graminis	0	2.5 ±0.4	8.5 ±0.9 *	2.0 ±0.4	3.4 ±0.5	1.9 ±0.3	2.8 ±0.5
Pythium ultimum	0	1.5 ±0.28	5.5 ±0.14*	2.4 ±0.18*	6.5 ±0.5*	3.15 ±0.45*	3.25 ±0.63*
Phytophthora capsici	0	3.5 ±0.71	3.45 ±0.64	4.5 ±0.71	2.5 ±0.71	4 ±0.14	6 ±0.21*
Sclerotinia sclerotiorum	0	7.75 ±1.18	8 ±1.35	8 ±1.35	8.75 ±1.38	7.33 ±1.86	11.25 ±0.75*
Sclerotinia minor	0	4 ±1.35	6.25 ±1.31	7.25 ±1.84*	5.25 ±1.11	2.75 ±0.95	4.75 ±1.31
Rhizoctonia solani	0	0	0.75 ±0.47*	0	1.25 ±0.75*	0	1 0.58*
Alternaria brassicae	1.2 ±0.3	6.5 ±0.7	8.5 ±1.41	6.5 ±0.7	8 ±1.41	9 ±1.41*	8 ±1.41
Botrytis cinerea	0	4.5 ±0.71	3.5 ±0.71	2.5 ±0.71	4.75 ±0.35	3.5 ±0.71	8.5 ±2.12*
Fusarium oxysporum	0	1.67 ±0.28	3.5 ±0.5*	1.75 ±0.25	2.25 ±0.48	3.75 0.28*	2.25 ±0.33
Fusrium graminearum	0	1.3 ±0.33	3.5 ±0.65*	2.15 0.25*	3 ±0.57*	1.38 ±0.24	1.33 ±0.33
Colletotrichum graminicola	0.1 ±0.21	6.25 ±1.32	9.5 ±0.95*	7 ±0.41	6.75 ±0.75	6 ±0.91	5.5 ±0.5

[a] Mean width of the zones of inhibition (mm) obtained from mean of 3 separate plates ± standard error
Asterisk (*) with gray highlighted columns indicate values that differ significantly from 30-84PCA.

The broad spectrum of inhibitory activity of phenazines against plant pathogenic organisms including bacteria, fungi and nematodes has been well documented, although

these studies typically compare the specificity of pathogen inhibition by different phenazine-producing strains rather than isogenic derivatives as in this study. For example, *P. chlororaphis* 30-84 (PCA, 2OHPCA and 2OHPZ producer) and *P. fluorescens* 2-79 (producing PCA only) both inhibit the growth of *G. graminis* var. *tritici* and suppress take-all disease (Thomashow & Weller, 1988, Pierson & Thomashow, 1992), however they failed to inhibit other pathogens such as *F. oxysporum* f. sp. *radicis-lycopersici* (Chin-A-Woeng *et al.*, 2001a). The inhibitory spectrum of PYO against phytopathogens has been less well studied probably because of the likely constraints on developing *P. aeruginosa,* an opportunistic pathogen, as a commercial biological control agent. However PYO production by *P. aeruginosa* LEC1 effectively inhibits growth of *Septoria tritici* (Flaishman *et al.*, 1990). In addition, the PCN producing strain *P. chlororaphis* PCL1391 has been shown to control *F. oxysporum* f. sp. *radicis-lycopersici*, *R. solani, P. ultimum*, and *Verticillium albo-atrum* (Chin-A-Woeng *et al.*, 1998). Our study showed a broader spectrum of inhibition for 2OHPCA and PYO than reported previously. However unlike previous reports for the PCN producing strain *P. chlororaphis* PCL1391, the PCN producing strain 30-84H failed to inhibit the growth of *R. solani* and had little effect on *F. oxyspoum* (Table 2.3). Moreover the introduction of *phzH* from PCL1391 into *P. chlororaphis* 30-84 (PCL1149,(Chin-A-Woeng *et al.*, 2001a) conferred the ability the suppress tomato foot rot caused by *F. oxysporum* f. sp. *radicis lycopersici*. It is likely that the conversion rate of PCA to PCN in 30-84H (which contains *phzH* from *P. aeruginosa* and not *P. chlororaphis* PCL1391) may be lower than in *P. chlororaphis* PCL1391 or the modified

30-84 strain PCL1149. Differences in the two *phzH* genes also may have contributed. However, these data support the hypothesis that different phenazines have specific antagonistic effects against pathogenic fungi.

The mechanisms for the antibiotic effects of phenazines have not been fully elucidated. However, previous reports suggest that antimicrobial activities are related to the chemical properties of each specific phenazine derivative, including their solubility, capacity to generate harmful reactive oxygen species (ROS), and activity under different pH conditions (Hassett *et al.*, 1992, Price-Whelan *et al.*, 2006, Gibson *et al.*, 2009, Liu & Nizet, 2009, Pierson & Pierson, 2010, Cezairliyan *et al.*, 2013). A previous study showed that *in vitro* antifungal activity was greatly influenced by physical factors such as pH (Ownley *et al.*, 1992). For example, the growth inhibition of *G. graminis* var. *tritici* due to purified PCA was greater at pH 6.0 than at higher pH values. In contrast, pathogen inhibition by PCN against *F. oxysporum* f. sp. *radicis-lycopersici* was 10 times greater than PCA at pH 5.7 (Chin-A-Woeng *et al.*, 1998). The authors speculated that antifungal activity might be influenced by the ionic form or solubility of different phenazines. Of interest is the recent finding that phenazines differentially effect the expression of other potential fungal inhibitory mechanisms (Wang *et al.*, 2016). Transcriptomic comparisons of biofilm populations of 30-84ZN, 30-84PCA, wild-type and 30-84O* revealed that 2OHPCA phenazine production was correlated with the expression of other potential fungal inhibitory mechanisms, including the production of exoenzymes and certain secondary metabolites (Wang *et al.*, 2016). These results suggest that altered phenazines may act both directly and indirectly (e.g. by synergistic

effects on the expression of other genes) in inhibiting fungal pathogens. Currently, screening of antifungal activities under various conditions is under investigation.

Phenazines play roles in biofilm formation

Previously, Maddula *et al*., (2008) showed that altering the ratio of phenazines produced by *P. chlororaphis* 30-84 affected cell attachment and biofilm architecture. We hypothesized that the production of other phenazines by isogenic derivatives also may alter aspects of biofilm formation. To test this hypothesis, 30-84 wild-type, 30-84ZN, and the altered phenazine-producing strains were growing in static plates in AB-C for up to 72 h. Cell attachment was measured by staining with crystal violet dye. After 48 h, the phenazine non-producing strain 30-84ZN was significantly impaired in biofilm formation compared to other phenazine producers (Fig. 2.4A). However, all of the phenazine-producing strains formed similar amounts of biofilm. After 72 h, biofilm formation by 30-84ZN remained impaired compared to the phenazine producers. Strains 30-84 wild-type, 30-84H, and 30-84S produced significantly more biofilm than 30-84PCA and 30-84M, whereas the amount of biofilm produced by 30-84MS was in between and not statistically different from any of the other phenazine-producing strains. The results indicated that loss of phenazine production significantly impaired biofilm formation, and modification of the type of phenazines produced by strain 30-84 affected the capability to form biofilms.

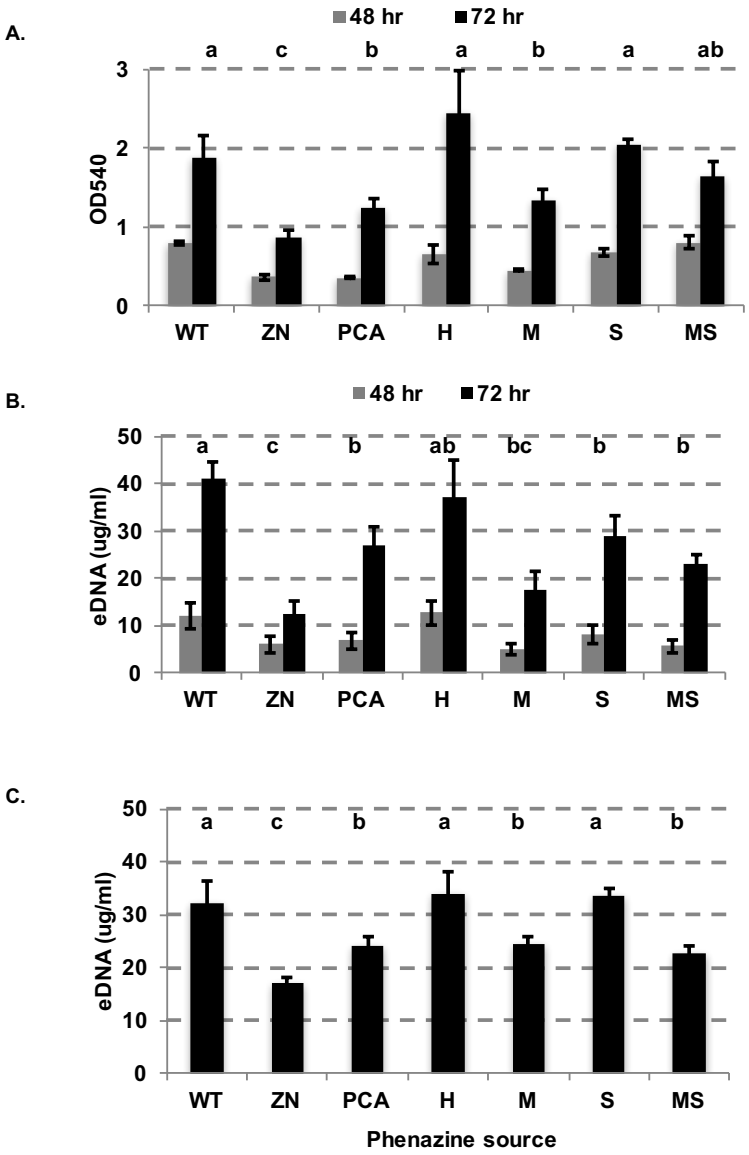


Figure 2. 4 Biofilm formation and eDNA production by *P. chlororaphis* 30-84 wild-type, 30-84ZN and the altered phenazine-producing strains 30-84PCA, 30-84H, 30-84M, 30-84S, and 30-84MS.
(A). Biofilm production by cultures grown in 96-well microtiter polystyrene plates containing AB-C for 48 and 72 hr at 28 °C without shaking. Attached cells were quantified based on crystal violet staining of cells adhering to the polystyrene after washing; staining was quantified at optical density 540. (B) Quantification of extracellular DNA produced by cultures grown in 24-well polystyrene plates containing AB-C for 48 and 72 hr at 28 °C without shaking; extracellular DNA concentration was measured using a Qubit (Invitrogen) fluorometer using a double stranded DNA fluorescent dye (Invitrogen). (C) Quantification of extracellular DNA produced by the phenazine non-producing strain 30-84ZN when grown for 72 hours in cultures containing supernatants derived from each of the phenazine-producing strains or 30-84ZN (negative control). The designation on the X axis indicates the source of the supernatants. Cultures were grown and eDNA measured as described in B. The data represent the average of 6 biological replicates and error bars indicate the standard error. Values with the same letter do not differ significantly as determined by a Fishers protected Least Significantly Difference (LSD) test (P>0.05).

Given the previous observations that biofilm formation is correlated with eDNA production, the amount of eDNA in cultures of *P. chlororaphis* 30-84 wild-type, 30-84ZN, and the altered phenazine-producing strains were measured (Fig. 2.4B). The amount of eDNA produced by 30-84ZN (12.63 ± 2.7 μg/ml) after 72 h typically was significantly lower than the amount produced by any of the phenazine-producing strains, demonstrating an important role of phenazine in eDNA release. eDNA production generally was correlated with biofilm production being highest for 30-84 wild-type (41.07 ± 3.6 μg/ml) and 30-84H (37.3 ± 7.7 μg/ml) and somewhat less for 30-84S (28.82 ± 4.3 μg/ml), 30-84PCA (26.96 ± 4.0 μg/ml), and 30-84MS (23.1 ± 1.2 μg/ml). The results suggest that the production of different phenazines affects the ability to promote eDNA release in a manner that is generally correlated with biofilm production.

To further verify the role of different phenazines in eDNA production, 30-84ZN was inoculated into media containing filter-sterilized supernatants from 30-84 wild-type, 30-84ZN, and the altered phenazine-producing strains. These supernatants were pre-treated with DNaseI, heated to in-activate the enzyme and then added as a 1:10 dilution (v:v) to fresh AB-C medium. For the negative control, supernatant of 30-84ZN was used after similar treatment. The amount of eDNA released was measured after 72 hr (Fig. 2.4C). Addition of 30-84ZN supernatant to the medium resulted in (17.13 ± 1.03 μg/ml) of eDNA which was similar to the amount produced by 30-84ZN in the absence of supernatant (Fig. 2.4B). Addition of supernatants from all of the phenazine-containing supernatants significantly increased the amount eDNA produced by 30-84ZN in a manner generally correlated with the amount of biofilm produced by the strains

comprising each supernatant. The amounts of eDNA produced by 30-84ZN cultures containing supernatants of 30-84 wild-type (32.2 ± 4.4 μg/ml), 30-84H (34.03 ± 4.1 μg/ml) or 30-84S (33.73 ± 1.5 μg/ml) were significantly higher than when 30-84ZN was grown in supernatants of 30-84PCA (24.1 ± 1.6 μg/ml), 30-84M (24.55 ± 1.48 μg/ml) or 30-84MS (22.78 ± 1.5 μg/ml). These results support the observation that different phenazines consistently promote the production of different amounts of eDNA.

A role for eDNA in the establishment of bacterial biofilms has been recognized for nearly a decade, however the source of the DNA remains controversial. One hypothesis is that eDNA in the biofilm matrix is a derived from chromosomal DNA resulting from cell lysis, which may be mediated by the generation of ROS (Wei & Ma, 2013, Okshevsky & Meyer, 2015, Wang *et al.*, 2016). Recently, it was demonstrated that release of eDNA by *P. chlororaphis* 30-84 and 30-84O* (overproducing 2OHPCA) was greater than by 30-84PCA or 30-84ZN (Wang *et al.*, 2016). Moreover, whole-transcriptomic analysis of each strain showed that the expression of genes encoding ROS detoxifying enzymes and DNA repair/modification enzymes were significantly higher for phenazine producing strains (especially 2OHPCA producers) than in the phenazine deficient strain. In that study, it was postulated that eDNA release may be related in part to the activity of a chromosomally encoded bacterial pyocin, which is increased with ROS generation (Wang *et al.*, 2016). Similarly, Das and Manefield (2012) showed that phenazine production (e.g. PYO) by *P. aeruginosa* is responsible for promotion of eDNA release via generation of H_2O_2*;* whereas PYO-deficient strains demonstrate lower amounts of cell lysis and eDNA release. They proposed that the phenazines serving as

electron shuttles can accept electrons from intracellular reductants (i.e. NAD(P)H) and donate to extracellular oxidants (i.e. molecular oxygen). During this process, the reduced molecular oxygen can become a ROS, which causes cell lysis by causing damage to the bacterial cell membrane, followed by release of chromosomal DNA into the extracellular space to form eDNA. Since different phenazines have different redox potentials (Price-Whelan *et al.*, 2006, Wang & Newman, 2008, Bellin *et al.*, 2014), it is logical to speculate that the efficiency of cell lysis and subsequent eDNA release might be dependent on what kinds of phenazines they produce and their effect on ROS generation.

Colonization of the wheat rhizosphere

To determine whether specific phenazines contribute the ecological persistence of *P. chlororaphis* 30-84 in the wheat rhizosphere, the population density of *P. chlororaphis* wild-type, 30-84ZN, and the altered phenazine-producing strains on wheat roots were compared after 30 days. Each of strains was inoculated separately onto the roots of pregeminated wheat seedlings and planted in natural wheat field soil, e.g. containing the native soil microbiota. The plants were harvested after 30 days and total bacterial populations were determined by serial dilution plating on LB with appropriate antibiotics. As expected, the bacterial population of the phenazine non-producing 30-84ZN were significantly lower than those of all phenazine producers (7.3 log units/g of root compared to ~ 8 log units/g of root, respectively) (Fig. 2.5). These findings are in agreement with previous findings in which the population of a phenazine deficient mutant declined more rapidly than wild-type over the repeating plant-harvest cycles (Mazzola *et al.*, 1992). The final populations of the different phenazine producers on

wheat roots were not significantly different (Fig. 2.5). These results demonstrate that phenazine production is important for persistence in the rhizosphere, however the type of phenazines produced by the isogenic strains of 30-84 did not significantly impact rhizosphere population size under these conditions.

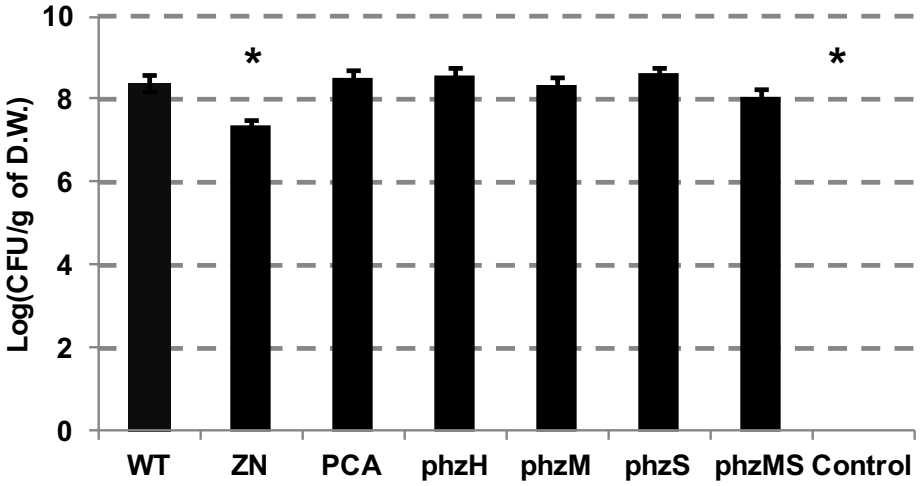


Figure 2. 5 Effect of altered phenazine production on root colonization.
Pregerminated wheat seeds were treated with a bacterial suspension of 30-84 wild-type, 30-84ZN, altered phenazine-producing strains 30-84PCA, 30-84H, 30-84M, 30-84S and 30-84MS and control without bacterial inoculation, and grown in natural soil mixed with sand (2:1, v:v). Thirty days after inoculation, plants were harvested and bacterial populations quantified by serial dilution on LB amended with Rif and Gm. Populations are expressed as the log10 value of the colony forming units (CFU) per gram of root dry weight. Data represent the means of six separate plants, and error bars indicate standard errors. The assay was repeated once, and asterisks indicate statistically significant differences as determined by Fishers protected Least Significantly Difference (LSD) tests ($p<0.05$).

Conclusion

In summary, my findings indicate that production of phenazines by *P. chlororaphis* 30-84 is important for pathogen inhibition, eDNA production, and biofilm formation. In addition, phenazine production is beneficial for root colonization, but the kind of phenazine bacteria produced is not as important. Despite evidence of differences

in the specificity of pathogen inhibition activity by different phenazines under *in vitro* condition, this is not always correlated with disease suppression *in situ*. Future research will test the ability of the altered phenazine producers to suppress disease against specific plant pathogens, which is the most important measure of effectiveness for biological control agents. This research will provide a better understanding of the roles specific phenazine derivatives play as well as potential applications for improving biological control via natural and introduced populations of phenazine producers.

Materials and Methods

Bacteria strains and growth condition

All bacterial strains and plasmids used in this study are described in Table 2.1, and oligonucleotides and primers are listed in Table 2.2. *Escherichia coli* strains were grown at 37 °C in Luria-Bertani (LB) medium. *Pseudomonas chlororaphis* strain 30-84 and its derivatives were grown at 28 °C in either LB medium containing 5 g of NaCl per liter, pigment production medium (PPMD) and AB minimal medium supplemented with 2% casamino acids (Difco, Franklin Lakes, NJ) (AB-C). Antibiotics were used where appropriate at the following concentration for *E. coli*: ampicillin (Ap) at 100 μg/ml, gentamycin (Gm) 15 μg/ml, Kanamycin sulfate (Km) at 50 μg/ml and tetracycline (Tc) at 25 μg/ml; for *P. chlororaphis*: Gm at 50 μg/ml, rifampicin (Rif) at 100 μg/ml, Km at 50 μg/ml, and Tc at 50 μg/ml.

DNA manipulations and sequence analysis

Standard methods were used for plasmid DNA isolation, transformation, cloning, restriction enzyme digestions, agarose gel electrophoresis, and T4 DNA ligation (Sambrook & Russell, 2001). Polymerase chain reaction (PCR) was carried out in a 50 µl reaction mixture containing 10X Standard *Taq* reaction buffer (New England BioLabs, Ipswich, MA), 10mM dNTPs, 10 µM of each primer, 1.2 U of *Taq* DNA polymerase (New England BioLabs), and 20 ng of DNA. The PCR cycle included a first denaturation for 5 minutes at 95 °C followed by 30 cycles of 30s at 95 °C, 30s at 55 °C (or recommend Tm for the primers) and 90s at 68 °C, and a final elongation step of 10 minutes at 68 °C. DNA sequencing was performed at the Laboratory for Genome Technology within Institute for Plant Genomics and Biotechnology, Texas A&M University using an ABI 3130xl Genetic Analyzer. Nucleotide and amino acid homology searches were conducted using the BLAST programs at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST).

Construction of the altered phenazine expression vectors

Altered phenazine-producing strains were created by individually introducing different terminal modifying genes *in trans* into 30-84PCA, which contains a disruption of *phzO* (*phzO*::Tn5) and is capable of producing only PCA (Table 3.1). The very stable, broad host range plasmid pPROBE-GT' (pGT2) was used as the vector (Miller *et al.*, 2000). This plasmid contains the pVS1 and p15a replicons (the former supports plasmids stability without antibiotic selection). It also contains a multiple cloning site upstream of a promoter-less *gfp* gene (encoding the green fluorescent protein with its own ribosome binding site (RBS) (Miller *et al.*, 2000). The different expression vectors were then

constructed by introducing each terminal modifying enzyme (with native ribosome binding site) driven by the phenazine biosynthetic promoter upstream of the *gfp*, resulting in the expression of the terminal modifying enzyme and *gfp* under the control of the phenazine biosynthetic promoter. A truncated version of the phenazine promoter, P_{PsP}, was used to obtain high expression of the introduced genes (Table 2.1). Previous work (Chapter III) indicated that P_{PsP} remains under quorum sensing control but results in higher expression of reporter genes (e.g. ~5-fold higher expression compared to the native *phz* promoter). The P_{PsP} promoter lacks a 185 bp negative regulatory region of the promoter (Chapter III).

To facilitate cloning, expression vectors with gentamicin resistance (suitable for cloning into the kanamycin resistant 30-84PCA) were constructed in stages using the unique *Eco*RI, *Bam*HI, and *Hin*dIII restriction enzyme sites in the multiple cloning locus upstream of *gfp*. First P_{PsP} was cloned from plasmid pKT2-PsP*lacZ* (where it had been studied previously, Chapter III) into the *Eco*RI and *Bam*HI sites. The resulting plasmid pGT2-pPsP was transformed into *E.coli* strain DH5α and transformants were screened using gentamycin resistance and confirmed by *gfp* expression. Plasmid pGT2-pPsP was used as the negative control (empty vector) in all experiments and subsequently used for the insertion of either *phzH, phzM, phzS* and *phzM* + *phzS* between the *Bam*HI and *Hind*III sites.

The coding (and flanking) sequences for *phzH*, *phzM,* and *phzS* from *P. aeruginosa* PAO1 were used to design primers to amplify each of the gene inserts (flanking sequences contained RBS sequences). Primer pairs phzH1-phzH2, phzM1-

phzM2, and phzS1-phzS2 were used to amplify the gene inserts of *phzH, phzM,* and *phzS* genes, respectively (Table 2.2). Following PCR, amplified genes were cloned separately into the plasmid pCR2.1-TOPO vector (Invitrogen Life Technologies, Carlsbad, CA) by TA cloning. TOPO-*phzH,* TOPO-*phzS,* TOPO-*phzM* were then digested with *Bam*HI-*Hin*dIII and the inserts cloned into the *Bam*HI-*Hin*dIII site in pGT2-pPsP. In order to create a plasmid containing *phzM* and *phzS* together, primer pair phzM1-phzM3 was used to create a *phzM* amplicon with *BamH*I restriction enzyme sites at both ends of the PCR product. This amplicon was cloned into the *BamH*I site in pGT2-*phzS* resulting in pGT2-*phzMS.* The direction of the inserted gene was confirmed via enzyme digestion and PCR amplification and sequencing. To confirm gene expression, all strains were checked for *gfp* expression. The empty vector pGT2pPsP also was introduced into 30-84 wild-type, 30-84ZN, and 30-84PCA as the empty vector control.

Triparental mating

Triparental matings were performed with *E. coli* strain (DH5α) as the donor and HB101 (pRK2013) as the helper. The donor, helper and recipient strains (*P. chlororaphis* 30-84) were grown overnight in 3ml LB broth with appropriate antibiotics. Equal amount of each donor, helper, and recipient were spotted onto sterile nitrocellulose filter membrane on a LB plate with no antibiotic selection, and incubated at 28 °C for 48 h. The filters were resuspended in 3 ml sterile water, and 100 µl of each were spread on plates with LB supplemented with gentamycin (to select for recipient cells harboring the plasmid) and rifampicin (to select against *E. coli*). And plates were

incubated at 28 °C. Successful conjugants were selected by antibiotic resistance (Gn^+, Rif^+), *gfp* expression, and the production of different colored pigments indicative of each phenazine.

RNA extraction and semi-quantitative PCR analysis

P. chlororaphis 30-84 and its isogenic derivatives were grown separately overnight at 28 °C at 3ml AB-C until OD_{620} of 1.2. Total RNA was extracted using the RNEasy bacterial RNA Protect mini kit (Qiagen Inc., Valencia, CA) following manufacturer's recommended protocol. Contaminating genomic DNA was removed using DNase-I on-column digestion (Qiagen). Purified RNA was quantified using GE Nanovue spectrophotometer (GE Healthcare, Pittsburgh, PA). Total 5µg of RNA was used for cDNA synthesis by reverse transcription using SuperScript™ III reverse transcriptase (Invitrogen) with random hexamers at 50 °C for 1 h and inactivated the reaction by 70°C for 15 min. For negative control, sterilized water used instead of reverse transcriptase enzyme.

To confirm the transcription, semi-quantitiative PCR (semi-qPCR) was performed using primer sets phzHRT1-phzHRT2, phzMRT1-phzMRT2, phzSRT1-phzSRT2 and rpoDRT1-rpoDRT2 for detecting the expression of *phzH, phzM, phzS* and *RpoD* (Table 2.2). Semi-qPCR is a slightly different than standard quantitative PCR in that target copy number is estimated relative to another transcript rather than a standard curve. The *rpoD* gene was used as the reference gene, and sterilized water was used as the negative control. The 2 ng of cDNA were used as template for semi-qPCR with the *Taq* DNA Polymerase (New England BioLabs). The semi-qPCR amplifications were

carried out at 95 °C for 5 minutes followed by 25 cycles of 95 °C for 30s, 55 °C for 30s and 68 °C for 60s and a final elongation step of 68 °C for 10 minutes. Amplified DNA fragments were separated on 1.4 % (wt/vol) agarose gels. Gel pictures were taken under UV light after ethidium bromide staining for 30 min.

Phenazine extraction

Phenazines were extracted from 30-84 wild-type, 30-84ZN and the altered phenazine-producing strains using HyperSep C18 SPE column (Thermo Scientific). Briefly, cultures were grown in 10 ml PPMD with appropriate antibiotics for two days. Cell-free supernatant was prepared by centrifugation (2,600 X *g*) for 15 min and filter sterilized using a 0.22 μm filter (VWR, Radnor, PA). The SPE column was washed with acetonitrile and equilibrated with sterilized water three times before supernatants were added. Phenazines were retained on the columns, which were washed three times with sterilized water. Phenazines were eluted from the columns with 1 ml of acetonitrile.

HPLC- mass spectrometry Analyses of phenazine compounds

LC and mass analysis was performed on a Surveyor HPLC system (Thermo Finnigan, San Jose, CA) interfaced with quadruple ion trap mass spectrometer (LCQ-DECA, ThermoFinnigan) in the Laboratory for Biological Mass Spectormetry at Texas A&M University. The chromatographic separation was performed on Aquasil C18 column (Thermo Hypersil-Keyston, Bellefonte, PA) (2.1 × 150 mm, 3 μm) with solvent A (water) and B (acetonitrile) both containing 1 mM acetic acid as mobile phase. The flow rate was set at 200 μL/min. Elution gradient was as follows: from 0 - 5 min solvent B was held at 5%, from 5-10 increased to 50% B; from 10-24 increased to 70% B, from

24-25 to 100% B; during 25-30% 100% B is maintained, from 30-31 solvent B was reduced to 5% and maintained for 4 min. Electrospray ionization (ESI) in positive ion mode was carried out by applying 4.5 kV at the spray needle to create positive ions. Sheath gas and auxiliary gas flow rates were 50 and 10 arbitrary units, respectively. Transfer capillary temperature was held at 250 °C.

Fungal inhibition assay

To determine the ability of each altered phenazine-producing strains to inhibit a variety of fungal pathogen, *in vitro* dual culture assays were conducted. Plant pathogenic fungal strains were obtained from the Texas A&M University Department of Plant Pathology and Microbiology Teaching Laboratory stock collection. Fungal strains were initially cultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI). After 5 - 7 days inoculation, a 3-mm-diameter plug was taken from the edge of the mycelial mat and transferred to the center of Waksman agar (WKA) (Berg *et al.*, 2001). WKA was prepared by adding 5 g protease-peptone, 10 g glucose, 3 g beef extract, 5 g NaCl, and 20 g agar to 1 L distilled water, and adjusting its pH to 6.8. The fungal strains were inoculated 2 days prior to the inoculation of bacteria except for the rapidly growing strain *Pythium ultimum*. Bacterial inoculum was prepared from overnight culture grown in LB medium at 28 °C with rapid agitation, and spotted at the edge of the plates (3.5 cm apart from the center). To determine antifungal activity to *P. ultimum,* bacteria were spotted 2 days before fungal inoculation. After 5-7 days, zones of inhibition were measured as the distance between edge of the bacterial colony and the fungal mycelium.

Microtiter biofilm formation assay

The effect of phenazine derivatives on biofilm formation was investigated using the crystal violet assay as previous described (O'Toole & Kolter, 1998, Maddula *et al.*, 2006). Strains of *P. chlororaphis* 30-84, 30-84ZN and the altered phenazine-producing strains were grown separately overnight in 3ml of AB-C medium at 28 °C with rapid agitation and adjusted to a final OD_{620} of 0.8 with fresh media (AB-C). Each strain was re-inoculated into equal media type with 10^{-2} dilution, and 150 µl was added to each well of a 96-well polystyrene plate. For the negative control, cell-free AB-C was used respectively. Plates were incubated at 28 °C for up to 72 h without shaking. Unattached cells were removed by inversion of plates followed by tapping on the paper towels. Plates were incubated at 50 °C for 20 min to fix the remaining attached bacteria into the wells of plates. Surface-attached cells were stained with 0.1% crystal violet and washed twice with sterilized distilled water, and decolorized with 20% acetone/80% ethanol solution for 5 min. Attached cells were quantified by optical density of 540.

Extracellular DNA (eDNA) quantification

The concentration of extracellular DNA (eDNA) was determined quantitatively using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) as previously described (Wang *et al.*, 2016). Briefly, cells were grown under the same conditions used for the biofilm assay except using 1.5 ml of culture in 24-well polystyrene plates. At specific time intervals, the entire 1.5 ml static cultures were transferred to 50 ml centrifuge tubes containing 15 ml sterilized distilled water and vortexed vigorously to thoroughly disrupt the biofilm matrix. The supernatants were collected by centrifugation (1,250 x g for 10 min), and transferred to new tubes. To remove the remaining bacterial cells, supernatants

were filtered using a 0.22 μm filter (VWR). The concentration of eDNA was quantified by mixing 10 μl of cell-free supernatant with DNA quantifying fluorescent dyes from Qubit (Invitrogen). The fluorescence of DNA-dye interaction was measure using Qubit 2.0 Fluorometer according to the manufacturer's protocol (Invitrogen). The amount of eDNA was determined via comparison to a standard curve and reported as μg/ml.

Exogenous phenazine treatment

To verify that each phenazine is responsible for eDNA production, 30-84ZN was grown in media inoculated with supernatants containing phenazines produced by either 30-84 wild-type, 30-84ZN and altered phenazine-producing strains; supernatants of 30-84ZN were used as the control. The supernatants were produced by inoculating single colonies of each strain into 20 ml PPMD media and growing cultures at 28 °C with rapid shaking until an OD_{620} of 2.4 was reached. After centrifugation for 20 min at 1230 x g, supernatants were transferred to 50 ml centrifuge tubes. Subsequently, supernatants were filter sterilized using 0.22 μm filter (VWR) and 30 units of water-dissolved DNase-I (Qiagen) were added. After 1 h incubation at room temperature, DNase-I treated supernatants were incubated at 65 °C in a water-bath for 10 min to deactivate the DNase-I. For the assay, 30-84ZN was prepared from overnight culture grown in AB-C at 28 °C with rapid shaking, and adjusted to a final OD_{620} of 0.8. The cell-free, DNase-I treated supernatants were diluted 1:10 in fresh AB-C and 30-84ZN was inoculated (at a 10^{-2} dilution) into the supernatant-containing media. Production of eDNA was measured as described above after 72 h growth.

Root colonization assays

Wheat seeds (cultivar TAM112) were surface sterilized with 0.6 % NaClO and germinated on moistened, sterile germination paper for 3 days. Bacterial inoculum was prepared in 20 ml KMB broth, and inoculum was normalized to OD_{620} of 0.8 with phosphate buffered saline (PBS). The roots of the newly germinated seedlings were soaked in the bacterial cell suspension for 10 min, briefly air dried, and planted in 25 x 200 mm Cone-tainers. Planting media was derived from soil (clay loam) obtained from a field continously cultivated in wheat (Uvalde, TX). Prior to plant wheat seedlings, soil were passed through 0.5-cm-mesh screen to remove plant debris and mixed with sand (2:1, v:v). The Cone-tainers were arranged in a completely randomized design, and plants were watered every other day with 5 ml of sterilized distilled water. After 30 days, plants were harvested, and entire root systems were rigorously washed to remove non-adhering bacteria. Roots were blotted dry on filter paper, weighed and immersed into 1 ml PBS. Bacteria were removed from whole roots by vortexing and sonication 3 times each for 10 seconds. Total populations were determined by serial dilution on LB agar amended with rifampicin.

Statistical Analysis

All data presented in this study are mean ± SEM from multiple determinations. Differences between strains were analyzed statistically using ANOVA and Fisher's protected Least Significant Difference (LSD) test ($P<0.05$).

CHAPTER III

ANALYSIS OF THE PHENAZINE PROMOTER REGION OF THE BIOLOGICAL CONTROL STRAIN *PSEUDOMONAS CHLORORAPHIS* 30-84 AND CHARACTERIZATION OF A NEGATIVE REGULATORY ELEMENT

Summary

Phenazines are broad host range antibiotics produced by several bacterial species, including *Pseudomonas* and *Streptomyces*. These compounds play a key role in the antagonistic activity of the biocontrol strain *P. chlororaphis* 30-84 towards *Gaeumannomyces graminis* var. *tritici*, the causative agent of wheat take all disease. Strain 30-84 contains a seven-gene operon (*phzXYFABCD*) and phenazine modifying enzyme (*phzO*) responsible for the biosynthesis of three phenazines. Expression of the phenazine operon is dependent on the PhzR/PhzI quorum sensing located immediatly upstream. The gene *phzI* encodes an AHL synthase responsible for the production of the signal molecule *N*-Acyl-homoserine lactone (AHL). PhzR is a transcriptional factor, and once AHL signal binds to PhzR, the PhzR-AHL complex binds to the phenazine operon promoter resulting in phenazine production. To delineate the phenazine promoter, the region between *phzR* and *phzX* was characterized. DNA sequence analysis identified several interesting features within *phz* promoter and downstream of transcriptional start site (TSS) of *phzX,* which is the first gene of phenazine biosynthetic operon. This includes sequence features that are potentially capable of providing significant secondary structures such as forming stem loops and thereby negatively controlling gene expression. These features were characterized by creating phenazine promoter subclones

with deletions of various sequences or with site-specific alteration. Deletion of a 185 bp region downstream of the TSS, including the start of translation resulted in 5-fold higher *lacZ* expression compared to the wild type promoter. A mutant (30-84 Enh) was generated that containing a 90 bp deletion of the bacterial chromosome designed to disrupt the potential stemloop structures contained within the 150 bp region. This deletion resulted in significantly higher phenazine production, and expression remained under quorum sensing control. Quantitative real-time PCR data indicated that 30-84Enh exhibited 1.7-fold higher expression of *phzR* and 1.3-fold higher expression of *phzI* compared to 30-84 wild-type. These data suggest that the stemloop region negatively regulates phenazine production at least partially through expression of *phzR* and *phzI.* Enhanced phenazine production also increased the capacity for fungal inhibition, biofilm formation and plant growth promotion. This work provides greater insight into the characterization of the quorum sensing controlled promoter as well as potential applications for improved biological control by this phenazine-producing strain.

Introduction

Pseudomonas chlororaphis 30-84 is a rhizosphere-colonizing bacterial species capable of producing an array of secondary metabolites with beneficial agronomic applications. *P. chlororaphis* 30-84 was isolated as a biological control for take-all disease of wheat, caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Ggt). Phenazines are the principal antifungal secondary metabolite produced by *P. chlororaphis* 30-84 and several other well-studied biological control agents (Thomashow

et al., 1990, Pierson & Thomashow, 1992, Thomashow *et al.*, 1993, Pierson *et al.*, 1995, Chin-A-Woeng *et al.*, 1998, Powell *et al.*, 2000, Tambong & Höfte, 2001, Hu *et al.*, 2005). Phenazines are brightly-colored, heterocyclic, nitrogen-containing molecules that are highly redox active and involved in a diverse array of biological functions (Mavrodi *et al.*, 2006, Pierson & Pierson, 2010). *P. chlororaphis* 30-84 produces three phenazine derivatives, phenazine-1-carboxylic acid (PCA), 2-hydroxy-phenazine-1-carboxylic acid (2OHPCA) and a small amount of 2-hydroxy-phenazine (2OHPZ). PCA is produced via expression of the phenazine biosynthetic operon *phzXYFABCD* and the phenazine modifying enzyme *phzO,* encoding an aromatic monooxygenase*,* is responsible for its conversion to the 2-hydroxy derivatives (Pierson & Thomashow, 1992, Delaney *et al.*, 2001, Mavrodi *et al.*, 2006).

The ecological benefits of phenazine production have been well documented (reviewed in Chin-A-Woeng *et al.*, 2003, Mavrodi *et al.*, 2006, Pierson & Pierson, 2010, Mavrodi *et al.*, 2013). These include their antibiotic characteristics, which facilitate survival in competition with other microorganisms (Mazzola *et al.*, 1992, Beifuss & Tietze, 2005, Mavrodi *et al.*, 2006, Dwivedi *et al.*, 2009, Pierson & Pierson, 2010). Phenazine production by *P. chlororaphis* 30-84 is primarily responsible for the inhibition of Ggt *in vitro* and *in situ* on roots as well as for its persistence on roots in competition with other rhizosphere microorganisms (Mazzola *et al.*, 1992, Pierson & Thomashow, 1992). Phenazines also contribute to biofilm formation (Maddula *et al.*, 2006, Dietrich *et al.*, 2008, Maddula *et al.*, 2008, Ramos *et al.*, 2010, Das *et al.*, 2013a, Wang *et al.*, 2016). In *P. chlororaphis* 30-84 this was demonstrated using a phenazine

biosynthetic mutant of *P. chlororaphis* 30-84, 30-84ZN (*phzB*::*lacZ*), which was found to be defective in cell attachment and biofilm development (Maddula *et al.*, 2006). However, rescue of 30-84ZN via the constitutive expression of the phenazine operon resulted in earlier and thicker biofilm production. Subsequently using isogenic derivatives of *P. chlororaphis* 30-84 producing only PCA or overproducing 2OHPCA, the roles of different phenazines in specific aspects of biofilm formation and architecture were demonstrated (Maddula *et al.*, 2008). More recently, differences in the ecological roles and transcriptional influence of each phenazine derivatives produced by *P. chlororaphis* 30-84 were demonstrated (Wang *et al.*, 2016). For example production of 2OHPCA more readily promotes the release of extracellular DNA, which becomes a key component of biofilm, and resulting in a greater structured biofilm matrix. Moreover, RNA-seq analyses revealed that phenazine production has broad impacts on gene expression patterns including the regulation of genes involved in biosynthesis of exoenzymes, secondary metabolites and other genes important for survival. Similar results have been shown for other phenazine producing species (Dietrich *et al.*, 2006). The results suggest that phenazines not only play roles in the ecological fitness of the producing bacteria, but also serve as signals that influence gene expression patterns.

Given the multiplicity of roles served by phenazines in all phenazine-producing strains studied to date, it is not surprising that phenazines are regulated at multiple levels. In most phenazine producing bacteria, including the plant growth promoting rhizobacteria *P. fluorescens* 2-79, *P. chlororaphis* PCL1391 and *P. chlororaphis* 30-84, phenazine biosynthesis is controlled directly by the PhzR/PhzI quorum sensing (QS)

system (Pierson *et al.*, 1994, Wood & Pierson, 1996, Chin-A-Woeng *et al.*, 2001a, Khan *et al.*, 2005, Khan *et al.*, 2007). Typically, *phzR* and *phzI* are located immediately upstream of the phenazine biosynthetic operon. The gene *phzI* encodes an *N*-Acyl homoserine lactone (AHL) synthase and *phzR* encodes a transcriptional regulator of the *phz* biosynthetic operon (Pierson *et al.*, 1994, Wood & Pierson, 1996, Khan *et al.*, 2007). Once AHL signals reach a threshold level, they activate PhzR by forming PhzR-AHL complex. This complex then binds to the specific sequence motif known as a "*phz* box" within the phenazine biosynthetic promoter resulting in the activation of the expression of the phenazine biosynthetic genes. The activated PhzR-AHL complex also binds to a *phz* box in the promoter region of *phzI* to induce the expression of *phzI* resulting in enhanced AHL signal production and accelerating the expression of phenazine biosynthetic genes. Previously, Khan *et al*. (2007) identified multiple products of the AHL synthases produced by *P. chlororaphis* strains 30-84 and PCL1391, and *P. fluorescens* 2-79, and suggested that *N*-(3-Hydroxylhexanol)-L-homoserine lactone is the major signal to activate PhzR, which in turns regulates the expression of phenazine operon. In addition to quorum sensing regulation, other genes have been shown to be involved in the regulation of phenazine biosynthesis in *P. chlororaphis* 30-84 including the Gac/Rsm network, sigma factor RpoS, the two component system RpeB/RpeA, and the transcription regulator Pip (Chancey *et al.*, 1999, Zhang & Pierson, 2001, Whistler & Pierson, 2003, Wang *et al.*, 2012a, Wang *et al.*, 2013).

Predictably, much attention related to the regulation of phenazine biosynthesis has focused on the promoter region of the phenazine biosynthetic genes, including the

regions that interact with quorum sensing-mediated regulation (e.g the AHL signals or *phz* box) or other phenazine regulatory systems. In the present study, bioinformatic analysis of the 759-bp intergenic region between *phzR* and *phzX* in *P. chlororaphis* 30-84 revealed the presence of functional elements in the promoter region, some of which are similar and some unique to the 30-84 phenazine biosynthetic promoter. These include the *phz* box (located at ca. -35) and -10 sequence, which are highly conserved to the previously identified features of 18-bp palindromic *phz* box and -10 hexamer in the phenazine operon promoter of *P. fluorescens* 2-79 and *P. chlororaphis* PCL1391 (Chin-A-Woeng *et al.*, 2001b, Khan *et al.*, 2005, Khan *et al.*, 2007). Interestingly, only *P. chlororaphis* 30-84 contains unique sequence repeat motifs located between the *phz* box and the ATG of the first gene of the phenazine operon (*phzX*), and these motifs are predicted to form significant secondary structures. Initially, the objective of this study was to understand the potential role of these features in phenazine regulation. To delineate the phenazine promoter, the region between *phzR* and *phzX* was characterized by creating phenazine promoter subclones with deletions of various sequences or with site-specific alteration. During this process a negative regulatory element was discovered via the deletion of a 150 bp region downstream of the transcriptional start sequence, resulting in significantly enhanced phenazine gene expression. Given that phenazine biosynthesis is exponentially upregulated under quorum sensing control, the finding of a significant negative regulatory element in the promoter was surprising. To further characterize this regulatory element a phenazine-enhanced derivative was created by

deletion of the negative regulatory element in the bacterial chromosome. The potential advantages of enhanced phenazine production for biological control were investigated.

Results

Analysis of the promoter for the phenazine biosynthesis operon

The promoter sequence of the phenazine biosynthetic operon, although similar to other phenazine producing strains, contains sequence repeat features that are unique to *P. chlororaphis* 30-84**.** The DNA sequence of the entire 759 base pair (bp) region between *phzR* and *phzX* (the first gene in the phenazine biosynthetic operon in *P. chlororaphis* 30-84), e.g. spanning the *Eco*RV-*Sal*I restriction enzyme sites is shown in Fig. 3.1. Sequence analysis revealed two palindromic regions, one centered on the *Pst*I site (5'-GCGGGCTGCAGCCAGCCCGC-3') and a second centered on the *Bgl*II site (5'-CACTACAAGATCTGGTAGT-3'). The sequence surrounding the *Bgl*II site is very similar to the consensus *lux* box (*phz* box) found near the -35 promoter region of numerous quorum sensing regulated promoters (Khan *et al.*, 2005). The -10 site is located 25 bp downstream from the -35 site in the promoter region with a good sequence match to consensus sequences. The sequence of -35 and -10 promoter regions of *P. chlororaphis* 30-84 were highly conserved compared to the *phzA* promoter region of *P. fluorescens* strain 2-79 and *P. chlororaphis* PCL1391 (Chin-A-Woeng *et al.*, 2001b, Khan *et al.*, 2005). A transcription start site was previously identified by RNA-seq analysis (Wang *et al.*, 2013) and is located 10 bp downstream from center of the -10 site in the promoter region. Located between base pairs 496 and 617 are two identical repeats

(Fig. 1, 5'-CACCCCCAA-3') that flank a 112 bp sequence that has 38% GC content (as compared to the 51% GC content in the rest of the region). This sequence is also somewhat repetitive, (e.g. containing two *Ssp*I sites). A similar region with low GC level content (40% or 38%) is present in the promoter region of *phzA* in *P. fluorescens* strain 2-79 and *P. chlororaphis* PCL1391, but both strains lack the direct repeat. In contrast, neither the direct repeat nor a similar region of low GC level content is present in the *phzI* promoter sequences of 30-84, 2-79 or PCL1391 (Fig 3.2). This is of interest since the promoters of *phzI* and the phenazine biosynthetic operon are regulated by quorum sensing, suggesting a feature that is unique to the regulation of the phenazine biosynthetic operon in 30-84.

In order to the identify the portion of the 759 bp region responsible for promoter activity and examine the possible roles of sequence motifs within the phenazine promoter, sequence fragments of the promoter region were cloned into the promoter trap vector pKT2-*lacZ* resulting in the plasmids: pKT2VS, pKT2PS, pKT2F1R1, pKT2F1R2, pKT2PsP and pKT2BsP (Table 3.1 and Fig 3.3). Each test plasmid as well as the empty plasmid (pKT2*lacZ,* no insert) was conjugated separately into strain 30-84Ice (*phzB::inaZ*) so as to minimize the interference of phenazine on the β-galactosidase assay. The β-galactosidase activity was determined after 24 h growth in either LB or M9 minimal medium (Fig. 3.3). As expected, the negative control plasmid having no promoter (pKT2lacZ) showed no activity. The promoter derivatives pKT2VS (containing all of the 759 bp *Eco*RV-*Sal*I sequence) and pKT2PS (containing only the *Pst*I to *Sal*I sequence, e.g. includes a disruption of the first palindromic sequence)

yielded 295 units of activity after growth in LB (45 and 32 units in minimal medium, respectively), indicating that neither the region upstream of the *Pst*I site nor the first palindromic sequence is required for promoter activity under standard growth conditions.

Figure 3. 1 DNA sequence of the phenazine promoter region between *phzR* and *phzX* in *P. chlororaphis* strain 30-84. The -35 and -10 sites of the *phzX* promoter and the ribosome-binding (RBS) site of *phzX* are labeled and indicated by orange underlining, whereas the -35 and -10 sites and the RBS of *phzR* are labeled and indicated by purple underlining. The transcription start sites, shown as an arrow labeled +1, for *phzX* (orange arrow) and *phzR* (purple arrow) were determined previously (*phzX,* (Wang *et al.*, 2013) or by comparison to *P. fluorescens* 2-79 (*phzR*, Khan et al., 2005). Orange-colored letters indicates sequence of *phzX*; purple indicates sequence belonging to *phzR.* All the restriction enzyme sites that were used to create *phz* promoter derivatives pKT2VS, pKT2 PS, pKT2PsP, and pKT2BsP are labeled with underlining. The red arrows indicate PCR primer sequences used to create *phz* promoter derivatives pKT2F1R1 and pKT2F1R2. This sequence contains multiple motifs. The green arrows/lettering indicate two sequences with dyad symmetry and the blue arrows indicate two direct repeat sequences flanking an A/T-rich region (38% G/C level) containing two *SspI* sites. The -10 and -35 promoter region and ribosome binding site of *phzR* were determined based on previous study by Khan *et al*. (1995).

In contrast, deletion of 128 bp between the PphzR1 primer site (Table 3.2) and *Sal*I (pKT2F1R1 and pKT2F1R2) resulted in 4.7 and 4.1 fold increases in *β*-galactosidase activity in LB and 1.4 and 2.4 fold increase in minimal medium, respectively. These data suggested that the 128 bp sequence between the PphzR1 primer site and *Sal*I (containing only a portion of the ribosome binding site and part of the *phzX* sequence) contains a negative regulatory region that suppresses phenazine promoter activity. The plasmid pKT2PsP containing the sequence between the *Pst*I and first *Ssp*I site (includes the putative *phz* box, has a 185 deletion including the negative regulatory region) produced the highest β-galactosidase activity in LB (1545 units) and minimal medium (254 units) respectively (Fig 3.3C). The *Bgl*II site is situated in the center of a putative *phz* box that is similar to the consensus *lux* box sequence (Fig. 3.1 and 3.2). The plasmid pKT2BsP that deleted half of the *Bgl*II site within the putative *phz* box resulted in no β-galactosidase activity, consistent with the involvement of this *phz* box sequence in promoter function, probably as the PhzR binding site. These findings suggested that 127 bp sequence between *Pst*I and the first *Ssp*I is sufficient for phenazine gene expression, and that the 185 bp sequence between the first *Ssp*I and *Sal*I (containing the second direct repeat, the ribosome binding site, and the start of *phzX*) contains a negative element as shown by the increased expression with its deletion.

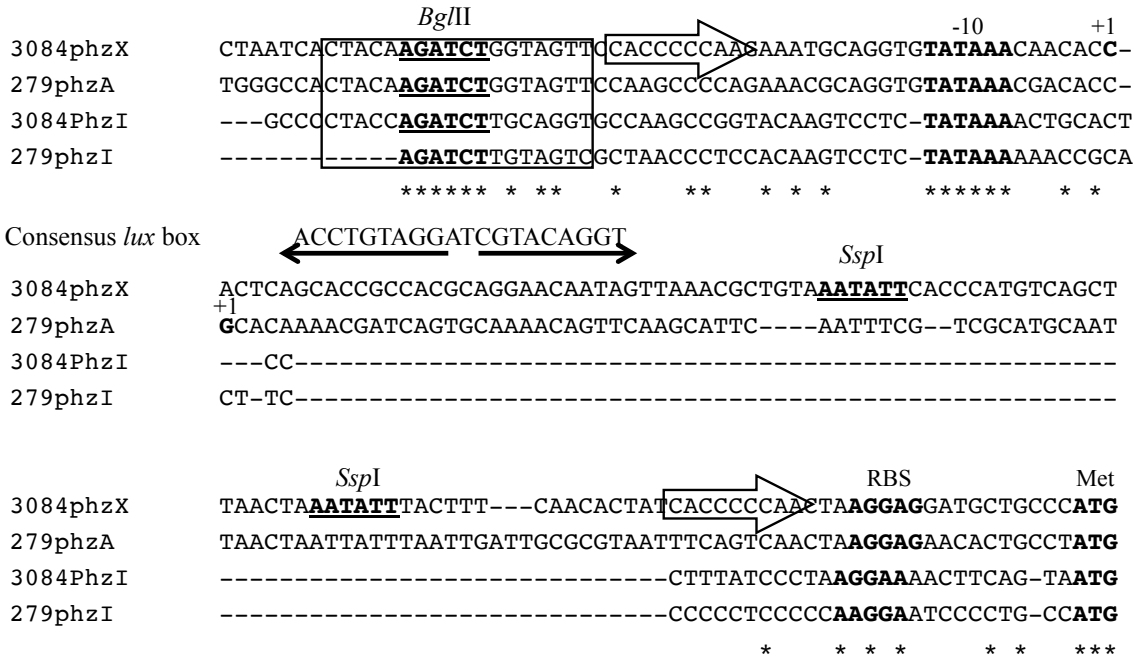


Figure 3. 2. Comparison of the nucleotide sequences of the phenazine biosynthetic promoter regions and the *phzI* promoter regions of *P. chlororaphis* strain 30-84 and *P. fluorescens* strain 2-79. The putative boxed region indicates the *phz* box sequences for the four promoters, and asterisks (*) indicate matches between 3 of the 4 sequences. Restriction enzyme sites are underlined. The bold sequences indicate the putative -10 consensus sequences, ribosome binding site sequences (RBS), and the ATG start. The hollow arrows indicate the direct repeat sequences (CACCCCCAA).

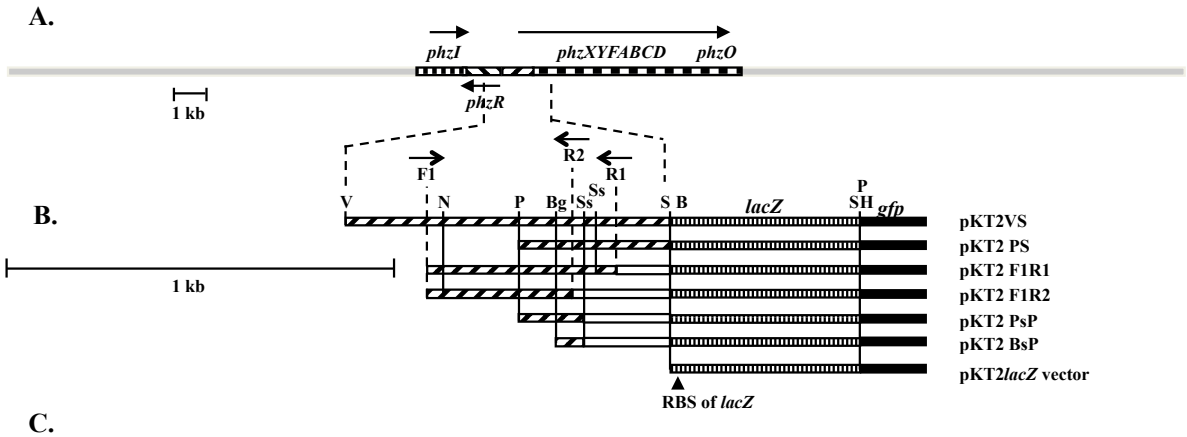
Table 3. 1 Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics	Reference or source
P. chlororaphis		
30-84 WT	Phz^+ Rif^R wild-type	Pierson and Thomashow (1995)
30-84 ZN	Phz^- Rif^R *phzB*::*lacZ* genomic fusion	Wood *et al*., (1997)
30-84 Ice	Phz^- Rif^R *phzB*::*inaZ* genomic fusion	Wood *et al*., (1997)
30-84 PCA	PCA^+ Rif^R *phzO*::Tn5	Maddula *et al.,* (2008)
30-84 WT-Enh	Phz^+ Rif^R Δ90bp at *phzX* promoter	This study
30-84 ZN-Enh	Phz^- Rif^R *phzB*::*lacZ* genomic fusion and Δ90bp at pPhzX	This study
30-84 Ice-Enh	Phz^- Rif^R *phzB*::*inaZ* genomic fusion and Δ90bp at pPhzX	This study
30-84 I/Z	Phz^- Rif^R *phzB*::*lacZ phzI*::KmR	Whistler and Pierson (2003)
E. coli		
DH5α	F^- *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*argF-lacZYA*) *I169* Φ80*lacZ* ΔM15λ^-	GIBCO-BRL
HB101	F^- *hsdS20*(r_B^- m_B^-) *supE44 recA1 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-5*λ^-	GIBCO-BRL
Plasmid		
pKT2*lacZ*	Km^R *lacZ* carried on promoter trap vector pPROBE-KT'	Wang *et al.,* 2012
pKT2 VS	Transcriptional *lacZ* fusion to promoter subclone VS carried on pKT2	This study
pKT2 PS	Transcriptional *lacZ* fusion to promoter subclone PS carried on pKT2	This study
pKT2 F1R1	Transcriptional *lacZ* fusion to promoter subclone F1R1 carried on pKT2	This study
pKT2 F1R2	Transcriptional *lacZ* fusion to promoter subclone F1R2 carried on pKT2	This study
pKT2 F1R4	Transcriptional *lacZ* fusion to promoter subclone F1R4 carried on pKT2	
pKT2 PsP	Transcriptional *lacZ* fusion to promoter subclone PsP carried on pKT2	This study
pKT2 BsP	Transcriptional *lacZ* fusion to promoter subclone BsP carried on pKT2	This study
pKT2 PS Var1	Site mutagenesis of the first direct repeat of *phzX* promoter region on KT2 PS	This study
pKT2 PS Var2	Site mutagenesis of the second direct repeat of *phzX* promoter region on KT2 PS	This study
pKT2 PS Var3	Site mutagenesis of the both direct repeats of *phzX* promoter region on KT2 PS	This study
pKT2 PS Var4	Site mutagenesis of the both ribosome binding site and ATG of *phzX* promoter region on KT2 PS	This study
pKT2 PS Var5	Site mutagenesis of deletion between *Ssp*I site repeats of *phzX* promoter region on KT2 PS	This study

Table 3. 1 Continued

Strains and plasmids	Relevant characteristics	Reference or source
pKT2pTac*lacZ*	pKT2*lacZ* containing constitutive promoter pTac	This study
pKT2pTac185*lacZ*	pKT2pTac*lacZ* containing 185bp *phz* promoter negative regulatory region	This study
pGT2*lacZ*	Gm^R *lacZ* carried on promoter trap vector pPROBE-GT'	This study
pGT2 WX*lacZ*	Transcriptional lacZ fusion to *phzX* promoter subclone carried on pGT2	This study
pGT2 EX*lacZ*	Transcriptional lacZ fusion to Enhaced-*phzX* promoter subclone carried on pGT2	This study
pGT2 WR*lacZ*	Transcriptional lacZ fusion to *phzR* promoter subclone carried on pGT2	This study
pLAFR3	IncP1 cos^+ rlx^+ Tc^R	Staskawicz *et al.,* 1987
pUC57-Enh	pUC57 containing part of phzR to phzY with 90-bp deletion of negative regulatory region on 1.5-kb BamHI fragment	GenScript
pLAF-Enh	pLAFR3 containing part of *phzR* to *phzY* with 90-bp deletion of negative regulatory region on 1.5-kb *BamH*I fragment	This study
pLSP259	pLAFR3 containing 30-84 genomic DNA, Tc^R	Pierson and Thomashow, 1992
pUCP18-RedS	Ap^R, pBAD *gam bet exo*	Lesic and Rahme, 2008
pUC18	ColE1, Ap^R	yanisch-perron *et al.,* 1983
pUC18-PS*lacZ*	pUC18 containing *phz* promoter fragment PS	This study
pIC20R	pUC-like pladmid with more restriction sites, Ap^r	Marsh *et al.,* 1984
pLSP202-0.8	pIC20R containing 1.1 Kb between *phzR* to *phzX*	This study

[a] Km^R, Ap^R, Gn^R and Rif^R = kanamycin, ampicillin, gentamycin and rifampin resistance, respectively.



C.

	30-84Ice (LB)		30-84Ice (M9)		DH5α (LB)	
Plasmid	MU ± SE	Fold	MU ± SE	Fold	MU ± SE	Fold
pKT2*lacZ*	6 ± 1	NA	28 ± 9	NA	6 ± 1	NA
pKT2 VS	295 ± 38	1	45 ± 24	1	110 ± 16	1
pKT2 PS	295 ± 73	1	32 ± 10	0.7	76 ± 23	0.7
pKT2 F1R1	1,386 ± 322	4.7	63 ±25	1.4	185 ± 83	1.7
pKT2 F1R2	1,207 ± 343	4.1	110 ± 27	2.4	175 ± 24	1.6
pKT2 PsP	1,545 ± 212	5.2	254 ± 88	5.6	527 ± 92	4.8
pKT2 BsP	ND		ND		ND	

Figure 3. 3 Analysis of the *P. chlororaphis* strain 30-84 *phz* promoter derivatives.
A. The region of the *P. chlororaphis* chromosome that contains the phenazine biosynthetic region, including *phzI*, *phzR* and the *phzXYFABCD* and *phzO* genes. B. Expanded view of the phenazine promoter region and subclones fused to *lacZ* in pKT2lacZ. Restriction site abbreviations are as follows: V = *Eco*RV, N = *Nhe*I, P = *Pst*I, Ss = *Ssp*I, S = *Sal*I, B = *Bam*HI, Bg = *Bgl*II, H = *Hin*dIII. F1, R1 and R2 indicate the relative positions of the PCR primers used to amplify different portions of the region and are described in the text. The diagonal lines indicate the promoter regions, the hollow rectangles indicate the regions not present in the subclones, the vertical and solid rectangle represent the *lacZ* and GFP genes, respectively. The remainder of the vector is omitted for clarity. C. The actives of *phz* promoter derivatives in 30-84 Ice and *E. coli* strain DH5α in different media. The β-galactosidase activities (Miller Unit) were determined in triplicate (Mean ± SE): MU = Miller Unit, SE = Standard Error, ND = not determined.

Table 3. 2. Oligonucleotides used for gene cloning and qPCR

Oligonucleotide	Sequence (5'-3')
pPhzF1	CGAATTCCCATAGACGGCTTCGCGCTA
pPhzR1	CGGGATCCCTTAGTTGGGGGTGATAGTGTTG
pPhzR2	CGGGATCCCTGAGTGGGTGTTGTTTATACACCT
phzXTXF	CGCGAATTCGAACCCGCCTCTTCACAATA
phzXTXR	CGCGGATCCATTAAAGCCGCTAGGGGAAA
phzXTRR	CGCTCTAGAGGGAAAGCGAAGCAGGCAT
phzRTRF	CGCGAATTCGAACCCGCCTCTTCACAATA
phzRTXR	CGCTCTAGAGGGAAAGCGAAGCAGGCAT
rpoD RT1	ACGTCCTGAGCGGTTACATC
rpoD RT2	CTTTCGGCTTCTTCTTCGTC
16s RT1	ACGTCCTACGGGAGAAAGC
16s RT2	CGTGTCTCAGTTCCAGTGTGA
phzX RT2	AACCACTTCTGGGTGGAAAG
phzX RT2	ATCTTGCCGTCATCCAGTTC
phzY RT1	CATATCTGGGTCGAGTGTGATG
phzY RT2	GCAGGAAGTGGTTCTCGTAATA
phzF RT1	CCTGGACCAGCCATGAAAT
phzF RT2	CAGTGGGTAGAACCGAGAAATAC
phzA RT1	CATTCCATCGATCGTCCCTTAC
phzA RT2	GGAAGTAGCGCTGCATGT
phzB RT1	CTACATGGTGGTGGATGAAGAG
phzB RT2	GGTCTTGCCTTCGATGAAGTA
phzC RT1	CGTCATCATCGACGCCTTT
phzC RT2	GGTTTCCGACAGGTTCATCTC
phzD RT1	CGCCAGAGTGAAGAACTCAA
phzD RT2	CCGTAACTCAAACACGCAATAAC
phzO RT1	CAGAGAGAAGAACCTGCTGATG
phzO RT2	GTGCGTGCTGTTCTTCAAAC
gacA RT1	GATGACCATGATCTCGTTCG
gacA RT2	CATCTTGACGTCCATGAGGA
gacS RT1	AAAACCTGGAAACCATCGAG
gacS RT2	GGTGGGTGAAACCGAGAATA
rpeA RT1	GTCGACGACGAACTGGAATC
rpeA RT2	CTGGATACGCTTTTCGCAAT

Table 3. 2. Continued

Oligonucleotide	Sequence (5'-3')
rpeB RT1	CATCCTTCTGGTCGAAGACG
rpeB RT2	AGGTCGAGAATCACCAGGTC
phzR RT1	CGCAAGGATAATCCCATCAG
phzR RT2	CACATTCCCTACCGCTGAAC
phzI RT1	CTACCTCCTGGCGTTCAATG
phzI RT2	GAAGCGAGTCATTTCCCAGA
pip RT1	AAAAGACCCGCGAGAACATT
pip RT2	ACGTACAGCTGCTCCTTGCT
rpoS RT1	ATCAGTGGCTTTCCGAATTG
rpoS RT2	GACCTTCGACCTGGATCTGA
csaR RT1	GATGCTCAAGCAACAGGAGA
csaR RT2	GCTTGCGGATAAGAGCCATA
csaI RT1	GCTACACCCTGGCACTGG
csaI RT2	AACGCGAGATTTCCCAGGT
hfq RT1	GTCAAAAGGGCATTCGCTAC
hfq RT2	CTGACGGTGTTCTTCAGCAG
rsmA RT1	GCGCAGAAAGCCTGATTATT
rsmA RT2	ATGGCTTGGTTCTTCGTCCT
rsmE RT1	GCTTGGATCCGTTGGTAGAT
rsmE RT2	ATCACCATTCTGGGCGTTAG
rsmZ RT1	TGTCGACGGATAGACACAGC
rsmZ RT2	GTCCTGATGAATCGCATCCT

[a] Underlined nucleotides are restriction sites added and the restriction enzymes are indicated at the end of primers.

To determine whether sequences containing the putative *phz* box were under quorum sensing control, time course assays were performed using these same promoter derivatives to examine β-galactosidase activity relative to cell density (Fig. 3.4A). Although several of the promoter derivatives produced greater *β*-galactosidase activity than was observed for the derivative containing the entire promoter sequence (KT2VS and KT2PS), the cell density at which *lacZ* expression was first observed remained

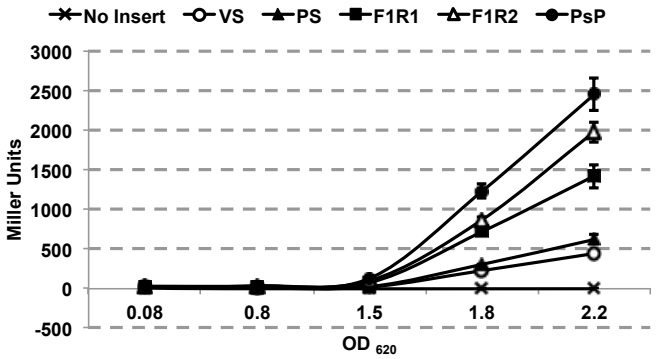
approximately the same for all derivatives (e.g. ~OD_{620}=1.2), demonstrating that all were regulated by quorum sensing. As above, pKT2PsP containing the 127 bp region of *Pst*I-*SsP*I produced the greatest activity, indicating this region is necessary and sufficient for high expression, but depends on quorum sensing.

To further demonstrate quorum sensing control of all promoter derivatives, a Tc^r version of each plasmid was introduced separately into strains 30-84I and 30-84I/I2 (with disruptions in *phzI*, and *phzI* and *csaI* together, respectively). In contrast to the expression levels observed when the plasmids were introduced into strain 30-84Ice, no significant β-galactosidase activity (data not shown) or *gfp* expression was found when they were introduced into any of the quorum sensing deficient mutants (Fig. 3.4A). These results confirm that quorum sensing regulation is required for promoter activity, regardless of the promoter fragment size.

In many pseudomonads, secondary metabolite production is dependent on the presence of a functional GacS/GacA two component regulatory system (TCST), and loss of either *gacA* or *gacS* resulted in complete loss of phenazine production (Pierson *et al.*, 1995, Chancey *et al.*, 1999, Zhang & Pierson, 2001, Chancey *et al.*, 2002, Haas & Défago, 2005, Driscoll *et al.*, 2011, Wang *et al.*, 2012a, Wang *et al.*, 2013). To determine whether any of the promoter derivatives are independent from GacS/GacA regulation, each promoter derivatives was conjugated separately into strain 30-84W, a spontaneous *gacA* mutant. None of the promoters had any detectable β-galactosidase activity. The results demonstrate that the 127 bp region of the promoter that is necessary

for phenazine gene expression requires both quorum sensing and a functional *gacA* for expression.

A.



B.

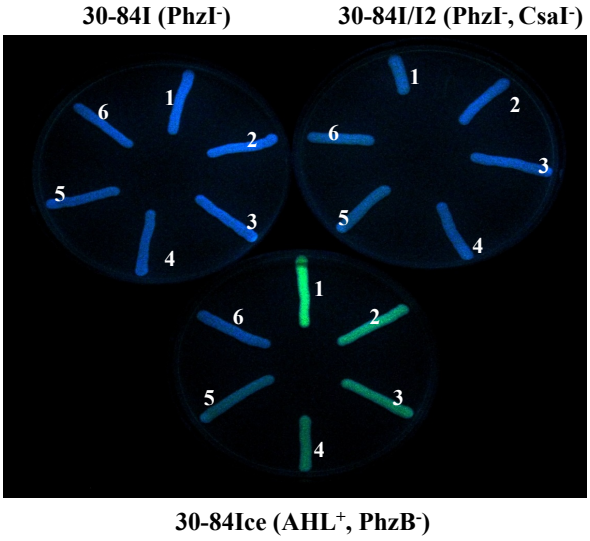


Figure 3. 4 Quorum sensing dependence of phenazine promoter derivatives in the *P. chlororaphis* strain 30-84.
A. Time course of expression of various phenazine promoter derivatives fused to *lacZ*. Strain 30-84Ice (*phzB::inaZ*) containing either pKT2*lacZ* (control) or the promoter subclones were grown in LB supplemented with Km^{50} and cell growth was measured by optical density (OD_{620} nm). Samples were taken periodically, and β-galactosidase activity was measured (Miller Units). Plasmids tested including; VS= pKT2VS, PS= pKT2PS, F1R1= pKT2F1R1, F1R2= pKT2F1R2, PsP= pKT2PsP and no insert= pKT2*lacZ*. B. Analysis of the dependence of promoter activity on AHL signals by comparing reporter activity in AHL signal producing and non-producing strains of 30-84. Promoter expression was qualitatively measured by GFP expression in AHL^+ (30-84Ice) and AHL^- mutants (30-84I and 30-84I/I2). Cultures were streaked onto LB plate and photographed under UV light. 30-84 Ice (*phzB::inaZ*), 30-84I (*phzI::*km), 30-84I/I2 (*phzI::*km and *csaI::uidA*-gm). 1= pKT2 PsP, 2 = pKT2 F1R2, 3 = pKT2 F1R1, 4 = pKT2 PS, 5 = pKT2 VS, 6 = pKT2*lacZ* no insert.

Characterization of promoter sequences potentially involved in repression

DNA sequence analysis identified several features within the *phz* promoter region that are potentially capable of providing significant secondary structures such as forming stem loops and thereby controlling gene expression via the formation of stem loops (Fig. 3.1). These include the two direct repeats, the two *Ssp*I sites within the region of low GC content, and the ribosome binding site and *phzX* translation start site. Analysis of the DNA sequence from the transcription +1 and the *Sal*I sites with the program Mfold (Zuker, 2003) also identified the potential for the formation of two loop structures: between the two *Ssp*I sites and between the predicted RBS and the translational start site of *phzX* (Fig. 3.5A). Analysis of the mRNA for that region predicted secondary structure between the second CACCCCCAA direct repeat and the predicted RBS for the *phzX* ORF (Fig. 3.5B). To investigate whether these sequences affect gene regulation, a series of promoters were synthesized with modifications to some of these sequences. To modify the potential for stem loop formation between the direct repeats bases CACCCCCAA of the direct repeat sequences were changed to CATATATAA (modifications to direct repeats 1, 2, or both: Var1, Var2, Var3, respectively, as shown in Fig. 3.6A, Table 3.1). To modify the potential for stem loops between and the ribosome binding site bases AGGAGGA of the ribosome binding site were changed to TATATTG and bases ATG of *phzX* start codon were changed to CTG (Var 4). The potential stem loop formation between two *Ssp*I sites was removed by digestion with *Ssp*I and re-ligated (Var5). These sequences were cloned into pKT2*lacZ* and introduced into 30-84Ice, and the expression of each promoter derivative was

quantified by β-galactosidase activity (Fig. 3.6B). Plasmids pKT2PS (sequence from the *Pst*I site to the *Sal*I site, containing all of the sequence motifs) and pKT2PsP (sequence from the *PstI* site to the first *SspI*, containing only direct repeat 1) were included in the assay as indicators of wild type and enhanced promoter activity. There were no significant differences in β-galactosidase expression between any of the promoter variants with modifications to the direct repeats (Var1-3) and the wild type control (Fig. 3.6B). However Var4 had -1.6 fold less expression than the intact *phz* promoter (pKT2PS) suggesting that modifications to sequences involved in translation had a slightly negative effect on expression. Interestingly, Var5 showed slightly higher expression (e.g. 1.4 fold) than the intact *phz* promoter, but significantly less expression compared to pKT2PsP indicating that the stem loop between *Ssp*I sites is at least partially responsible for the *phz* promoter attenuation. We expected that altering the potential for stem loops in these regions could alleviate repression of expression by unfolding secondary structure. These results indicate that single structural change could not relieve transcriptional repression to the same level as observed when the entire region was deleted.

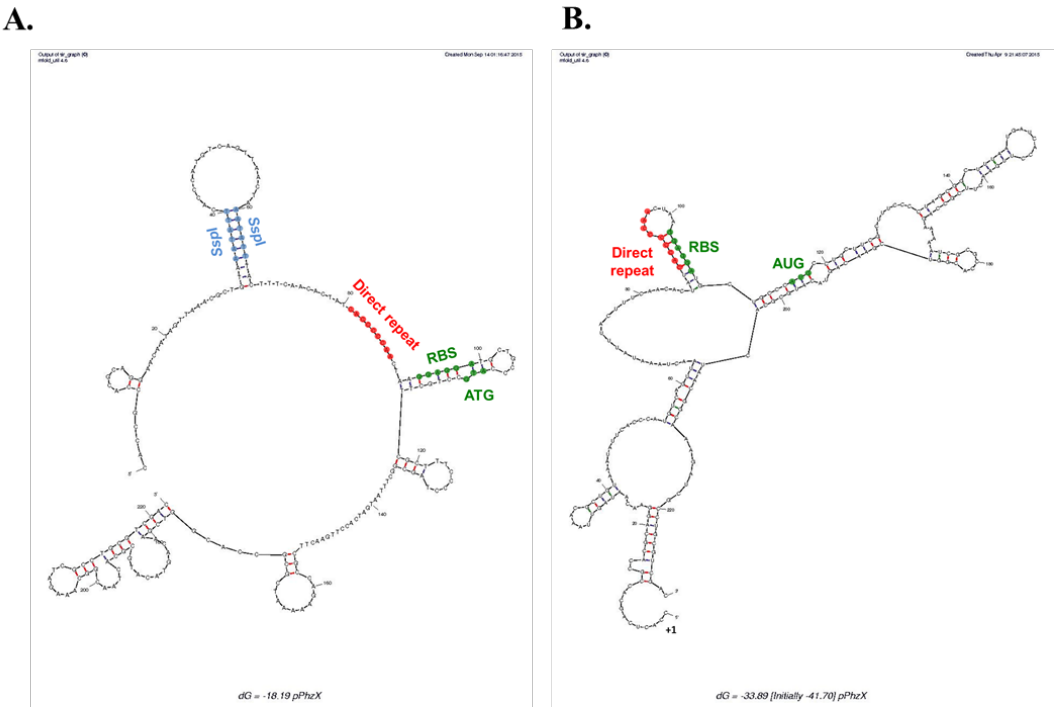
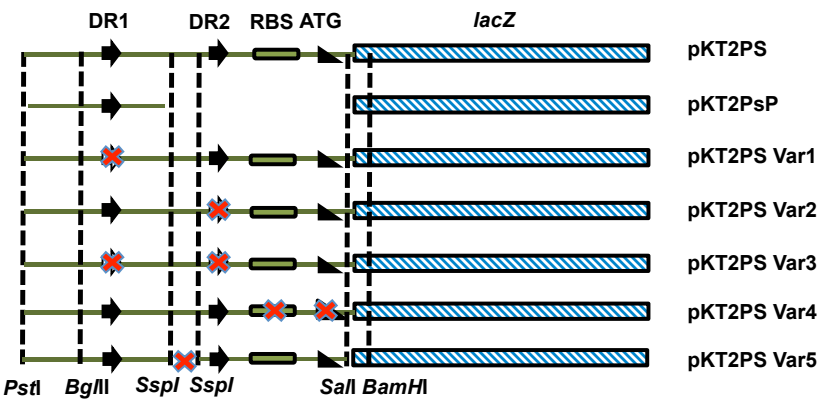


Figure 3. 5 Analysis of secondary structure predicted by the MFold software (Zuker, 2003).
A. Potential secondary structure predicted from the DNA sequence spanning the transcription start site (+1) site to *Sal*I site of *phzX*. Two *Ssp*I sites were marked with blue highlights, the second direct repeats are marked with red highlight, and the putative *phzX* RBS and start codon are marked with green highlight. B. Potential secondary structure predicted from the mRNA sequence spanning the transcription start site (+1) site to *Sal*I site of *phzX*. The second direct repeat, ribosome binding site, and AUG start codons are indicated.

A.



B.

Plasmid in 30-84 Ice	MU ± SE	Fold
pKT2 PS	318.9 ± 24.6 c	-
pKT2 PsP	1219 ± 82.03 a	3.8
pKT2 PS Var1	385.1 ± 40.15 c	1.1
pKT2 PS Var2	350.4 ± 29.35 c	1.2
pKT2 PS Var3	389.9 ± 35.96 c	1.0
pKT2 PS Var4	220.5 ± 5.67 d	-1.4
pKT2 PS Var5	493.8 ± 43.48 b	1.6

Figure 3. 6 Construction of pKT2 PS promoter and derivatives.
A. The pKT2 PS promoter and derivatives with specific sequence alterations fused to *lacZ*. The black solid arrows represent the two direct repeat (5'-CACCCCCAA-3'), DR1 and DR2. The green rectangle and black triangle represent the RBS and start codon of *phzX,* respectively. The hollow symbols with red-cross represent modified sequence motifs. The blue-hatched rectangles represent *lacZ*, and dotted lines indicate restriction enzyme sites. The modified sequences of promoter region were synthesized and cloned into pKT2*lacZ* and plasmids were introduced separately into 30-84Ice B. The β-galactosidase activity of pKT2 PS promoter and each derivative in 30-84Ice. Promoter activity is expressed in Miller Units as the standard error of 6 replicates. Values with the same letter do not differ significantly as determined by a Fishers protected Least Significantly Difference (LSD) test (P>0.05).

To determine whether this 185 bp region was a general repressor of gene expression (e.g. represses the activity of a heterologous promoter), we introduced the 185 bp sequence downstream of the promoter *pTac* and upstream of the *lacZ* reporter,

resulting in pKT2pTac185*lacZ* (Table 3.1). Plasmids pKT2pTac*lacZ* (no insert) and pKT2pTac185*lacZ* were introduced into *E.coli* (DH5a), strains 30-84Ice and 30-84W (a spontaneous 30-84 *gacA* mutant). The presence of the 185 bp region resulted in a significant reduction of *lacZ* expression in *E. coli* strain DH5α and in both strains 30-84Ice and 30-84W (*gacA*) (Table 3.3). These results indicate that repression of promoter activity occurs in both *Pseudomonas* and *E. coli* and is not related specifically to interaction with the upstream sequence of the phenazine promoter, or influenced by *gacA*.

Table 3. 3 Effect of the presence of the 185 bp region on lacZ expression from *Ptac* promoter.

Plasmid/Strain	DH5α	30-84Ice	30-84W (*gacA*)
pKT2Tac*lacZ*	836 ± 70	2,052 ± 407	3,037 ± 286
pKT2Tac185*lacZ*	14 ± 2	9 ± 2	7 ± 3
Fold Decrease	59X	228X	434X

Generation of enhanced phenazine-producing strain

To confirm that the sequence that repressed promoter activity in our plasmid-borne reporter system also represses chromosomal phenazine promoter activity, we generated a deletion sequence for marker exchange into the chromosome to create a deletion of the negative regulatory region. This construct was designed to delete 90 bp of sequence starting at the +8 bp (from the transcription start site) to the ribosome binding site (deletion includes both *Ssp*I sites and the second direct repeat, Fig. 3.7A). The fragment included flanking sequence upstream from the *Eco*RV site in the middle of *phzR* and downstream to the *Bam*HI site at the 3'-end of *phzY* (the second gene in the

phenazine biosynthetic operin in *P. chlororaphis* 30-84) to facilitate homologous recombination. To insure this construct would function correctly, expression was tested *in trans* prior to the chromosomal replacement. For this purpose, the 1575 bp fragment was cloned into the unique *Bam*HI site in the pKT2*lacZ* (to create plasmid pKT2-pPhzEnh, Table 3.1), introduced into 30-84Ice, β-galactosidase activity measured. As shown Fig 3.7B, pKT2-pPhzEnh resulted 3.4-fold higher expression compared to the intact phenazine promoter on pKT2PS, and similar levels of expression level compared to the strongest promoter derivative, pKT2PsP. This result confirmed that deletion of the 90bp sequence functioned properly *in trans* and was suitable for marker exchange.

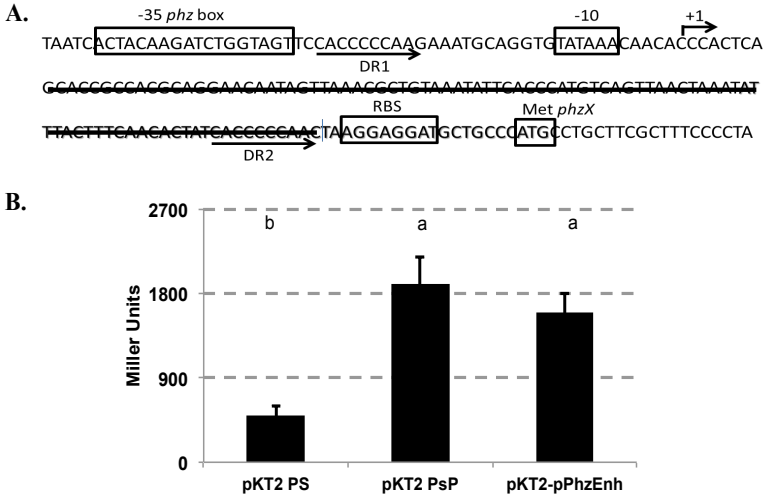


Figure 3. 7 Developing the pPhzEnh promoter.
A. DNA sequence of *phz* promoter and the 90bp deleted region containing the putative promoter inhibitory region (indicated by strikethrough). The deletion includes the two *Ssp*I sites required to form the potential stem loop in the DNA (see Fig. 3.5A) and DR2, which is required to form the potential RBS-AUG stemloop in the mRNA (see Fig. 3.5B). Boxed nucleotides indicate the *phz* box and -35, -10 (RNA polymerase binding site), the +1 transcription start site (TSS), the ribosome binding site (RBS), and *phzX* translation start site. B. Promoter activity was quantified from overnight cultures of 30-84 Ice containing promoter derivatives pKT2PS (contains wild-type promoter), pKT2PsP (truncated promoter), and pKT2-pPhzEnh (phenazine enhanced promoter). The β-galactosidase activity of each derivative was expressed Miller Units as the average of triplicate samples with standard error (Mean ± SE). Values with the same letter do not differ significantly as determined by a Fishers protected Least Significantly Difference (LSD) test (P>0.05).

The 1575 bp fragment was cloned into pLAFR3 for marker exchange into the chromosomes of wild type 30-84, 30-84ZN and 30-84Ice strains via modified λ-Red cloning technique (Lesic & Rahme, 2008) to create derivatives having the enhanced promoter (WT-Enh, ZN-Enh, Ice-Enh, respectively) (Fig. 3.8). The impact of the deletion of 90 bp of the *phz* promoter on phenazine production was determined both visually and quantitatively. On PPMD plates, the WT-Enh strain exhibited higher phenazine production (darker orange color) than strain 30-84 wild-type (Fig. 3.8A). The overnight culture of ZN-Enh also showed higher β-galactosidase activity (darker blue) than 30-84ZN on LB supplemented with 2% X-Gal (Fig. 3.8B). To quantify phenazine production, wild-type and WT-Enh were grown for 24 h in three types of media: PPMD, LB, and AB minimal medium, and total phenazines were extracted. Compared to wild-type, WT-Enh produced significantly more phenazine in all media types (i.e. 3.1-fold, 1.9-fold, and 2.4-fold in AB, LB and PPMD, respectively, Fig. 3.8C). In order to determine whether enhanced phenazine production is related to enhanced expression of the *phz* biosynthetic genes, strain 30-84ZN and ZN-Enh were grown overnight in 3ml LB media and β-galactosidase activity was measured. As shown in Fig. 3.8D, β-galactosidase activity of ZN-Enh was 2.1-fold higher than that of 30-84ZN. The results confirm that the 90 bp sequence of the bacterial chromosome containing possible stem loop forming regions acts as a negative regulator of phenazine promoter activity.

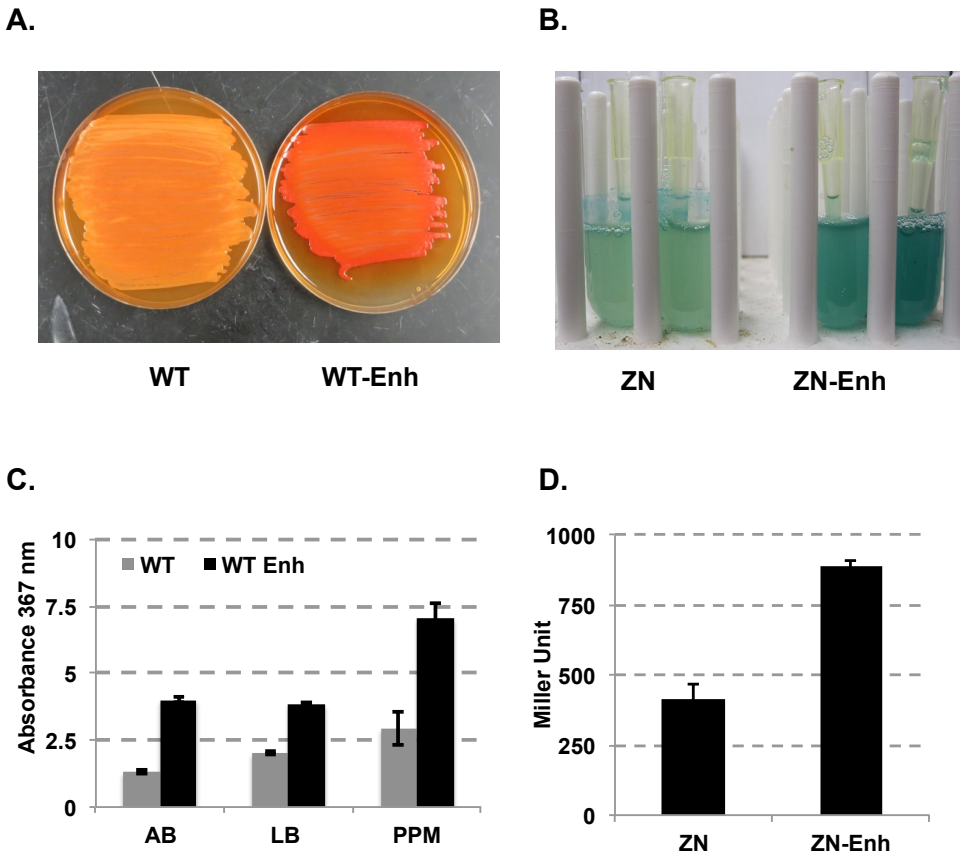


Figure 3. 8 Characterizing phenazine enhanced strains WT-Enh and ZN-Enh.
A. Phenazine production by 30-84 wild-type and 30-84WT-Enh after 48 h on PPMD agar plates. B. Overnight culture of 30-84ZN and 30-84ZN-Enh on LB broth supplemented with 2% X-Gal. C. Phenazine production. The 30-84 wild-type and WT-Enh were grown in different media for 24 h at 28 ºC with shaking. Phenazine was extracted in equal volume of benzene and the amount of phenazine was quantified at 367 nm. Bars represent means ± SE of six replicates from at least two independent experiments. D. Expression of the *phz* biosynthetic operon. 30-84ZN and ZN-Enh were grown in LB broth for 24 h at 28 ºC with shaking. Expression of *phz* was quantified by β-galactosidase assay. Each bar represent means ± SE of six replicates from at least two independent experiments.

In order to confirm findings based on the plasmid reporter that this deletion did not affect quorum sensing control of promoter activity, the relationship between the cell growth and the production of both primary phenazine derivatives was examined using a time course assay. As shown in Fig. 3.9A, 30-84 wild-type and WT-Enh had similar growth rates, indicating that alteration of the promoter did not alter growth rates. As

shown in Fig. 3.9B and 3.9C neither strain produced detectable amounts of phenazine before 5 hours of growth, indicating a similar inhibition of phenazine production at low cell density. Both strains produced detectable amounts once the cell densities reached an OD_{620} of 1.3 (6.5 hours post-inoculation). By the time the cell growth reached OD_{620} = 1.8 (9.5 hours after inoculation), both strains were producing both phenazine derivatives, but strain WT-Enh produced significantly more than the wild type (Fig. 3.9B and 3.9C). From that time point on phenazine production by WT-Enh was significantly greater than wild-type, and by the late stationary phase, PCA concentration from strain WT-Enh was 5.8-fold higher, and 2OHPCA concentration was 4.4-fold higher than from the strain 30-84 wild-type (Fig. 3.9B and 3.9C). The results confirmed that even though strain WT-Enh produces much more phenazines than wild type, it is still regulated by quorum sensing. Furthermore once the required signal density is present WT-Enh is more efficient in the production of both phenazines than wild type.

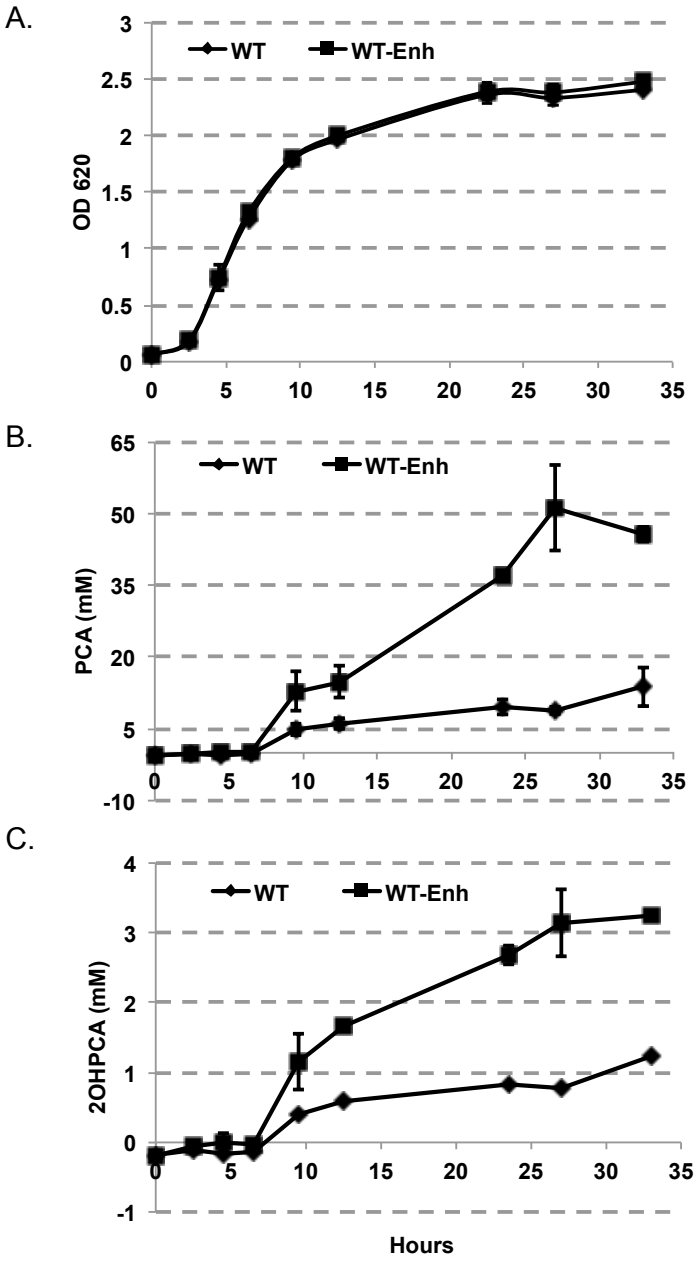


Figure 3. 9 Bacterial growth and time course of phenazine production.
A. Growth curve of strain 30-84 wild-type and 30-84 WT-Enh in AB-minimal media supplemented with 2% casamino acids at 28 ºC. B. Time course of PCA and C. 2OHPCA production by 30-84 wild-type and WT-Enh. During the growth curve, samples were taken periodically and total phenazines were extracted in benzene. The relative amount of PCA and 2OHPCA was calculated by multiplying their absorption maxima by their standard extinction coefficients (Maddula et al., 2008). Data points represent means of three replicates ± standard error. Similar results were obtained in at least two independent experiments.

Effect of phenazines on phenazine promoter activity

The significant enhancement in both the rate and total amount of phenazines produced by WT-Enh compared to wild-type suggested that the deleted region may contain sequences capable of negatively interacting with metabolites (e.g. substrates or products of phenazine biosynthesis), similar to a riboswitch. Typically a riboswitch is located on the 5' leader sequence of the bacterial mRNA coding sequence, and gene expression is regulated by the binding of target metabolites or molecules such as amino acids, nucleotides, sugars, metal ions and other small ligands to mRNA (Naville & Gautheret, 2009, Hollands *et al.*, 2012). In order to determine whether the deleted region contained a riboswitch, a fragment of the sequence (from *Pst*I site to 121 bp downstream of the translational start site of *phzX*) was PCR amplified from 30-84 wild-type and WT-Enh and inserted into the promoterless vector pGT2*lacZ*, resulting in pGT2*WXlacZ* and pGT2*EXlacZ* (Fig. 3.10A). The construct pGT2*WXlacZ* is similar to pKT2PS (Fig. 3.3B), but the gentamicin plasmid was used to facilitate introduction into the kanamycin resistant derivative 30-84PCA. These promoter derivatives were then conjugated into strains 30-84 wild-type, 30-84PCA, and 30-84Ice, and promoter expression measured as β-galactosidase activity. These strains (30-84 wild-type, 30-84PCA, and 30-84Ice) are useful for comparing the effect of different phenazines on *phz* promoter expression, because wild-type produces both PCA and 2OHPCA, whereas 30-84PCA produces only PCA (due to deletion of *phzO, phzO*::Tn5), and 30-84Ice produced no phenazine (*phzB*::*inaZ*) (Wood *et al.*, 1997, Maddula *et al.*, 2008). As shown in Fig. 3.10B, the expression of both pGT2*WXlacZ* and pGT2*EXlacZ* in 30-84 PCA and 30-84 Ice were

not significantly different, suggesting that PCA production did not alter phenazine promoter expression and more specifically did not interact with the 90 bp region to control promoter expression. Interestingly the expression of both pGT2*WXlacZ* and pGT2*EXlacZ* were slightly reduced in wild type suggesting that 2OHPCA production may interact with promoter expression.

These data led to two possible hypotheses: that 2OHPCA may act as a negative regulatory signal molecule via its influence on the phenazine biosynthetic promoter or that because of the orange color of 2OHPCA in the culture perhaps the sensitivity of β-galactosidase assay was affected. To prevent a misleading conclusion, experiments were carried out using addition of exogenous phenazine derivatives. Strains of 30-84Ice containing either pGT2*WXl*acZ or pGT2*EXlacZ* were grown in LB supplemented with each phenazine derivatives (e.g. final concentration of 100 μg/ml). Samples were taken periodically and β-galactosidase activity was measured until 27 h. As shown in Fig. 3.10C, the expression of both promoters was unaffected by the addition of either phenazine to the culture. Taken together, our results suggest that neither of the primary phenazines produced by strain 30-84 are negative regulatory signal molecules.

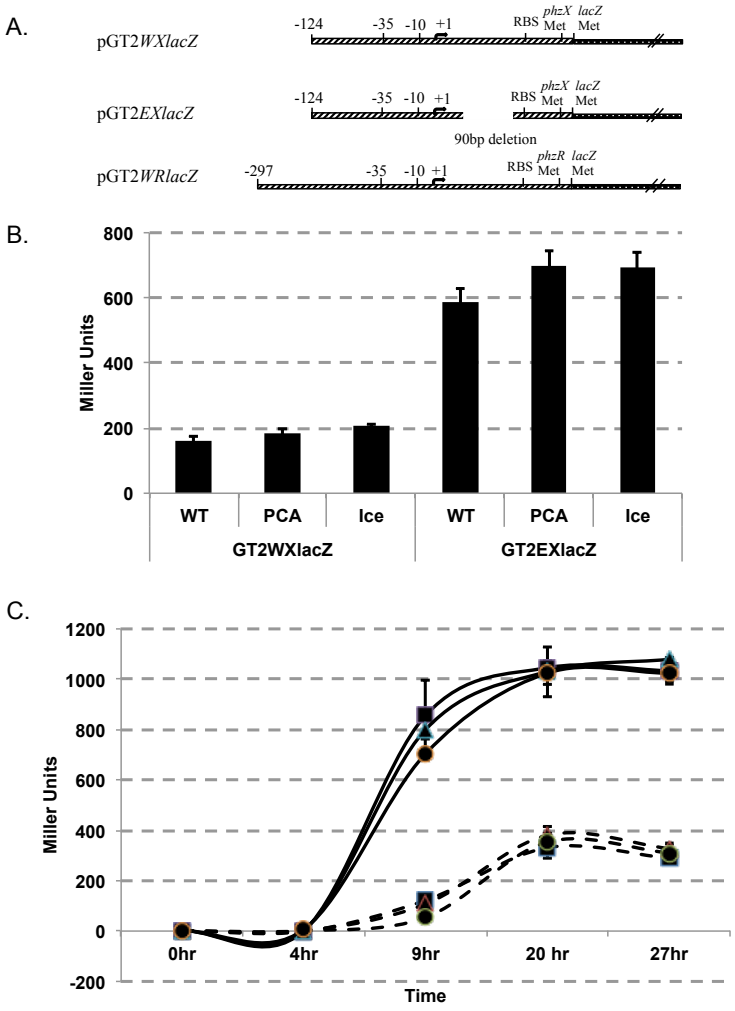


Figure 3. 10 Activities of *phzX-lacZ* and enhanced *phzX-lacz* transcriptional fusion in strain 30-84 and its derivatives.

A. Map of the promoter sequences used to construct the transcriptional fusion reporters. The reporter plasmids pGT2*WX*lacZ and pGT*EX*lacZ contained flanking sequence from -124 bp to 29 bp downstream of *phzX* translation start site and were amplified from genomic DNA of 30-84 wild-type and WT-Enh using primer set phzXTXF-phzXTXR. These fragments contain *phzX* promoter region, ribosome binding site, transcription and translation start site and the first 29 bp of the *phzX* gene. The reporter plasmids pGT2*WRlacZ* contained flanking sequence from -297 bp to 62 bp downstream of *phzR* translation start site was amplified from genomic DNA of 30-84 wild-type using primer set phzRTXF-phzRTXR. This 443-bp fragment contains *phzR* promoter region, ribosome binding site, transcription and translation start site and the first 19 bp of the *phzR* gene. B. Assay for β-galactosidase activities expressed from the pGT2WXlacZ and pGTEXlacZ transcriptional fusion plasmids in strain 30-84 wild-type, 30-84PCA (*phzO::*Tn5, PCA only producer), and 30-84Ice (*phzB::inaZ*, non-phenazine producer). Data represent means of four replicates ± standard error. Similar results were obtained in at least two independent experiments. C. Time course assay of β-galactosidase activities expressed from the pGT2WXlacZ (dashed lines) and pGTEXlacZ (solid lines) transcriptional fusion plasmids in strain 30-84Ice grown in LB supplemented with either 100 µg/ml of PCA or 2OHPCA. Samples were taken periodically and β-galactosidase activity was measured (Miller Units). Symbols in Fig. 12C describe culture amendments; square: 0.2N NaOH as a negative control; triangle: exogenous PCA; circle: exogenous 2OHPCA.

Expression of the quorum sensing genes phzR and phzI and phzO are up-regulated in the phenazine enhanced strain

To determine the effect of the 90 bp deletion on the gene expression of phenazine biosynthetic and regulatory genes, the transcript abundances of these genes in 30-84 wild-type and WT-Enh were compared using qPCR. Consistent with the increase in phenazine production in WT-Enh relative to 30-84 wild-type, deletion of 90 bp from the promoter region resulted in 1.5- to 2.5-fold increases in the transcript abundances of genes in the phenazine biosynthetic operon as well as the terminal modifying gene, *phzO* (Fig. 3.11A). These data indicate that the deleted 90-bp region negatively regulates the production of PCA and 2-OH-PC via direct or indirect (via availability of PCA to *phzO*) regulation of the transcript abundance of *phzXYFABCD* and *phzO*.

The effect of the deletion on the *phzR/phzI* quorum sensing system also was examined. Given that *phzR* is located immediately upstream of *phzX* and divergently transcribed, regulatory elements in the intergenic region may have important consequences for the transcription of either *phzR* or *phzX,* or both. qPCR analysis demonstrated that the transcript abundances of *phzR* and *phzI* were 1.7- and 1.3-fold greater in WT-Enh compared to 30-84 wild-type (Fig. 3.11A). These data suggest that the 90 bp deletion results in a slight increase in *phzR* and *phzI* expression.

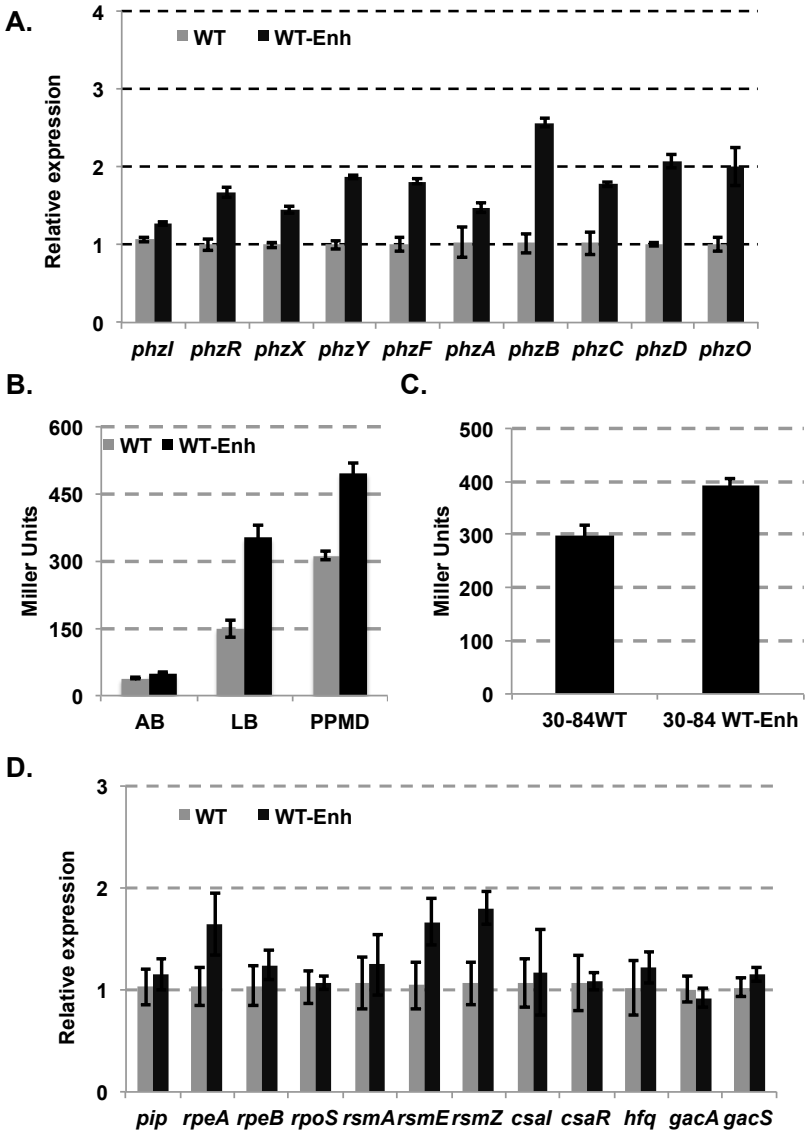


Figure 3. 11 Expression of the phenazine regulatory genes in phenazine enhanced strain.
A. Relative expression of the phenazine quorum sensing genes *phzI/R,* the phenazine biosynthetic genes *phzXYFABCD* in the *phz* operon, and the terminal modifying enzyme *phzO* in 30-84WT and WT-Enh. Transcript abundances were determined via qPCR and standardized to 30-84WT. B. AHL production by 30-84 wild-type and WT-Enh. AHLs obtained from overnight cultures of 30-84 wild-type and WT-Enh were quantified using the AHL-specific reporter strain 30-84I/Z (*phzI*⁻ *phzB*::*lacZ*). C. Comparison of the expression of quorum sensing regulator *phzR* in 30-84 wild-type and WT-Enh using a *phzR::lacZ* transcriptional fusion on plasmid pGT2*RXlacZ*. D. Relative expression of phenazine regulatory genes *pip, rpeA, rpeB, rpoS, rsmA, rsmE, rsmZ, csaI, csaR, hfq, gacA,* and *gacS* in 30-84 wild-type and 30-84 WT-Enh. Transcript abundances were determined via qPCR and standardized to 30-84WT. A and D: Cells were grown in AB minimal medium + 2% casamino acid for 18 h with shaking to an OD600 of 1.2. Data points represent means of three replicates ± standard error. Statistical analysis of ΔCt values confirmed that for each gene tested, non-overlapping error bars indicate significant differences between strains in gene expression.

To determine whether changes in *phzI* transcript abundance resulted in changes in AHL production, the AHL produced by 30-84 wild-type and WT-Enh was quantified using an AHL-specific signal reporter strain 30-84I/Z (Δ*phzI*, *phzB::lacZ*) in various media (AB, LB and PPMD). Consistent with qPCR data, WT-Enh produced significantly more AHL than that of the 30-84 wild-type in LB and PPMD media (Fig. 3.11B). This result indicates enhanced phenazine production in 30-84 WT-Enh was due at least partially to increased AHL production. To test whether the increased phenazine production also was due to an increase in *phzR* expression, we measured *phzR* expression in AB-C media using transcriptional fusion vector pGT2*WRXlacZ* in 30-84 wild-type and 30-84 WT-Enh (Table 3.1). As shown in Fig. 3.11B, the expression of *phzR* was significantly higher in the 30-84 WT-Enh compared to 30-84 wild-type in AB-C media (391.26 ± 14.45 MU versus 296.91 ± 19.92 MU). These results suggest that the 90 bp of negative regulatory region is also involved in modulating AHL production and *phzR* transcription.

Gene expression patterns of other phenazine regulatory systems

In strain 30-84, phenazine biosynthesis is controlled by a number of other regulatory genes including a second QS system (*csaR/csaI*); the two-component systems *rpeA/rpeB* and *gacS/gacA*, sigma factor *rpoS,* transcriptional regulator *pip*, post-transcriptional regulatory protein *rsmE*, and the small non-coding RNA (ncRNA) *rsmZ* (Chancey *et al.*, 1999, Zhang & Pierson, 2001, Whistler & Pierson, 2003, Wang *et al.*, 2012a, Wang *et al.*, 2013). The qPCR analysis of transcript abundance showed that the expression of *rpeA, rsmE,* and *rsmZ* transcript were slightly higher in the 30-84 WT-Enh

compared to 30-84 wild-type, whereas the abundances of *pip, rpeB, rpoS, rsmA, csaI, csaR, hfq, gacA* and *gacS* were not different (Fig. 3.11D). These results suggest that enhanced phenazine production by 30-84 WT-Enh compared to 30-84 wild-type alters the expression of the phenazine regulators, *rpeA* and *rsmE,* and ncRNA, *rsmZ.*

Unknown protein is directly binding to 90bp promoter negative regulatory region

To determine whether an unknown protein is interacting with the 90 bp region, wild-type and enhanced biotinylated DNA probes (identical to the wild type and deletion-containing inserts in pGT2*WXlacZ* and pGT2*EXlacZ,* respectively) were generated by PCR amplification. These probes contained the putative -35 and -10 boxes and downstream sequence with or without 90bp promoter negative regulatory elements described above (Fig. 3.12A). Since phenazine biosynthesis is regulated directly by quorum sensing, total proteins were isolated from crude extract at two different time point: OD_{620}= 0.4 (cell density below level required for quorum sensing mediated phenazine production) and 1.0 (cell density sufficient for quorum sensing mediated phenazine production). Both DNA probes (WT=272 bp and Enh=182 bp) were mixed with total proteins for the band-shift analysis. At low cell density, both DNA probes showed same binding patterns, producing three bands (Fig. 3.12B). However at higher cell density, the 182 bp DNA probe produced only two bands whereas the 272 bp DNA probe produced 3 bands (Fig 3.12B). These data suggest that 90 bp promoter regulatory region may interact with an unknown protein, present at low cell density, which has a repressor function. Whereas at higher cell density (e.g. when the QS system is activated),

this unknown protein has reduced affinity for the enhanced promoter with the deletion of the 90 bp region.

Ecological consequences of enhanced phenazine production

Phenazines have been studied extensively due to their ability to inhibit fungi by generating and accumulating reactive oxygen species, which are toxic to fungal cells (Britigan *et al.*, 1997, Chin-A-Woeng *et al.*, 2001a, Mavrodi *et al.*, 2001, Babitzke & Romeo, 2007, Wang *et al.*, 2012a, Wang *et al.*, 2013). The ability of strain 30-84 wild-type and WT-Enh to inhibit growth of the plant pathogen *G. graminis* var. *graminis* was measured using an *in vitro* dual culture plate assay. The zone of fungal growth inhibition for strain 30-84 wild-type was 8.3 ± 0.02 mm (Fig. 3.13A), compared to little or no inhibition by the non-phenazine producing control strains 30-84ZN and 30-84 ZN-Enh (1.25 ± 0.6 and 1.5 ± 0.6 mm, respectively).

Enhanced phenazine production also affects biofilm formation when strains are grown in static culture. Assays were conducted with strain 30-84 wild-type and WT-Enh in three types of medium, AB-C, LB and PPMD, and showed that 30-84 WT-Enh formed significantly more biofilm than wild type in minimal, LB, and rich media (Fig. 3.13B). The results suggest that enhanced biofilm production, correlated with enhanced phenazine production, may have important consequences for bacterial attachment and survival in niches where biofilm formation is important.

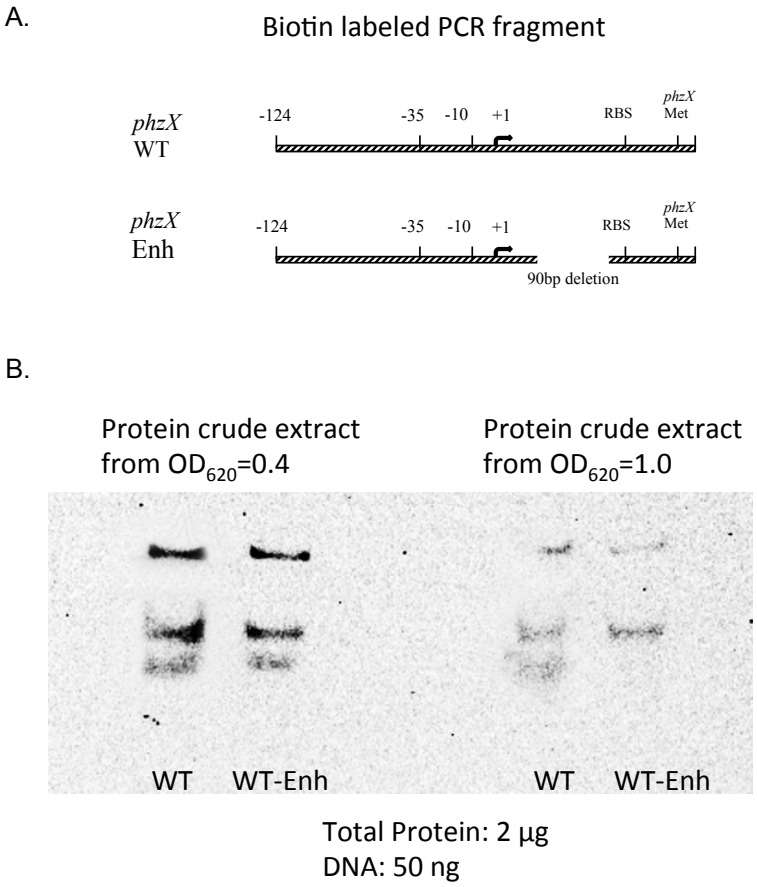


Figure 3. 12 Binding of unknown protein to *phzX* promoter region.
A. 272 bp (*phzX* WT) and 182 bp (*phzX* Enh) of biotin labeled DNA probes, corresponding to the *phzX* promoter region. Each DNA probe was amplified by PCR using genomic DNAs from 30-84 wild-type and WT-Enh as template. B. Direct binding of unknown protein(s) detected by band-shift assay. Fifty nanogram of biotinlyated DNA fragments was used as probe with 2 µg of total protein isolated from crude cell extract from cultures grown to different cell density (OD620=0.4 and 1.0). Samples were separated by 6% native polyacrylamide gel electrophoresis at 100V for 1 h.

However strain 30-84 WT-Enh produced a significantly larger zone (10.2 ± 0.2 mm) than 30-84 wild-type (Fig. 3.13A). These data demonstrate that phenazine production is a major factor in controlling fungal growth inhibition by *P. chlororaphis* 30-84, and that enhanced phenazine production results in more control.

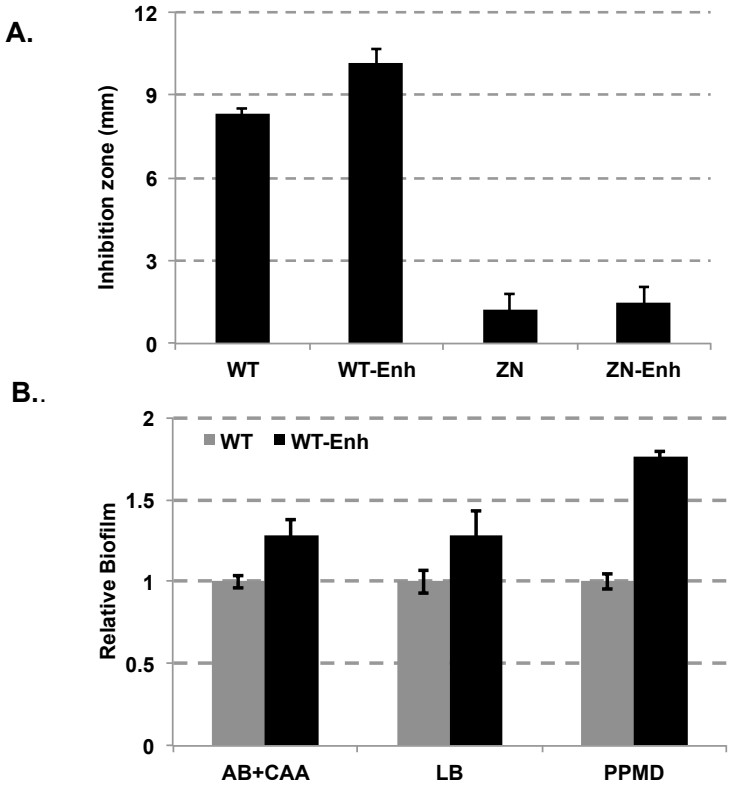


Figure 3. 13 Effect of enhanced phenazine production in pathogen inhibition and biofilm formation. A. A plug of *G. graminis* var. *graminis* taken from 5-day old PDA plate was transferred to the center of a fresh PDA plate. Overnight cultures of each strain were spotted at the edge of each plate. The zones of fungal growth inhibition (mm) were measured at 5 days post-inoculation. Data represent average of three replicates with standard errors. B. Biofilm formation by 30-84 wild-type and WT-Enh. Bacteria were grown in AB-C, LB and PPMD in static plates for 48 h. Attached cells were stained with crystal violet and quantified by optical density 540 mm. Relative biofilm was calculated by standardizing to 30-84 wild-type. Data represent average of three replicates with standard errors.

Ecological consequences of enhanced phenazine production for interactions with the plant

Previously, quorum sensing mutants of strain 30-84 exhibited reduced colonization of the wheat rhizosphere in natural soil (Zhang & Pierson, 2001, Maddula *et al.*, 2006). To determine whether enhanced phenazine production could improve

wheat root colonization, WT-Enh was compared to strain 30-84 wild-type and 30-84ZN in the natural wheat rhizosphere soil mixed with sand (v:v, 2:1). Bacterial populations of 30-84 wild-type and WT-Enh (9.60 and 9.48 log units/g of roots, respectively) isolated from the roots were significantly higher than populations of 30-84ZN (8.54 log units/g of root, Fig. 3.14A). However 30-84 wild-type and WT-Enh established similar colonized population rates on wheat roots. These results suggest that although wild-type levels of phenazine production contributed to root colonization, enhanced phenazine production does not improve root colonization under these conditions.

In addition to inhibitory secondary metabolites, many plant growth-promoting rhizobacteria also produce or modulate plant hormones. While testing root colonization, I noticed that roots colonized by WT-Enh had significantly more root dry weight compared to 30-84 wild-type (14.6 mg compared to 10.5 respectively, Fig. 15B). However, WT-Enh showed similar levels of dry weight compared to 30-84ZN (12.08 mg). The data suggest that strain WT-Enh stimulated plant growth but not due to the enhanced phenazine production.

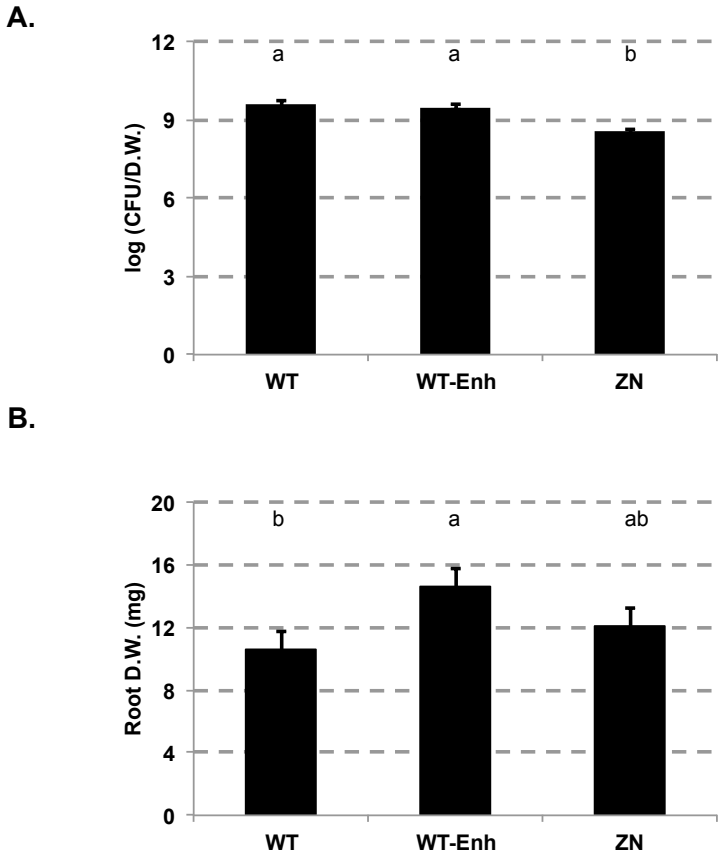


Figure 3. 14 Effect of enhanced phenazine production in root colonization and plant growth promotion.
A. Bacterial populations on wheat roots. B. Plant growth promotion by 30-84 derivatives. A and B. Pregerminated wheat seeds were treated with bacterial suspension of 30-84 wild-type, WT-Enh, and 30-84 ZN, and sown in natural soil mixed with sand (2:1, v:v). Thirty days after inoculation, plants were harvested, and fresh and dry root weights were measured. Bacterial populations are calculated as the log10 value of the colony forming units per gram of dry roots. Data represent average of eight separate plants with standard errors. These experiments were repeated once. Values with the same letter do not differ significantly as determined by a Fishers protected Least Significantly Difference (LSD) test (P>0.05).

Discussion

That phenazine production is regulated at multiple levels in all phenazine-producing strains studied to date is consistent the multiplicity of roles served by phenazines and thus potentially the multiplicity of cues regulating production. In *P. chlororaphis* 30-84, phenazines play a central role in the rhizosphere colonization,

pathogen inhibition and disease suppression, and biofilm formation (Pierson & Thomashow, 1992, Whistler & Pierson, 2003, Maddula *et al.*, 2006, Maddula *et al.*, 2008). Predictably, much attention related to the regulation of phenazines has focused on the promoter region of the phenazine biosynthetic genes, including the regions that interact with quorum sensing-mediated regulation (e.g the AHL signals or *phz* box) or other phenazine regulatory systems (e.g. transcriptional/translational regulatory proteins or small non-coding RNAs) (Khan *et al.*, 2005, Khan *et al.*, 2007, Li *et al.*, 2011, Selin *et al.*, 2012, Wang *et al.*, 2012a, Wang *et al.*, 2013, Ren *et al.*, 2014). In the present study, the functional elements of the promoter region, some of which are similar and some unique to the 30-84 phenazine biosynthetic promoter, were identified. Of particular interest was the identification of a negative regulatory element within the promoter region. To further characterize this regulatory element, I created a phenazine-enhanced derivative by deletion of the negative regulatory element and investigated the potential advantages of enhanced phenazine production for the application as a biological control perspective.

Analysis of the entire nucleotide sequence located between the translational start of the *phzR* and *phzX*, the first gene in the phenazine biosynthetic operon identified several features that may relate to gene regulation, including two palindromic sequences, two direct repeats (5'-CACCCCCAA-3') that flank a 112 bp sequence with low GC contents, and two *Ssp*I restriction enzyme sites [5'-AATATT-3' (Fig. 3.1)]. The nucleotide sequences of the promoter regions of *phzX* in strain 30-84 and *phzA* in *P. fluorescens* strain 2-79 are highly conserved, but sequence repeat features (direct repeats

and *Ssp*I repeats) were unique to 30-84, (Fig. 3.2, Khan *et al.,* 2005). Based on analysis of promoter fragments fused to *lacZ,* the first palindromic sequence (centered around a *Pst*I restriction enzyme site) is not required for promoter activity, but the second palindromic sequence (centered around a *Bgl*II restriction enzyme site) is required for promoter activity (Fig. 3.1 and 3.3). This region contains the putative *phz* box, with high sequence conservation to previously identified *phz* box sequences located near the -35 promoter region of *phz* operon in *P. fluorescens* strain 2-79 (Khan *et al.*, 2005) and *P. chlororaphis* strain PCL1391 and PA23 (Chin-A-Woeng *et al.*, 2001b, Selin *et al.*, 2012) (Fig. 3.1 and 3.2). The same sequence motif also can be found in the promoter region of *phzI* (Fig. 3.2). Validation of the requirement of the *phz* box sequence for promoter activity was confirmed by the absence of expression of a phenazine promoter derivative pKT2 BsP, which contains only half of the *phz* box (Fig. 3.3).

Interestingly, all phenazine promoter derivative plasmids had a low level of expression in *E. coli* strain DH5α (Fig. 3.3), even though DH5α does not contain a quorum sensing system comparable to PhzR/PhzI and does not regulate the expression of promoter derivatives in a quorum sensing dependent manner (data not shown). Although gene expression of all plasmids was low in *E. coli*, the fold-change in expression of the different promoter derivatives were similar those observed in *P. chlororaphis* 30-84, suggesting the mechanisms by which alterations of the promoter sequences affected gene expression may be related to general promoter functionality. Moreover, the complete absence of a need for quorum sensing activation for gene expression in *E. coli* suggests the interesting hypothesis that *P. chlororaphis* and other

Pseudomonas species with *phzR/phzI* quorum sensing have a mechanism to prevent gene expression prior to quorum sensing system activation. In *P. aeruginosa*, the repressor protein RsaL negatively regulates transcription of the *lasI* (*luxI* homolog, AHL synthase) by binding to promoter of the *lasI.* Mutation of the *rsaL* resulted in significantly higher production of AHL signal as well as other secondary metabolites (e.g. pyocyanin and hydrogen cyanide), which are regulated by quorum sensing system (Rampioni *et al.*, 2006, Rampioni *et al.*, 2007). The authors suggested that RsaL may act as a global regulator and controls biosynthesis of secondary metabolites by directly binds to target promoters or controls the amount of AHL signals production for their quorum sensing dependent transcription (Rampioni *et al.*, 2007). The gene, *rsaL* is located between *lasR* (*luxR* homolog, transcription regulator) and *lasI*, and shares a promoter region with *lasI* (Rampioni et al., 2006). However, there are no additional gene between *phzR* and *phzI* in *P. chlororaphis* 30-84*,* and blast search of both amino acid sequence and nucleotide sequence failed to reveal an RsaL homolog. Identifying a candidate protein that may have similar function in *P. chlororaphis* 30-84 is under investigation.

One of the unique findings of this study was the identification of a negative regulatory element in the *phz* promoter region. Many of the subsequent analyses were focused on trying to determine the mechanism(s) by which this regulatory element controls phenazine production. Similar to the phenazine biosynthetic genes, the expression of many secondary metabolites and their precursors is tightly orchestrated by multiple regulatory systems. Often the formation of secondary structure formation in the DNA or RNA are important for interactions with various regulatory elements (Brantl,

2004). My studies considered the possibilities that secondary structure played a role in transcriptional regulation, such as a riboswitch, or was involved in interactions with other regulatory proteins.

One of the best examples of transcriptional attenuation related to secondary structure is in the regulation of the tryptophan biosynthetic operon (*trpEDCBA*) in *E. coli.* This operon has a leader sequence region that contains imperfect two inverted repeat sequence sets (Yanofsky, 1981). These inverted repeats can self-hybridize to form stem-loop secondary structures when transcription begins resulting in either read-through or premature transcription termination depending on the environmental tryptophan concentration. Similar to this example, sequence analysis of the 5' untranslated region (UTR) of the *phz* operon of strain 30-84 predicted two loop structures between the *Ssp*I repeat sites and between the RBS and translation start site for DNA (Fig. 3.5A) and a stem loop structure between the second direct repeat and RBS for mRNA (Fig. 3.5B). Although site-specific mutagenesis to modify the stem loop structures showed no significant affect in β-galactosidase expression between promoter variants (Fig. 3.6), the possibility that these repeats play a role in transcriptional regulation cannot be ruled out. It is possible that these site-specific changes created other secondary structure, other regions are involved in loop structure formation, or that more than one motif is responsible for the repression that is alleviated by truncation of the entire negative element region.

The possibility that the negative regulatory region contained a riboswitch also was investigated. A riboswitch is a common bacterial regulatory component consisting

of a nucleotide sequence typically located in the 5' UTR of mRNAs. It regulates gene expression in response to the concentration of specific molecules (Wang *et al.*, 2012c), resulting in conformational changes to the mRNA structures and thus control of gene expression (Hollands *et al.*, 2012). Several studies have suggested that phenazines are capable of serving as signal molecules, altering the transcriptomic patterns of gene expression (Dietrich *et al.*, 2006, Wang *et al.*, 2016). Previous work also reported that phenazines may regulate phenazine production via interactions with 5' UTR of *phz* biosynthetic operon (Li *et al.*, 2011). Investigation of the transcriptional control of phenazine production by endogenously produced or supplying exogenous phenazines revealed no significant changes in expression levels of *phzX*, as measured by transcriptional fusions (Fig. 3.10). These results are not consistent with the hypothesis that phenazines serve as a signal molecule to regulate the expression of *phzX*.

A key phenotype of the 30-84 WT-Enh mutant is that enhanced phenazine production (Fig. 3.8A and 3.8C) is still regulated by the *phzR/phzI* quorum sensing system (Fig. 3.9B and 3.9C). This observation suggested that enhanced phenazine production may result from alterations in *phzR/phzI* mediated gene regulation. Given that the *phzR* gene and phz biosynthetic operons are divergently transcribed (Pierson *et al.*, 1994, Khan *et al.*, 2005, Khan *et al.*, 2007), it is possible that changes in the promoter region affected the expression of both genes. Greater phenazine production by WT-Enh relative to wild-type was correlated with higher transcript abundance of the *phz* biosynthetic genes as well as, *phzO.* Transcript abundance of *phzI* and *phzR* also were significantly higher in the WT-Enh than wild-type and higher expression of *phzR* in the

transcriptional fusion vector *in trans* also was observed (Fig. 3.11C). These results are consistent with PhzR quorum sensing mediated regulation of both *phzI* and the phenazine biosynthetic operon. These results suggest that the negative regulatory element also likely modulates the expression of the divergently transcribed *phzR,* resulting in higher levels of AHL signals and enhanced phenazine production.

The possibility exists for additional interaction between the negative element and other components of the complex network that regulates phenazine biosynthesis in 30-84, including the second quorum sensing system CasR/CsaI, the Gac/Rsm system and other known phenazine regulators including RpeA/RpeB, sigma factor RpoS, Pip and IopA/IopB (Pierson & Thomashow, 1992, Pierson *et al.*, 1994, Pierson *et al.*, 1995, Chancey *et al.*, 1999, Zhang & Pierson, 2001, Whistler & Pierson, 2003, Wang *et al.*, 2012a, Wang *et al.*, 2013). Comparison of the transcript abundance of these regulatory genes in wild-type and WT-Enh revealed that only the transcript abundance of *rpeA*, *rsmE*, and *rsmZ* were slightly increased. RpeA has been identified as a putative TCST sensor kinase that generally serves as a phenazine repressor (Whistler & Pierson, 2003, Wang *et al.*, 2012a). It is not clear that higher expression of RpeA could be involved. RsmE is a post-transcriptional regulatory RNA binding proteins, which in many other *Pseudomonas* species has been shown to be a negative regulator of gene expression by blocking target ribosome binding sites or targeting mRNA for degradation (Heeb *et al.*, 2002, Reimmann *et al.*, 2005, Lapouge *et al.*, 2008, Humair *et al.*, 2010). RsmE interacts with the small non-coding RNAs (ncRNA), *rsmX*, *rsmY*, and *rsmZ,* which have multiple stemploop structures that attract and sequester the RsmE protein, relieving repression of

translational initiation (Reimmann *et al.*, 2005, Sonnleitner *et al.*, 2006, Babitzke & Romeo, 2007, Wang *et al.*, 2013, Duss *et al.*, 2014). A recent study showed that in *P. chlororaphis* strain PA23, the *phzR/phzI* QS system both directly regulate *rsmZ* transcription by binding at a consensus *phz* Box in the promoter region of *rsmZ,* and indirectly activate *rsmE* transcription (Selin *et al.*, 2014). Similar to *P. chlororaphis* strain PA23, a *phz* box was found in the promoter region of *rsmZ* in strain 30-84 (Fig. 3.15). Therefore, the phenomenon of higher transcriptomic abundance of *rsmE,* and *rsmZ* in WT-Enh may be due to the higher expression of *phzR* and AHL signal production, which in turn could lead to further enhancement in phenazine production.

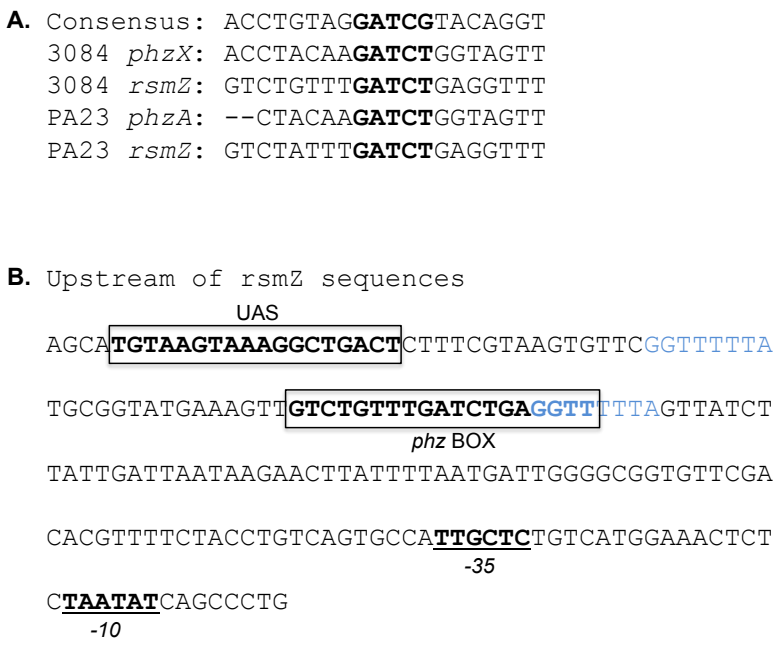


Figure 3. 15. Identification of *phz* box sequences in the *rsmZ* promoter region.
A. Alignments of the *phz* box sequences in the *phzX* and *rsmZ* in 30-84 compared to consensus sequence of *lux* box and promoter regions of *phzA* and *rsmZ* in *P. chlororaphis* strain PA23. The conserved sequences within the *phz* boxes are bolded (*Bgl*II enzyme site). B. Upstream of *rsmZ* sequences. The GacA recognition site, upstream activation sequences (UAS) was identified by previous study (Wang *et al.*, 2013) and labeled with bold. The -35 and -10 promoter elements are also indicated.

Lastly, a novel quorum sensing anti-activator protein QslA was identified recently in *P. aeruginosa* strain PAO1 (Seet & Zhang, 2011, Fan *et al.*, 2013). The mutation of *qslA* elevated AHL-dependent quorum sensing signaling, and increased the expression of quorum sensing dependent genes involved in bacterial virulence factors including pyocyanin (Seet & Zhang, 2011). QslA is hypothesized to function by interrupting the dimerization of LasR, and preventing LasR from binding to its target promoter of genes. In this manner the quorum sensing threshold and responses are tightly tuned by QslA (Fan *et al.*, 2013). That a QslA homolog may exist in *P. chlororaphis* 30-84 and differentially interact with the promoter fusions was an intriguing hypothesis. However a blast search of amino acid sequence failed to reveal a QslA homolog in *P. chlororaphis* 30-84, but identifying a candidate protein that may have a similar function is under investigation.

Phenazine production by *P. chlororaphis* 30-84 contributes in multiple ways to its ecological fitness and biological control capabilities including pathogen inhibition, biofilm formation, root colonization and rhizosphere persistence (Mazzola *et al.*, 1992, Pierson & Thomashow, 1992, Whistler & Pierson, 2003, Maddula *et al.*, 2006, Maddula *et al.*, 2008). We hypothesized that enhanced phenazine production should result in enhanced biological control capabilities. Consistent with this hypothesis, *in vitro* dual culture assays revealed that 30-84 WT-Enh has greater ability to inhibit fungal growth of the *G. graminis* than 30-84 wild-type (Fig. 3.13A). Microtiter biofilm assay also revealed better biofilm formation by strain 30-84 WT-Enh compared to 30-84 wild-type

(Fig. 3.13B). Although enhanced phenazine production did not provide better colonization of wheat roots (Fig. 3.14A), it did result in enhanced root growth. Interestingly, strain 30-84ZN also slightly improved root biomass compared to 30-84 wild-type (Fig. 3.14B). These results are consistent with metabolic analysis, showing 30-84ZN produced indole-3-acetic acid (IAA) 6 times the 30-84 wild-type levels (Pierson *et al.,* unpublished data). However, how 30-84WT-Enh improved plant growth is currently under investigation.

The regulation of secondary bacterial metabolite biosynthesis is likely to be tightly regulated to enable bacteria to control the production of optimum levels under different environmental conditions. In this study, we showed that the *phz* promoter in *P. chlororaphis* 30-84 contains multiple unique sequence motifs that may contribute to phenazine gene regulation. This study revealed the presence of a negative regulatory element that significantly limits the amount of phenazines that are produced. Encouragingly for biological control applications, generation of an enhanced phenazine-producing strain via the deletion of this region resulted in a strain with improved ability to form biofilms, inhibit target fungi, and promote wheat root growth. Future studies with the enhanced biological control strain will consider whether these capabilities result in better disease suppression.

Materials and Methods

Bacteria, plasmids and media

Bacterial strains and plasmids are described in Table 3.1, and primer sets that used in this study are listed in Table 3.3. A spontaneous rifampicin-resistant derivative of *P. chlororaphis* strain 30-84 was used and all mutants were derived from this parental strain. Unless otherwise indicated, all media formulations are as described previously (Maddula *et al.*, 2006, Maddula *et al.*, 2008, Wang *et al.*, 2012a). *P. chlororaphis* 30-84 and its derivatives were grown at 28 °C in Luria-Bertani medium (LB) containing 5 g of NaCl per liter, King's B medium (KMB), M9 minimal media, AB minimal media amended with 2% casamino acids (AB-C) (Difco, Franklin Lakes, NJ), or pigment production medium-D (PPM-D). Where applicable, antibiotics were used at the following concentrations (μg/ml) for *Escherichia coli*: ampicillin (100), gentamycin (15), kanamycin (50), and tetracycline (25); for *P. chlororaphis*: gentamycin (50), kanamycin (50), rifampicin (100), tetracycline (50) and piperacillin (50).

DNA manipulations, sequence analysis, and PCR

Standard methods were utilized for plasmid DNA isolation, restriction enzyme digestion, ligation, transformation, and agarose gel electrophoresis (Sambrook & Russell, 2001). Plasmids were introduced into *P. chlororaphis* 30-84 and derivatives using triparental matings as described previously (Pierson & Thomashow, 1992, Pierson *et al.*, 1994, Maddula *et al.*, 2008). Standard PCR reactions were carried out using *Taq* DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA) in a final volume of 50 μl. Reaction conditions consisted of 95^{o}C for 5 min, followed by 25-30 cycles of 95 °C for 30 s, 55 °C (or recommended primer's Tm temperature) for 30 s, 72 °C for 90 s and a final extension period of 72^{o}C for 10 min. DNA sequencing was performed by the

Laboratory for Genome Technology within Institute for Plant Genomics and Biotechnology, Texas A&M University using an ABI 3130xl Genetic Analyzer. Nucleotide and amino acid sequence data was analyzed using BLAST programs on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST).

Quantification of phenazine production

To quantify phenazine production, 3 colonies were selected from different plates and grown overnight in PPMD, LB or AB-C broth at 28 °C with rapid aeration. Optical densities were adjusted to $OD_{620} = 0.8$ and 10 microliters of the adjusted culture was re-inoculated into 10 ml of fresh medium. As described previously (Pierson & Thomashow, 1992, Maddula *et al.*, 2008), after 24 h growth at 28 °C with rapid aeration, cell-free supernatants were harvested by centrifugation and supernatants were acidified to approximately pH 2 with concentrated HCl. Total phenazines were extracted in an equal volume of benzene, and the separated benzene phase was evaporated under air. Extracted phenazines were dissolved in 0.2 N NaOH and quantified by UV-visible spectroscopy. The relative amounts of phenazines were calculated by their absorption maxima and standard extinction coefficients as described previously (Olson & Richards, 1967, Jayatilake *et al.*, 1996, Maddula *et al.*, 2008).

Quantification of β-galactosidase activity

Qualitative β-galactosidase activity was measured by streaking colonies onto selective medium amended with 2% X-gal and visually scoring the plates for blue after 24-48 h. Qunatitative β-galactosidase activity was carried out from 3 ml of cultures

grown at 28 °C for 24 h with shaking in LB, AB-C or M9 minimal media broth as described previously (Miller, 1972). Briefly, the optical densities (OD_{620}) of overnight cultures were measured and 500 µl of cultures were mixed with equal volume of Z-buffer (final concentration of 0.06M $Na_2HPO_4.7H_2O$, 0.04M $NaH_2PO_4.H_2O$, 0.01M KCl, 0.001M $MgSO_4$ and 0.05M β-mercaptoethanol). To permeabilize the cells, 60 µl of $CHCl_3$ was added and cultures were vortexed for 10 sec. The assay was started by adding 200 µl of 4 mg/ml ONPG (*o*-Nitrophenyl-β-galactoside) and the reaction was stopped by adding 500 µl of stop buffer (1M Na_2CO_3) when the mixtures turned sufficiently yellow. After the cell debris was pelleted by centrifugation for 10 min, supernatants were collected and the optical density was measured at OD_{415} and OD_{540}. Miller units were calculated by the following formula 1000 x [(OD_{415}-1.75x OD_{540})] / (Time x Volume x OD_{620}) as described previously, (Miller, 1972).

Time course assay of phenazine quantification and β-galactosidase Assays

Time course assays were performed by inoculating fresh AB+2% CAA broth (20 ml) at a 10^{-2} dilution with cells from an overnight culture grown with shaking at 28 °C, and growing the cells with rapid aeration at 28 °C. Measurement of cell density, phenazine production and β-galactosidase activity were performed periodically until cells entered late stationary phase as described above.

Construction and analysis of plasmids containing the phz promoter derivatives

Analysis of promoter activity was carried out by cloning various sequence fragments from the promoter region into the promoter trap vector pKT2-*lacZ* (Wang *et al.*, 2012a), resulting in the plasmids: pKT2VS, pKT2PS, pKT2F1R1, pKT2F1R2,

pKT2BsP and pKT2PsP (Fig. 3.3 and Table 3.1). The vector pKT2*lacZ* contains a promoterless *lacZ* gene with its own ribosome binding site (RBS) located downstream of multiple cloning locus containing unique *Eco*RI and *Bam*HI sites. Plasmids were conjugated into 30-84Ice via triparental mating and the promoter activity of the various promoter sequence fragments was determined by β-galactosidase activity in 30-84Ice after 24 h growth in LB and M9 medium. 30-84Ice contains a *phzB::inaZ* insertion (Table 3.1) and as a consequence does not produce phenazine, which interferes with the β-galactosidase assay.

Sequences tested for promoter activity include a 0.8-kb *Eco*R1-*Bam*HI fragment containing intergenic region between *phzR* to *phzX* as determined from subcloning derivatives of cosmid pLSP259, which contains quorum sensing system *phzI/phzR* and phenazine biosynthetic operon (*phzXYFABCD*) (Pierson & Thomashow, 1992). The plasmid containing this fragment is designated pKT2VS (Fig. 3.3). Using pKT2VS as a template, a 356 bp fragment containing the *Pst*I and *Bgl*II sites, the two direct repeats and the AT-rich region with unique *Eco*RI and *Bam*HI was amplified using PphzF1 and PphzR1 primers to construct pKT2F1R1 (Fig. 3.3). Amplification using primers PphzF1-PphzR2 yielded a 245 bp fragment containing the *Bgl*II and *Pst*I sites and the first direct repeat with unique *Eco*RI and *Bam*HI ends that were used to construct pKT2F1R2 (Fig. 3.3). A subclone that contained half of the *Bgl*II site to the PphzR1 primer site was constructed by inserting the *Bgl*II fragment containing the *Bgl*II-*Sal*I region and *lacZ* from pKT2F1R1 into the *Bam*HI and *Bgl*II sites of pKT2. The resulting plasmid was named pKT2BgS (Fig. 3.3). The 0.3 kb *Pst*I-*Sal*I region was cloned by digestion of

pLSP202-0.8 with *Pst*I and insertion of the 4.3 kb fragment into the *Pst*I site in pUC18 creating pUC18-PS*lacZ*. Plasmid pUC18-PS*lacZ* was digested with *Eco*RI and *Hin*dIII and inserted into the *Eco*RI-*Hin*dIII sites of pKT2*lacZ* resulting in pKT2PS (Fig. 3.3). Located within the A/T-rich region flanked by the two direct repeats are two *Ssp*I sites at bases 574 and 599, respectively. The promoter sequence from *Pst*I to the first *Ssp*I site with additional *Eco*RI and *Bam*HI sequences at the 5' and 3' ends was synthesized and inserted into the *Eco*RI-*Bam*HI site of pUC57 (GenScript, Piscataway, NJ). The 127 bp fragment containing the *Pst*I to the first *Ssp*I sites, the first direct repeat with unique *Eco*RI and *Bam*HI ends were used to construct pKT2PsP (Fig. 3.3). A list of plasmids and primers are given in tables 1 and 2.

Characterization and disruption of 185 bp negative regulatory sequence

To disrupt the proposed stemloop within *phzX* promoter region, variants of the *phzX* promoter sequence between *Pst*I and *Sal*I sites were synthesized (GenScript) each having flanking *Eco*RI and *Bam*HI restriction enzyme sites. Each promoter variations has a modification to at least one sequence motifs shown in Fig 3.6A: modifications included changes to either the direct repeat sequence (5'-CACCCCCAA-3' to 5'-CACTATCAT-3'), the ribosome binding site sequence (5'-GGAGGA-3' to 5'-TATATTG-3'), or the translation start site (5'-ATG-3' to 5'-GCC-3'). The 0.3-Kb *Eco*RI – *Bam*HI fragments of pUC57-PS Var1, pUC57-PS Var2, pUC57-PS Var3 and pUC57-PS Var4 were cloned into pKT2*lacZ* resulting in pKT2-PS Var1, pKT2-PS Var2, pKT2-PS Var3 and pKT2-PS Var4, respectively (Fig. 3.6A, Table 3.1). To disrupt the stem loop formation between two *Ssp*I sites, 0.3-Kb fragment containing *Pst*I and *Sal*I

sites was PCR amplified using pKT2-PS as a template with primer set pPhzPS1 and pPhzPS2 (Table 2). Subsequently this fragment was cloned into the plasmid pCR2.1-TOPO vector (Invitrogen) by TA cloning. Plasmid TOPO-PS was digested with *Ssp*I and re-ligated to remove 22 bp flanked by *Ssp*I sites. The plasmid TOPO-PSΔ*Ssp*I was digested with *EcoR*I – *Bam*HI and ligated into the *Eco*RI – *Bam*HI site in the pKT2*lacZ* resulting pKT2-PS Var5 (Fig. 3.6A, Table 3.1). The promoter activities of these plasmids were determined by β-galactosidase activity in 30-84Ice after 24 h growth in LB supplemented with Km.

To further characterize the effect of a 185 bp negative regulatory sequence identified in the promoter analysis, the sequence was expressed from the quorum sensing-independent promoter pTac. The 185 bp sequences from the *Ssp*I site at base 599 to the *Bam*HI site at base 763 was cloned as a *Bam*HI-*Bgl*II fragment into the *Bam*HI site within pKT2pTac*lacZ*. This construct placed the 185 bp region between the pTac promoter and a *lacZ* gene having its own ribosome binding site. Plasmids pKT2pTac*lacZ* and pKT2pTac185*lacZ* (Table 3.1) were introduced into strains 30-84Ice and 30-84W, a spontaneous *gacA* mutant, and β-galactosidase activity measured.

Generation of a phenazine enhanced mutant.

In order to generate a derivative with enhanced phenazine production, a deletion sequence for marker exchange into the chromosome was created a mutation of the negative regulatory region (Fig. 3.7A). This construct lacks the 90 bp of sequence starting at the +8 bp (from the transcription start site) to the ribosome binding site (deletion includes both *Ssp*I sites and the second direct repeat). To facilitate homologous

recombination, the fragment included flanking sequence upstream from the *Eco*RV site in the middle of *phzR* and downstream to the *Bam*HI site at the 3'-end of *phzY*. The resulting 1575 bp fragment was cloned into the unique *Bam*HI site in the pKT2*lacZ* (to create plasmid pKT2-pPhzEnh, Table 3.1) and introduced into 30-84Ice for measuring expression *in trans*.

The fragment also was cloned into pLAFR3 at the *BamH*I site for maker exchange into the genome of 30-84, respectively. The pLAFR3 plasmid containing pPhz-Enh was introduced into the strain 30-84WT and 30-84ZN containing pUCP18-RedS via triparental mating and the 90bp deletion of *phzX* promoter was marker exchanged into each via homologous recombination with the support of λ phage recombinases (Lesic & Rahme, 2008, Wang *et al.*, 2012a). A dark orange (for 30-84WT) or dark blue (30-84ZN) colony was chosen, and pUCP18-redS plasmid was cured by streaked out to the LB with 5% sucrose. A Tc^s, Pip^s, and $Sucrose^R$ colony was chosen and mutation was verified by PCR and sequencing.

Construction of phzX-lacZ and Enh-phzX-lacZ and phzR-lacZ transcriptional fusions

In order to test the possibility that the 90 bp fragment deleted from the enhanced promoter functions as transcriptional regulator, transcriptional fusions were constructed. The reporter plasmids pGT2*WX*lacZ and pGT2*EX*lacZ contained flanking sequence from -124 bp to 29 bp downstream of *phzX* translation start site and were amplified from genomic DNA 30-84 wild-type and 30-84 WT-Enh using primer set phzXTXF-phzXTXR (Fig 3.10A, Table 3.1 and 3.2). These fragments contain *phzX* promoter region, ribosome binding site, transcription and translation start site and the first 29 bp of

the *phzX* gene (Fig. 3.10A). Following amplification purified PCR products were ligated into TOPO vector (Invitrogen) by TA cloning. Then TOPO vectors containing 268 bp and 178 bp fragments of *phzX* promoter region were digested by *Eco*RI and *Bam*HI and ligated into the promoter trap vector pGT2*lacZ.* The transcriptional fusions were introduced into strain 30-84 wild-type, 30-84 PCA and 30-84 Ice by triparental mating.

Supplementation with exogenous phenazine

To determine whether phenazine derivatives have a role as a transcriptional signal molecule, purified PCA and 2-OH-PCA was added to LB attain a final concentration of 100 μg/ml. Bacterial overnight cultures of strain 30-84Ice harboring pGT2*WXlacZ* or pGT2*EXlacZ* were re-inoculated with 10^{-2} dilution into 10 ml of LB supplemented with each phenazine derivatives. Samples were taken from each time point, and subsequently promoter activity was measured by β-galactosidase activity. Separation of PCA and 2OHPCA were achieved by modifying the pH during the extraction process and equal volume of 0.2N NaOH was used for the negative control of exogenous phenazine (Wang *et al.*, 2016).

RNA preparation for quantitative PCR

Single colonies of strain 30-84 from three separate AB-C plates were chosen and grown overnight at 28 °C in AB-C with rapid aeration. Thirty microliters of overnight cultures (1:100 dilution) were re-inoculated into 3 ml of fresh AB-C broth and grown at 28 °C with shaking (200 rpm) until OD_{620}=1.2 was reached. Prior to RNA extraction, the RNA was stabilized by the addition of 2 ml Qiagen RNA Protect reagent to 1 ml samples. Cells were harvested by centrifugation for 10 min at 4000 rpm. Total RNA was

extracted using a Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's recommended protocol. The genomic DNA was removed using on-column DNase-I digestion (Qiagen), and purified total RNA was quantified using a GE Nanovue spectrophotometer (GE Healthcare). The cDNA was synthesized by reverse-transcriptase reaction using SuperScript III reverse transcriptase (Invitrogen) with random primers (Invitrogen) at 50 °C for 1 h and inactivated the reaction by 70°C for 15 min. Negative controls for the qPCR analysis consisted of either sterilized water or extracted RNAs that were subjected to the same reaction conditions without adding reverse transcriptase enzyme.

qPCR methods and analysis

SYBR Green reactions were performed using the ABI 7900 HT Fast System (Applied Biosystems, Foster City, CA) in 384 well optical reaction plates. qPCR was performed at the at the Texas A&M Genomics and Technology Laboratory using a previously described method with a few modifications (Wang *et al.*, 2012a). Aliquots (1 μl) of cDNA (2 ng/reaction) or negative controls were used as template for qPCR reactions with Fast SYBR Green PCR Master Mix (Applied Biosystems) and primers (500 nM final concentration). Primer pairs used to detect the expression of *phzI, phzR, phzX, phzY, phzF, phzA, phzB, phzC, phzD, phzO, gacA*, *gacS*, *rpeB*, *rpeA*, *csaI*, *csaR*, *pip*, *hfq*, *rsmA, rsmE, rsmZ, rpoD*, 16S rDNA and *rpoS* are provided in Table 3.2. qPCR amplifications were carried out at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, and a final dissociation curve analysis step from 65°C to 95°C. Technical replicate experiments were performed for each of the

triplicate biological samples. Amplification specificity for each reaction was confirmed by the dissociation curve analysis. Determined Ct values were then used for further ΔΔCt analysis. The 16S rDNA and *rpoD* genes were used as the reference genes to normalize samples and similar results were obtained. A relative quantification (RQ) value was calculated for each gene with the control group as a reference (Wang *et al.*, 2012a, Wang *et al.*, 2013).

AHL extraction and quantification

Total AHLs were extracted from 5 ml cultures, which were grown at 28 °C with rapid agitation in 3 different media, AB-C, LB and PPMD. When cell density reached OD_{620} of 1.6, the cultures were centrifuged for 10 min at 4000 rpm and supernatants were mixed with an equal volume of acidified ethyl acetate. The ethyl acetate phase was evaporated in the hood and the dried extracts containing AHLs were suspended with an equal volume of same media (AB-C, LB and PPMD) to the original culture. Followed by filter-sterilization, AHL production was quantified by inoculating the extracted AHLs with the AHL-specific reporter strain 30-84I/Z (*phzI*⁻*,phzB::lacZ*). 30-84I/Z is deficient in AHL production due to mutation of AHL synthase gene *phzI,* but responds to AHL by producing the reporter gene *lacZ.* The β-galactosidase activity was determined on cultures grown at 28 °C for 24 h with shaking.

Construction of phzR transcriptional reporter

The *phzR* transcriptional reporter was constructed to compare *phzR* expression in 30-84 wild-type and WT-Enh. The PCR fragment that contained flanking sequence with unique *Eco*RI and *Bam*HI from -297 bp to 62 bp downstream of *phzR* translation start

site was amplified using primer set phzRTXF-phzRTXR (Table 3.2). This 443-bp PCR fragment contains *phzR* promoter region was ligated into TOPO vector (Invitrogen) by TA cloning. Then TOPO-phzWR were digested by *Eco*RI and *Bam*HI and ligated into the promoter trap vector pGT2*lacZ* to make the final *phzR* transcriptional fusion, pGT2*WRlacZ* (Fig. 3.10A and Table 3.1). This reporter was introduced into strain 30-84 wild-type and WT-Enh by triparental mating.

DNA probe and total protein preparation for band-shift assay

The DNA probes for band-shift assay were obtained by PCR amplification using the primer set phzXTXF-phzXTXR (Fig. 3.12A and Table 3.2) with genomic DNAs from 30-84 wild-type and 30-84 WT-Enh as templates and FideliTaq DNA polymerase (Affymetrix) with 10 mM biotin-labeled dCTP (Biotium). Subsequently PCR products were gel purified using Wizard SV gel and PCR clean-up system (Promega). The concentration and purity of DNA probes were determined using GE NanoVue Plus spectrophotometer (GE:Healthcare Bio-Sciences Corp).

Total proteins for binding assay were obtained from crude extracts of bacterial cultures. Overnight cultures of 30-84 wild-type and WT-Enh in LB were re-inoculated in 20 ml fresh LB with 10^{-2} dilution. Bacterial cells were harvested at two time points: before and after QS system activation (OD_{620} = 0.4 and 1.0) by centrifugation at 4000 rpm for 20 min. The pellets were resuspended with 20 mM Tri-HCL (pH 7.5), followed by sonication for 30 sec intervals, 5 times. The suspensions were centrifuged at 12000 rpm for 10 min and supernatants were collected. The concentration of total protein from crude extract was determined by standard curve from Bradford assay.

Band-shift assay of DNA-protein interaction

DNA-protein binding reactions were prepared in a 20 µl final volume, containing 2 µg of crude extract, 50 ng of biotinlyated DNA probe, 0.01 mg/ml of poly [d(I-C)] in the binding buffer (10 mM HEPES at pH 7.4, 20 mM KCl, 1 mM $MgCl_2$, and 1 mM DTT). After the addition of DNA probe, binding reaction mixtures were incubated at room temperature for 30 min. Following incubation, 5 µl of 5X loading dye was added to the reaction mixtures, and 20 µl of binding reactions were loaded on a native 6% acrylamide gel. Electrophoresis was performed in 0.5 X TBE buffer at 100 V for 1 h. DNA-protein binding bands from the gel were transferred to nitrocellulose membrane and detected with Lightshift™ Chemiluminescent Kit (Thermo Scientific, Waltham, MA) according to manufacturer's instruction.

Fungal inhibition assay

To quantify the ability of strains 30-84 wild-type and WT-Enh to inhibit the plant pathogen *G. graminis,* the *in vitro* dual culture assay was conducted. Fungal strain was cultured for 5 days on potato dextrose agar (PDA), and a 3-mm-diameter plug was taken from the edge of the colony and transferred onto the center of fresh PDA plate. After 2 days of growth, bacterial overnight cultures of strains 30-84 wild-type, WT-Enh were spotted onto edge of plate. Non-phenazine producing strains 30-84ZN and ZN-Enh were used as negative control. After 10 days, zone of inhibition was measured as the distance between edge of the bacterial colony and the fungal mycelium.

Microtiter plate biofilm assay

To measure the ability of strains 30-84 wild-type and WT-Enh to form a biofilm, static biofilm assays were conducted in 24-well polystyrene microtiter plates. Bacterial cultures were grown at 28 °C with rapid agitation in 3 different media (AB-C, LB and PPMD), and the overnight cultures were diluted with equal media that culture was grown to final cell density of OD_{620} was 0.8. Each strain was re-inoculated subsequently into equal media type with 10^{-2} dilution, and then 1.5 ml of each inoculum was transferred into 24-well plates. Plates were incubated at 28 °C for 48 h without shaking. Plates were inverted and tapped vigorously to remove non-attached cells, and remaining adherent cells were fixed by incubation for 20 min at 50 °C. Fixed bacterial cells were stained with 0.1% crystal violet and washed twice with sterilized distilled water. Adherent cells were decolorized with 20% acetone/ 80% ethanol solution for 5 min, and biofilm was quantified by optical density of OD_{540}. Each medium without cell inoculation was used as negative control. In order to reduce the variation in the retention of crystal violet between three separate experiments, data were normalized to the value of 30-84 wild-type in each media (e.g. values are expressed as relative expression to 30-84 wild-type)

Root colonization assay

To determine the ability of colonizing to the host plant, wheat seeds (cultivar TAM112) were surface sterilized and the bacterial strain 30-84 wild-type and WT-Enh were inoculated as previously described (Wood *et al.*, 1997). Briefly bacterial cultures were grown in 10 ml KMB with appropriate antibiotics for 24 h, and inoculum was prepared OD_{620} = 0.8 with 1X PBS. Seeds were surface sterilized using 0.6 % NaClO

(10% of commercial bleach) for 10 min, followed by rinsing in sterile-distilled water for five times. Surface sterilized seeds were pregerminated on water agar for two days to ensure sterility before planting. The seedlings were mixed with the bacterial inoculums for 10 min, and sown in 25 × 200 mm cone-tainers containing a natural wheat rhizosphere soil mix (soil: sand, 2:1, v:v). Plants were harvested after 30 days and entire root systems were rigorously washed to remove non-adhering bacteria. Roots were blotted dry on filter paper, weighed and immersed into 1 ml PBS. Bacteria were removed from whole roots by vortexing and sonication, and total populations were determined by serial dilution on LB agar amended with rifampicin.

Statistical Analysis

All data presented in this study are mean ± SEM from multiple determinations. Differences between strains were analyzed statistically using ANOVA and Fisher's protected Least Significant Difference (LSD) test ($P<0.05$).

CHAPTER IV

FUNCTIONAL ANALYSIS OF MIAA, THE TRNA MODIFICATION ENZYME IN THE BIOLOGICAL CONTROL AGENT *PSEUDOMONAS CHLORORAPHIS* 30-84

Summary

Pseudomonas chlororaphis 30-84 is a rhizosphere-colonizing biological control bacterium, with strong antifungal and plant disease suppression capabilities due mainly to the biosynthesis of phenazines. Expression of the phenazine biosynthetic operon in *P. chlororaphis* 30-84 is regulated directly by the PhzR/PhzI quorum sensing system as well as the Gac/Rsm signal transduction pathway. During the screening of a Tn5 plasposon library of *P. chlororaphis* 30-84, an additional regulatory element that positively influences phenazine production was identified. Sequence analysis revealed that the Tn5 inserted into the *miaA* gene, encoding a tRNA isopentenyltransferase. The objectives of this study were to characterize the role of MiaA in phenazine biosynthesis as well as its relationship to bacterial fitness and biological control activity, including bacterial growth, antifungal activity, exoenzyme production and soil persistence. Compared to wild type, production of phenazines was significantly decreased in the *miaA* mutant along with the production of the quorum sensing *N*-acyl-homoserine lactone signal. Moreover transcript abundances of *phzI* and *phzR* as well *pip*, as the transcriptional regulator previously shown to regulate quorum sensing, were significantly reduced in the *miaA* mutant. Use of transcriptional fusions to the promoters of these genes, as well as AHL quantification, and over expression of the genes *in trans*

in the *miaA* mutant confirmed these findings. However, transcript abundances and over expression of other key regulatory genes previously shown to regulate *pip* including RpoS the stationary sigma factor and RpeB a two component response regulator indicated that these were not transcriptionally regulated by MiaA. Given the well described effects of the *miaA* mutation on translation, translational fusions of RpoS and RpeB to the *lacZ* gene were constructed and indicated that MiaA affects phenazine production via a reduction in the translational efficiency of these two important phenazine regulatory genes. Furthermore, noticeable changes in bacterial growth and survival rate in soil were observed in the *miaA* mutant, suggesting a general reduction in the translational efficiency of genes dependent on MiaA. Consistent with decreased phenazine production, mutation in *miaA* resulted in reduced antifungal activity. These results suggest MiaA influences the production of secondary metabolites and other traits involved niche adaptation in *P. chlororaphis* 30-84 via its tight control of the translation of key regulatory genes.

Introduction

The ability to produce secondary metabolites is key component of biological control activity against plant pathogenic organisms in the rhizosphere. Many beneficial *Pseudomonas* species produced a variety antimicrobial products, including 2,4-diacetylphloroglucinol, phenazines, pyoluteorin, pyrrolnitrin, hydrogen cyanide, and exoenzymes, including chitinases and exoproteases. Studies have shown that the secondary metabolites produced by plant-benefical microbes aid in plant protection

either by direct inhibition of pathogen growth, competition with pathogens, plant growth promotion, or induction of host defenses (Bloemberg & Lugtenberg, 2001, Raaijmakers *et al.*, 2002, Haas & Défago, 2005, Lugtenberg & Kamilova, 2009). Therefore, understanding the specific roles of secondary metabolites and how they are regulated are crucial for improving their application for biological control.

Pseudomonas chlororaphis 30-84 was isolated from the wheat rhizosphere as a biocontrol agent against take-all disease of wheat caused by the ascomycete *Gaumannomyces graminis* var. *tritici* (Pierson & Thomashow, 1992). Although *P. chlororaphis* 30-84 produces a variety of secondary metabolites with antibiotic properties, phenazines have been shown to be are essential for pathogen inhibition and disease suppression (Pierson & Thomashow, 1992). Phenazines belong to a group of nitrogen-containing, heterocyclic ring-structured compounds (Mavrodi *et al.*, 2006). *P. chlororaphis* 30-84 produces three phenazine derivatives, phenazine-1-carboxylic acid (PCA), 2-hydroxy-phenazine-1-carboxylic acid (2OHPCA) and some 2-hydroxy-phenazine (2OHPZ) (Pierson & Thomashow, 1992). Phenazine- producing pseudomonads including *P. chlororaphis* 30-84, share a conserved phenazine biosynthetic operon (annotated as *phzXYFABCD* in *P. chlororaphis*), which encodes the seven enzymes required for the biosynthesis of the core phenazine PCA (Pierson *et al.*, 1995, Mavrodi *et al.*, 2006, Mentel *et al.*, 2009). PCA is then converted into other derivatives by one or more accessory genes encoding terminal modifying enzymes such as *phzO* in 30-84 (Delaney *et al.*, 2001, Mavrodi *et al.*, 2006). The many ecological roles of phenazines have been described elsewhere (reviewed in Chin-A-Woeng *et al.*, 2003,

Mavrodi *et al.*, 2006, Price-Whelan *et al.*, 2006, Pierson & Pierson, 2010, Mavrodi *et al.*, 2013). In *P. chlororaphis* 30-84, in addition to pathogen inhibition, phenazines are essential for bacterial rhizosphere persistence (Mazzola *et al.*, 1992), biofilm formation (Maddula *et al.*, 2006), and extracellular DNA release (Wang *et al.*, 2016). Phenazine production regulates the expression of many genes, including genes involved in oxidative stress response, cell autolysis, the production of other secondary metabolites and exoenzymes, phenazine and iron transport, and other efflux genes (Dietrich *et al.*, 2006, Wang *et al.*, 2016). Additionally, phenazines can induce plant defense responses (Leeman *et al.*, 1996b, Audenaert *et al.*, 2002, De Vleesschauwer *et al.*, 2006).

Pseudomonas species share a conserved and complex phenazine regulatory network (Mavrodi *et al.*, 2006, Gross & Loper, 2009, Mentel *et al.*, 2009). As in other *Pseudomonas* species, phenazine biosynthesis in *P. chlororaphis* 30-84 is regulated by the PhzR/PhzI quorum sensing system, which is located directly upstream of the *phz* operon (Pierson *et al.*, 1994, Wood & Pierson, 1996). PhzR is a LuxR type transcriptional regulator that activates *phz* gene expression in response to the accumulation of *N*-acyl-homoserine lactone (AHL) signals. PhzI is a LuxI homolog, AHL synthase that produces the AHL signals (Pierson *et al.*, 1994, Wood *et al.*, 1997). Previous work demonstrated that phenazines and the quorum sensing genes are regulated via a hierarchy of integrated regulatory networks including the Gac/Rsm signal transduction pathway (Chancey *et al.*, 1999). Additionally, Pip, a transcriptional regulator was shown to control the transcriptional regulation of both the quorum sensing genes and the phenazine biosynthetic operon. In turn, *pip*, is transcriptionally regulated

by a two-component signal transduction system (RpeA/RpeB), and a stationary sigma factor (RpoS) (Whistler & Pierson, 2003, Wang *et al.*, 2012a, Wang *et al.*, 2013). Another quorum sensing system present in *P. chlororaphis* 30-84, CsaI/CsaR was shown to be important in exoprotease production and cell aggregation, but plays a minor role in phenazine production (Zhang & Pierson, 2001).

This study was motivated by the identification of a Tn5 transposon-generated mutant that exhibited significantly reduced orange pigmented production (e.g. the mutated region positively regulates phenazine production). Sequence analysis of the mutant indicated that the Tn5 transposon genes were inserted into the gene annotated as *miaA* in the bacterial chromosome. The *miaA* gene encodes a tRNA prenyltransferase, which modifies the adenosine at the position next to the 3' side of the anticodon (position 37) in the tRNA species (Fig. 4.1) (Leung *et al.*, 1997). The tRNA modification enzyme catalyzes nucleosides during the tRNA modification process after the primary transcript is made. The major modifications can be found in the anticodon region in positions 34 (the wobble position) and 37 (Bjork, 1984). In *E. coli,* the tRNA prenyltransferase enzyme encoded by *miaA* catalyzes the addition of a Δ^2-isopentenyl group from dimethylally diphosphate (DMAPP) to the N^6-nitrogen of adenosine in position 37 of tRNA species (i^6A-37) (Fig. 4.1) (Bjork, 1984, Bjork *et al.*, 1987). After i^6A-37 is formed by MiaA, the tRNA is further methylthiolated by the action of the MiaB (thiolation) and MiaC (methyl transfer) enzymes to form 2-methylthio-N^6-(Δ^2-isopentenyl) adenosine (ms^2 i^6A-37) (Esberg & Björk, 1995, Esberg *et al.*, 1999). The *miaA* mutation causes a lack of ms^2 i^6A-37 in the tRNA, which results in defects in

translation efficiency, codon context sensitivity, and fidelity (Li *et al.*, 1997). Many studies have shown that tRNAs deficient in $ms^2 i^6A$-37 (*E. coli*) or $ms^2 io^6A$-37 (*Salmonella typhimurium*) caused broad pleiotropic phenotypes including decreased growth rates and yield (Ericson & Björk, 1986), altered sensitivity to amino acid analogs (Ericson & Björk, 1986), increased aromatic amino acid biosynthesis and transport (Buck & Griffiths, 1981), reduced *leu* operon expression (Blum, 1988), altered utilization of primary carbon sources (Tsui *et al.*, 1994), suppression of tetracycline resistance (Burdett, 1993), reduced *vir* gene expression in *Agrobacterium tumefaciens* (Gray et al., 1992), and reduced translational expression of *rpoS* (Thompson & Gottesman, 2014).

The objectives of this study were to characterize the role of MiaA in phenazine biosynthesis as well as its effects related to bacterial fitness and biological control activity, including bacterial growth, antifungal activity, exoenzyme production and soil persistence.

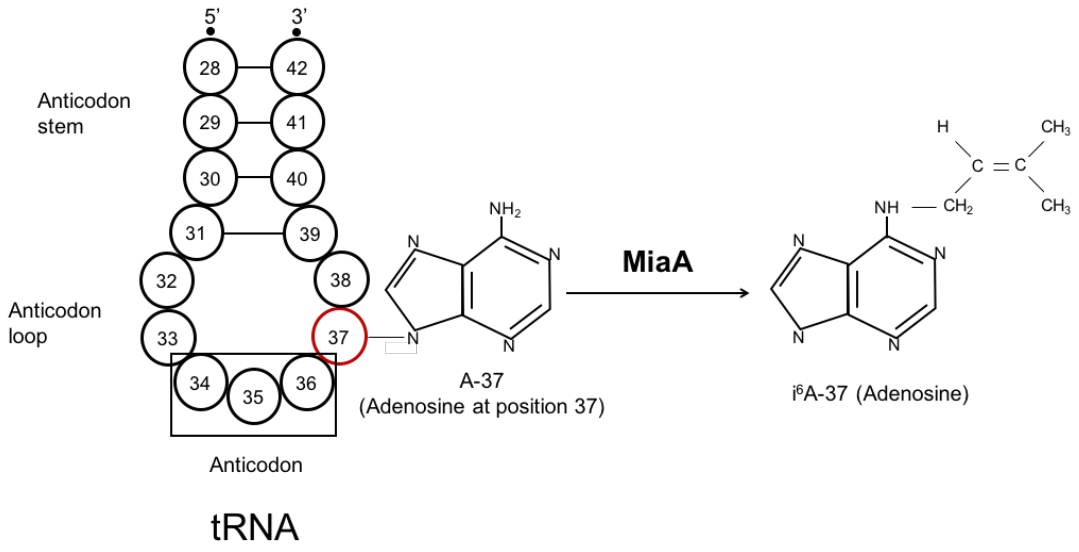


Figure 4. 1 Position 37 in tRNA species and biosynthesis of i^{6}A-37 mediated by MiaA.
A. Partial structure of tRNA species showing the anticodon stem, anticodon loop, and the site of anticodon (boxed). Red circled is represented of adenosine at position 37, which is the position modified by the enzyme, tRNA prenyltransferase. B. MiaA product, tRNA Δ^2-isopentenlypyrophosphate transferase catalyze adenosine at position 37 to make N^6-(Δ^2-isopentenyl)-adenosine to synthesis i^{6}A-37. Figure was modified from (Connolly & Winkler, 1989).

Results

Isolation of phenazine reduced mutant

During the screening of a Tn5 plasposon library of *P. chlororaphis* 30-84 created using pRL27::Tn5Km^R, a colony (ID: 30-84::19-30, Table 4.1) was isolated that appeared significantly reduced in orange pigment production, characteristic of 2-OH-PCA production. The reduction in phenazine production was confirmed in liquid and solid PPMD media, where the mutant exhibited less phenazine production (light orange color) than the wild type (dark orange color) (Fig. 4.2A and 4.2B). Sequence analysis of genomic DNA isolated from the mutant revealed that the transposon was inserted into a gene annotated as *miaA* (973 bp) at a position 785 bp downstream from the ATG start

codon. The *miaA* gene encodes a tRNA delta(2)-isopentenlypyrophosphate tansferase and is located between of *mutL* (DNA mismatch repair protein) and *hfq* (RNA chaperon protein) in the *P. chlororaphis* 30-84 genome (Fig. 4.2C). Complementation of the mutant via the introduction of multiple copies of the *miaA* gene (on pucMiaA, Table 4.1) restored the wild type phenotype, indicating that disruption of *miaA* was responsible for the phenotype (Fig. 4.2A and 4.2B).

The predicted protein encoded by *miaA* is 323 amino acids in length, and showed 94% amino acid identity to the MiaA protein in *Pseudomonas protegens* Pf-5 (GenBank accession: AAY95971). In addition, protein alignment revealed that the *P. chlororaphis* 30-84 MiaA shares 85%, 80%, 80%, 63% and 60% of amino acid identity with homologs in *Pseudomonas syringae* DC3000 (AAO58371), *P. aeruginosa* PAO1 (AAG08330), *P. putida* KT2440 (NP_746998), *Escherichia coli* K12 (BAE78172), and *Samonella enterica* serova. *Typimurium* (ADX20128). The multiple sequence alignment also revealed that the MiaA homologs were highly conserved between *Pseudomonas* species and other Gram negative bacterium (Fig. 4.3).

Table 4. 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
P. chlororpahis		
30-84WT	Phz^+ Rif^R wild-type	Pierson and Thomashow (1992)
30-84ZN	Phz^- Rif^R *phzB*::*lacZ* genomic fusion	Wood et al., (1997)
30-84ΔMiaA	Phz^+ Rif^R *miaA*::Tn5 Km^R (ID:30-84:19-30)	This Study
30-84I/Z	Phz^- Rif^R *phzB*::*lacZ phzI*::Km^R	Whistler and Pierson (2003)
E. coli		
DH5α	F^- *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*argF-lacZYA*) *I169* Φ80*lacZ* ΔM15λ^-	GIBCO-BRL
HB101	F^- *hsdS20*(r_B^- m_B^-) *supE44 recA1 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-5*λ^-	GIBCO-BRL
Plasmid		
pUCP20-Gm	Gm^R, pUCP20 with *Sma*I-flanked Gm^R cassette inserted into unique *Sca*I site within *bla*	Chiang and Burrows (2003)
pKT2pTac*lacZ*Tc^R	Km^R, Tc^R, pPROBE-KT2pTac*lacZ* containing constitutive promoter pTac with *Eco*RI-flanked Tc^R cassette inserted	Yu et al., unpublished
pGT2pTac*lacZ*	Gm^R, pGT2*lacZ* containing constitutive promoter pTac	Pierson et al., unpublished
pucMiaA	1106-bp DNA fragment containing *miaA* gene in pUCP20-Gm	This study
pucHfq	299-bp DNA fragment containing *hfq* gene in pUCP20-Gm	This study
pucRpoS	1405-bp DNA fragment containing *rpoS* gene in pUCP20-Gm	This study
KT2Pip	784-bp DNA framgnet containing *pip* gene in pKT2pTaclacZTc^R	This study
GT2RpeB	1.2-Kb DNA frament containing *rpeB* gene in pPROBE-GT'	Wang et al., (2012)
pGT2*lacZ*	Gm^R *lacZ* carried on promoter trap vector pPROBE-GT'	Yu et al., unpublished
pGT2*phzX-lacZ*	Transcriptional *lacZ* fushion to *phzX* promoter carried on pGT2*lacZ*	Yu et al., unpublished
pGT2*phzR-lacZ*	Transcriptional *lacZ* fushion to *phzR* promoter carried on pGT2*lacZ*	Yu et al., unpublished
pGT2*phzI-lacZ*	Transcriptional *lacZ* fushion to *phzI* promoter carried on pGT2*lacZ*	This study
pRL27	Plasposon vector of Tn5-RL27 (Km^R-oriR6 K)	Larsen et al., (2002)
pRK2013	IncP-I, *tra*RK2+, *rep*RK2, *rep*E1 Km^R	Ditta et al., (1980)
pME6015	pVS1-p15A shuttle vector for translational *lacZ* fusions, Tc^R	Blumer and Haas (2000)
pME6015-RpoS	968 bp fragment of 503 bp upstream and 465 bp downstream from translation start site of *rpoS* in pME6015	This study
pME6015-RpeB	463 bp fragment of 133 bp upstream and 330 bp downstream from translation start site of *rpeB* in pME6015	This study

[a] Km^R, Gm^R, Rif^R and Tc^R = kanamycin, gentamycin, rifampin and tetracycline resistance, respectively.

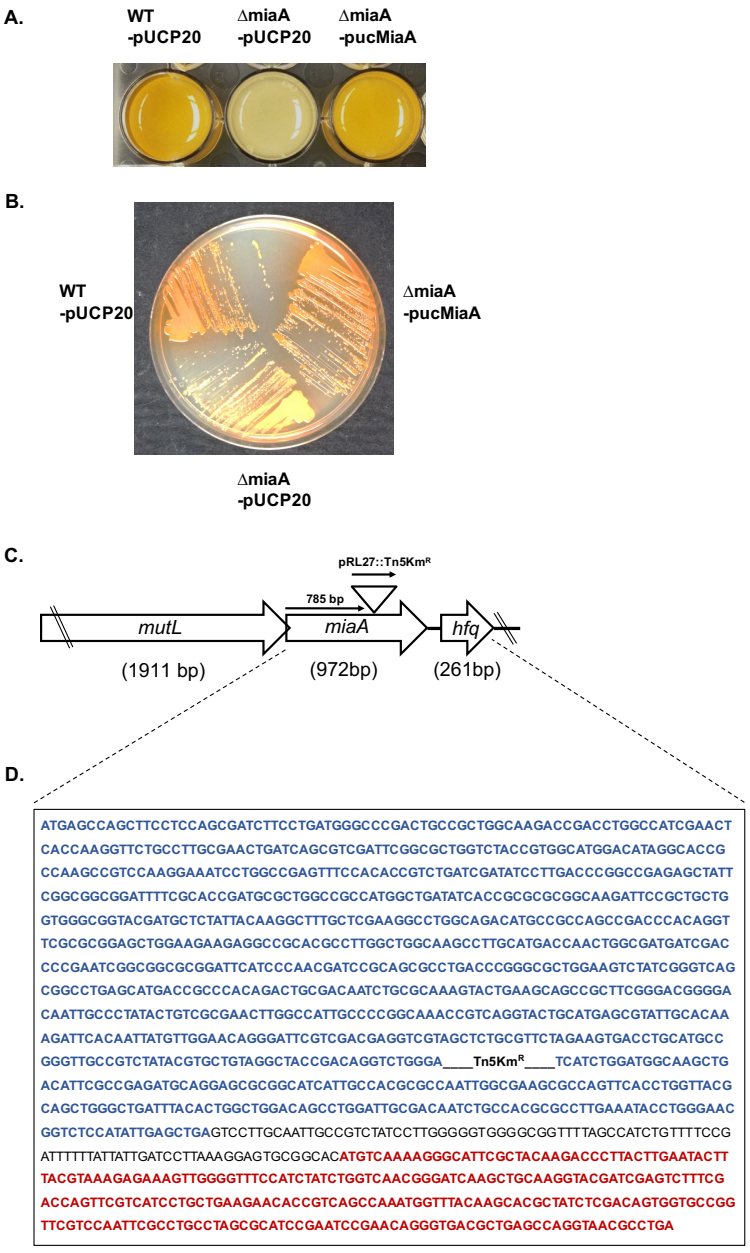


Figure 4. 2 Genomic organization of the chromosomal region of *P. chlororaphis* 30-84 surrounding *miaA* and phenotypes of the wild type, the *miaA* mutant and the complemented *miaA* mutant.
A. Pigment production by the wild type (containing the empty vector pUCP20-Gm), the *miaA* mutant (containing pUCP20-Gm), and the *miaA* mutant complemented by plasmid pucMiaA after growing in PPMD for 24 h at 28 °C with shaking. B. Pigment production by the same when grown on PPMD agar plates 36 h at 28 °C. C. The position of *miaA* and flanking genes in the chromosome of *P. chlororaphis* 30-84. Each open reading frame is represented by an arrow (indicating the transcriptional direction) and the sizes of the coding sequences are shown in parenthesis. An arrowhead indicates the position of the Tn5 insertion located at 785 bp from start codon of *miaA*. D. DNA sequences between *miaA* and *hfq* in *P. chlororaphis* 30-84. Blue-colored sequences indicate ORF of *miaA* and red-colored sequences indicate ORF of *hfq*. The location of the Tn5::Km^R is labeled.

Mutation in miaA results in decreased phenazine biosynthesis

Phenazine production by the *miaA* mutant was significantly lower than the wild type in all medium types, especially LB and AB-C medium where almost no phenazine was detected (Fig. 4.4A). For this analysis, bacterial cells were grown to an OD_{620} = 2.4 in three types of media: PPMD, LB and AB-C and quantitative spectrophotometry was used to determine the levels of phenazine produced. The complemented *miaA* mutant produced equivalent or slightly more phenazines than the wild type, indicating that the mutation in *miaA* mutant is responsible for the decreased phenazine production. The results demonstrate that a functional MiaA is required for robust phenazine production in *P. chlororaphis* 30-84.

The expression of the phenazine biosynthetic operon in wild type and the *miaA* mutant was quantified using a transcriptional fusion to the promoter of *phzX*, the first gene in the phenazine biosynthetic operon. The β-galactosidase activity of wild type containing *phzX-lacZ* was 218.44 ± 30.36 MU compared to 67.94 ± 25.21 MU in the *miaA* mutant (Fig. 4.4B). These results demonstrate that the reduction in phenazine production in the *miaA* mutant compared to the wild type is a result of reduced expression of the phenazine biosynthetic operon.

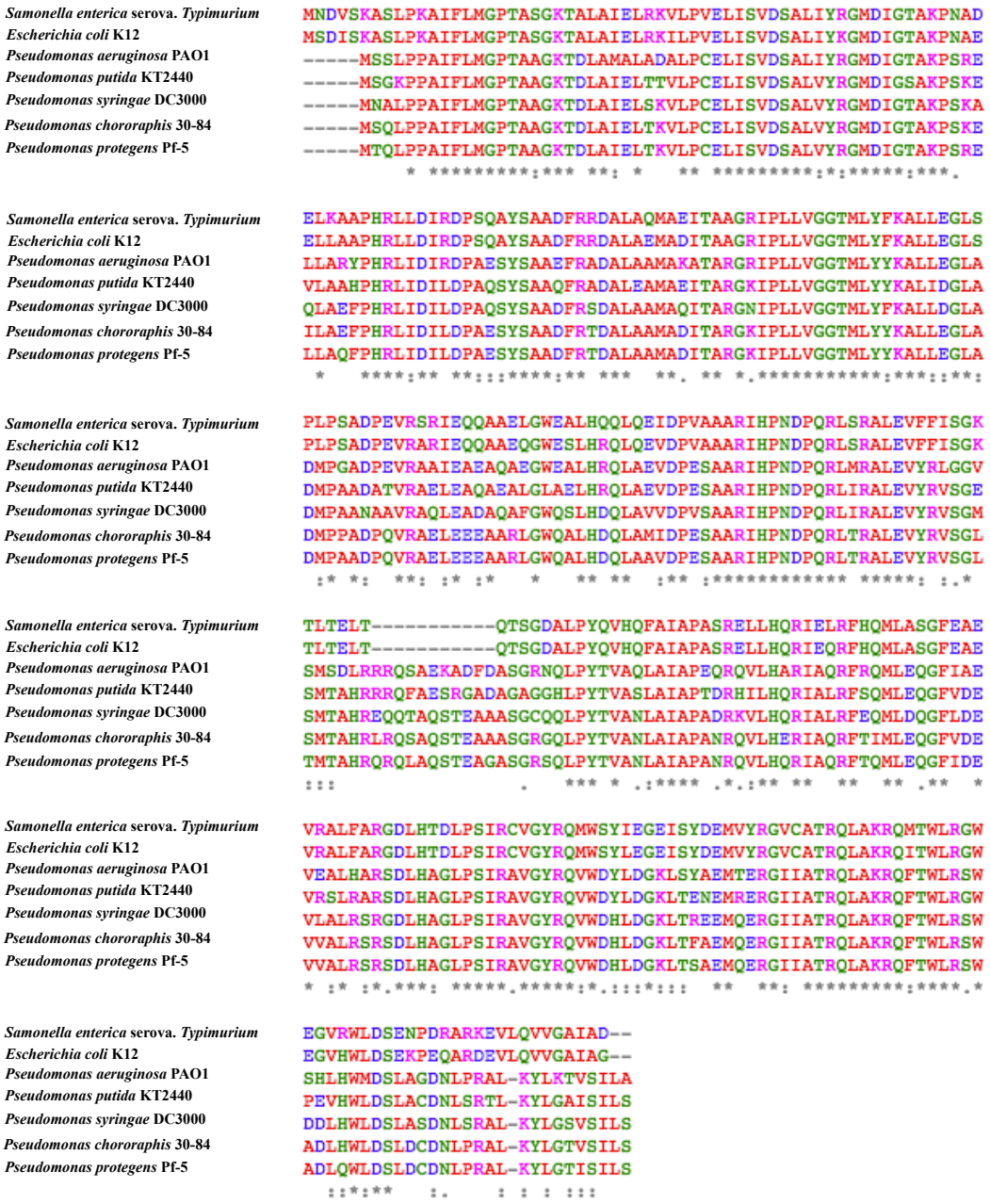


Figure 4. 3 Protein sequence alignments of MiaA homologues.
Amino acid sequences were obtained from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Identical residues (*), conserved (:) and semi-conserved (.) substitutions are indicated by the symbols located beneath each amino acid. Gaps introduced for alignment are indicated by dashes (-).

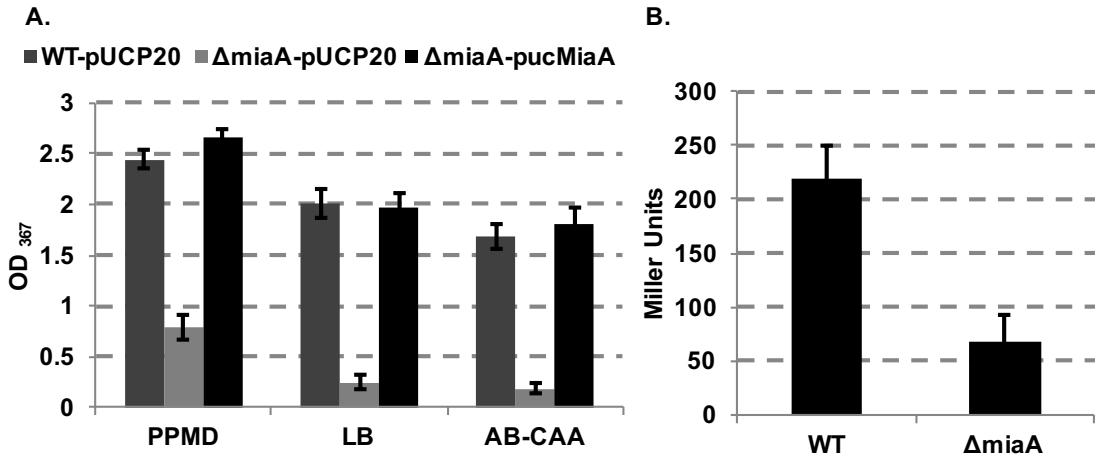


Figure 4. 4 Phenazine production for the wild type, the *miaA* mutant, and the complemented *miaA* mutant and the promoter activity of the phenazine biosynthetic gene *phzX*.
A. Phenazines were extracted from the wild type (containing the empty vector pUCP20-Gm), the *miaA* mutant (containing pUCP20-Gm), and the *miaA* mutant complemented by plasmid pucMiaA after growing in in different media (PPMD, LB and AB-C) at 28 °C with shaking until OD of 620 was reached 2.4. Phenazines were quantified by UV-visible light spectroscopy. Data are the average of 9 replicates and error bars represent standard errors. B. The activities of the *phzX* promoter was determined using transcriptional fusions to the *lacZ* reporter. Plasmids pGT2*phzX-lacZ* was introduced into the wild type and the *miaA* mutant and the expression of each promoter was measured as β-galactosidase activity. Data are the average of 9 replicates and standard errors are indicated.

MiaA affects growth of P. chlororaphis 30-84

The *miaA* mutant also grows more slowly than wild type and took significantly more time than wild type to reach OD_{620} = 2.4 for the phenazine production assays. Growth curves demonstrated that the *miaA* mutant exhibited a serious growth impairment compared to the wild type, even though specific growth patterns (entering exponential growth or doubling times) were similar (Fig. 4.5). In AB-C medium, the final cell densities of the *miaA* mutant (OD_{620} = 8.55 ± 0.076) also were significantly lower than observed for wild type (OD_{620} = 9.84 ± 0.174) at stationary phase (Fig. 4.5A). However, the growth of the complemented *miaA* mutant was fully restored to the wild

type level (OD_{620} = 9.7 ± 0.112, Fig. 4.5A). Similar patterns were observed for the growth rate of wild type and *miaA* mutant in M9 minimal medium with 2 % casamino acid (data not shown). The largest growth defect could be observed in the comparatively rich PPMD media, where the final cell densities of the wild type and *miaA* mutant were 11.25 ± 0.435 and 6.57 ± 0.645, respectively (Fig. 4.5B). Similarly, the impaired growth of the *miaA* mutant was fully restored to the wild type level in PPMD by complementation with the plasmid pucMiaA (10.98 ± 0.513). These results suggest functional MiaA is required for bacterial proper growth and that the growth defect is more pronounced in relatively rich media.

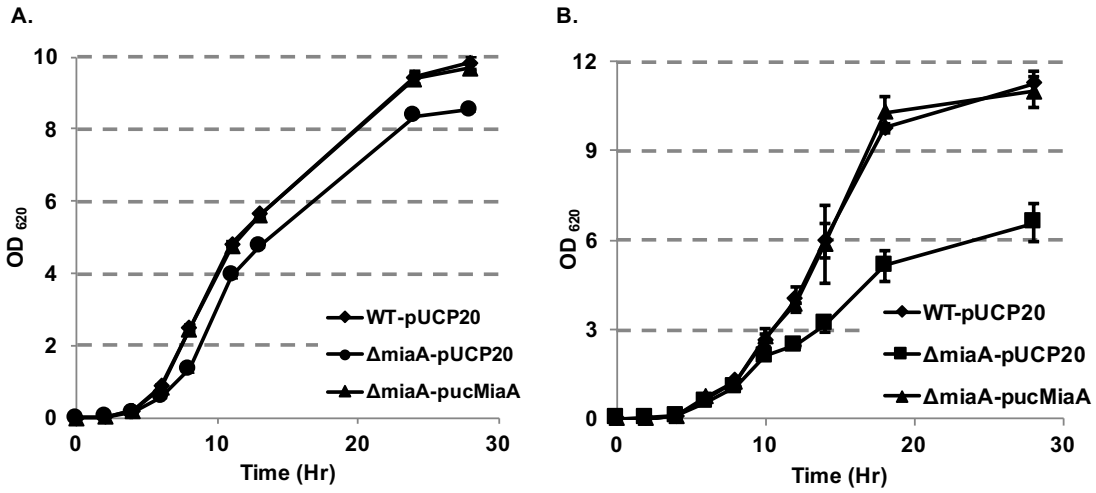


Figure 4. 5 Growth curves for the wild type, the *miaA* mutant and the complemented *miaA* mutant. Growth curves for the wild type (containing the empty vector pUCP20-Gm), the *miaA* mutant (containing pUCP20-Gm), and the *miaA* mutant complemented by plasmid pucMiaA in (A) AB-C and (B) PPMD. Cell densities of overnight cultures were normalized to an OD620 of 0.8, and 200 ⌠l of each culture was added to 20 ml of the same medium. The cultures then were grown at 28°C with rapid aeration and sampled once every 2 to 3 h until cells entered stationary phase. Data points are the average of 3 replicates with standard errors. The experiments were repeated twice and similar results were obtained.

Effect of miaA on expression of phenazine on quorum sensing and other regulatory genes

Regulation of phenazine biosynthesis in *P. chlororaphis* 30-84 is controlled by interactions between several regulatory systems/genes including the PhzR/PhzI quorum sensing system, the Gac/Rsm signal transduction pathway, *rpeA/rpeB,* the *rpoS, pip,* and *hfq* (Girard et al., 2006, Girard & Rigali, 2011, Wang et al., 2012a, Wang et al., 2013). To determine whether MiaA affects the expression of these phenazine regulatory genes, qPCR was used to measure transcript abundances. First the transcript abundances of *miaA* in the *miaA* mutant and the wild-type were determined. Transcript abundances of *miaA* in the *miaA* mutant were detectable, but significantly less than observed in the wild type (Fig. 4.6). The detection of a reduced amount of *miaA* transcript is consistent with the disruption of *miaA* by insertion of Tn5 (rather than deletion). The transcript abundances of the phenazine regulatory genes *phzX*, *phzI, phzR, gacA, gacS, rpeA, rpeB, rpoS, pip* and *hfq* also were determined in the *miaA* mutant relative to the wild type. Consistent with other findings in this paper, the transcript abundance of *phzX* was significantly lower in the *miaA* mutant than the wild type (Fig. 4.6).

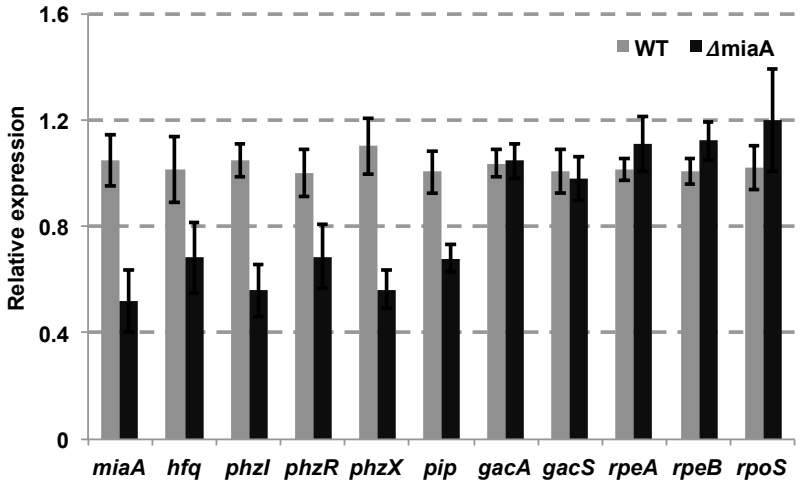


Figure 4. 6 The transcript abundance of several phenazine regulatory genes were measured in the wild type and *miaA* mutant.
The relative expression of *miaA, hfq, phzI, phzR, phzX, gacA, gacS, rpeA, rpeB, rpoS,* and *pip* in the wild type and the *miaA* mutant were determined by qPCR using *rpoD* as the reference gene. Cells were grown in AB-C medium to OD_{620} of 1.2 at 28 °C with shaking. Data points are the means of three biological replicates with three technical repeats ± standard error. Statistical analysis of ΔCt values confirmed that for each gene tested, non-overlapping error bars indicate significant differences in gene expression between strains. This experiment was repeated three times, and similar results were obtained.

Importantly the transcript abundances of *phzI* and *phzR* also were significantly decreased compared to wild type. The qPCR results were confirmed using transcriptional reporters with fusions between either the *phzI* or *phzR* promoter to *lacZ* and quantified gene expression as β-galatosidase activity. The expression of *phzI-lacZ* was significantly lower in the *miaA* mutant compared to the wild type (β-galactosidase activity of wild type was 304.24 ± 37.62 miller units (MU), whereas *miaA* mutant activity was 100.11 ± 15.51 MU, respectively) (Fig. 4.7A). The expression of *phzR* also was significantly lower in the *miaA* mutant compared to wild type (β-galactosidase activity of wild type was 239.30 ± 51.24 MU, whereas *miaA* mutant activity was 150.74

± 15.99 MU, respectively) (Fig. 4.7A). By comparison when the control plasmid having a constitutively expressed *lacZ* gene (pKT2pTac*lacZ*Tc^{R}) was used, no difference in the expression of *lacZ* in the wild type or *miaA* mutant was observed (981 ± 68.78 MU versus 969.1 ± 24.09 MU, respectively). These results confirm that in *P. chlororaphis* 30-84, MiaA specifically influences the transcription of both *phzI* and *phzR* and thereby phenazine biosynthesis, but has no effect on other promoter such as pTac.

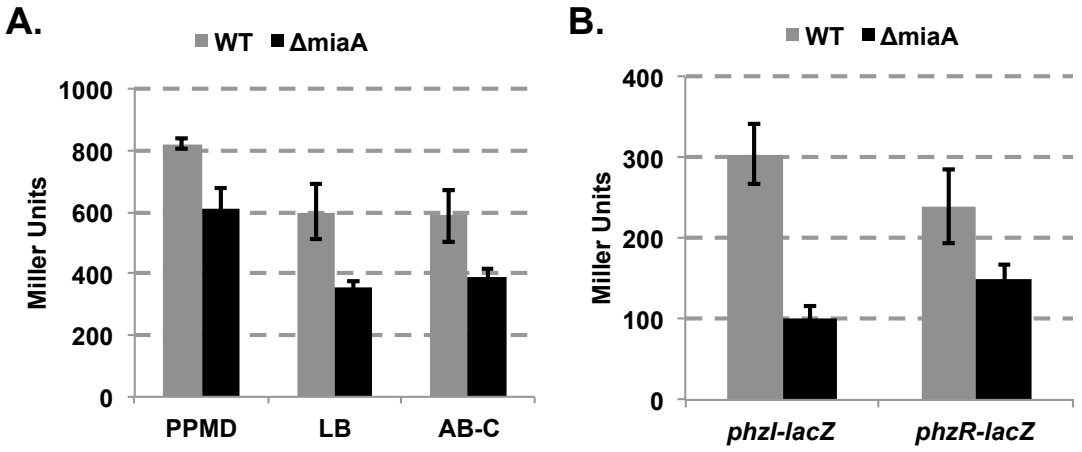


Figure 4. 7 Promoter activity and AHL production of the quorum sensing system, *phzR/phzI*.
A. The activities of the *phzI* and *phzR* promoters were determined using transcriptional fusions to the *lacZ* reporter. Plasmids pGT2*phzI-lacZ* and pGT2*phzR-lacZ* were introduced separately into the wild type and the *miaA* mutant, and the expression of each promoter was measured as β-galactosidase activity. Data are the average of 9 replicates and standard errors are indicated. B. Total AHLs were extracted from the wild type and the *miaA* mutant after growing in different media (PPMD, LB and AB-C) at 28 °C with shaking until OD620 = 1.6. AHLs were quantified based on β-galactosidase activity using the AHL-specific reporter 30-84I/Z (phzI-, *phzB*::*lacZ*) and are reported in Miller units. Data are the average β-galactosidase activity for 30-84I/Z based on 6 replicates and standard errors indicated.

To verify the amount of AHL produced by *P. chlororaphis* 30-84 and the *miaA* mutant, *P. chlororaphis* 30-84I/Z (*phzI*¯, *phzB*::*lacZ*) was used as a reporter of AHL abundance. For this assay AHL was extracted from cultures of the wild type and the *miaA* mutant, and the β-galatosidase activity of the *P. chlororaphis* 30-84I/Z reporter strain grown in the AHL-treated medium was quantified. The amount of AHL (measured as Miller units) produced by the *miaA* mutant was significantly lower than that produced by the wild type in all three media types, confirming that the reduction in phenazine expression in the *miaA* mutant was due in part to the decreased AHL accumulation (Fig. 4.7B).

To verify that the reduced phenazine production in the *miaA* mutant was due to reduced amounts of the quorum sensing regulator PhzR, multiple copies of *phzR* behind a constitutive promoter (GT2PhzR) were introduced separately into the wild type and *miaA* mutant, and phenazine production was quantified. As expected, constitutive expression of *phzR* by pGT2PhzR was able to fully rescue phenazine production in the *miaA* mutant (Fig. 4.8A and 4.8B).

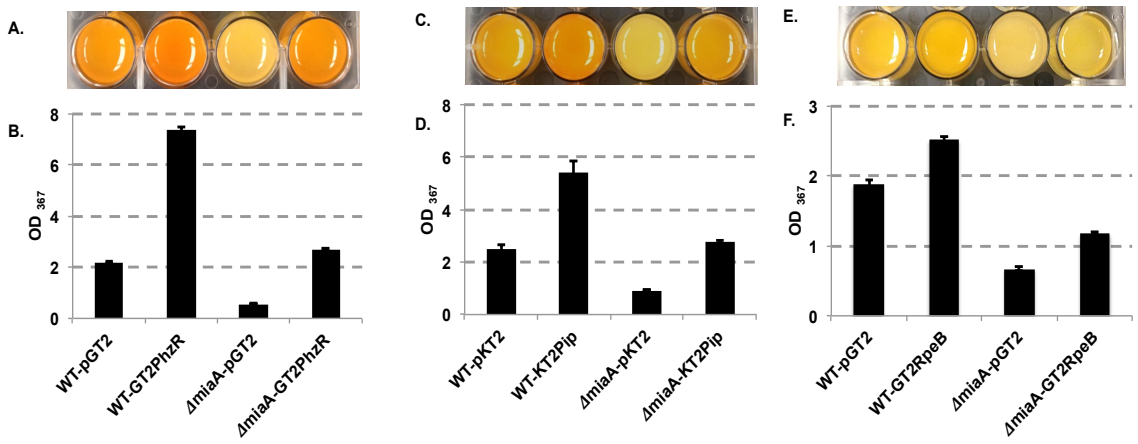


Figure 4. 8 Phenazine production by the wild type and *miaA* mutant with additional copies of *phzR, pip* and *rpeB.*
A. Pigment production and B. Quantification of phenazines produced by wild type and the *miaA* mutant containing either the empty vector pGT2pTac*lacZ* or pGT2PhzR in AB-C liquid medium. C. Pigment production and D. Quantification of phenazines produced by wild type and the *miaA* mutant containing either the empty vector pKT2pTac*lacZ*TCR or pKT2Pip in AB-C liquid medium. E. Pigment production and F. Quantification of phenazines produced by wild type and the *miaA* mutant containing either the empty vector pGT2pTac*lacZ* or pGT2RpeB in AB-C liquid medium. For all experiments, each strain was inoculated into AB-C liquid medium and grown at 28 °C with shaking at 200 rpm. After overnight, bacterial cultures were adjusted OD620 of 0.8 and sub-cultured 1:100 dilution to 5 ml fresh AB-C. Cultures were incubated at 28 °C with shaking at 200 rpm until an OD620 of 2.4 was reached. Phenazines were extracted with acidified benzene and quantified at OD 367. Data are the average of 8 replicates and standard errors are indicated.

Disruption of *miaA* also resulted in decreased *hfq* and *pip* transcripts in the *miaA* mutant compared to the wild type, but the transcript abundance of *rpeA, rpeB, gacA, gacS* and *rpoS* did not differ between the mutant and wild-type (Fig. 4.6). These results indicate that MiaA may influence the regulation of phenazine biosynthesis and quorum sensing via transcriptional control of *hfp, pip,* or both.

Reduced phenazine biosynthesis was not caused by a polar effect on hfq

The *miaA* gene is the last gene in an operon that consist of 5 genes (Pchl3084_0549, Pchl3084_0550, Pchl3084_0551, *mutL* and *miaA*) and is located

directly upstream of the *hfq* gene (Fig. 4.2C). Hfq is an RNA chaperon protein, which acts as a global post-transcriptional regulatory protein interacting with target mRNAs or facilitating the complementary base-pairing between small non-coding RNAs (sRNA) and mRNAs (Brennan & Link, 2007). Hfq was previously shown to positively regulate the production of bacterial secondary metabolites including antibiotics of 2,4-diacetylphloroglucinol (DAPG) and phenazine in *Pseudomonas* species (Sonnleitner *et al.*, 2003, Sonnleitner *et al.*, 2006, Wu *et al.*, 2010, Wang *et al.*, 2012b). In *Escherichia coli* K-12, the *hfq* gene contains three functional promoters, and two of them are located inside the open reading frame of *miaA* (Tsui et al., 1994, Tsui et al., 1996, Thompson & Gottesman, 2014). Therefore, it seemed possible that the reduction in phenazine production might be due to a polar effect of the Tn5 insertion, e.g. reduced expression of *hfq*.

To test this hypothesis, multiple copies of *hfq* (on pucHfq) were introduced into the wild type and the *miaA* mutant, and the amount of phenazine production (PPMD and AB-C) was quantified in different medium. In the wild type additional copies of *hfq* significantly increased phenazine production compared to the wild type (with the empty vector), demonstrating both the functionality of the construct and the positive role of Hfq in phenazine production (Table 4.2). However, no significant phenazine production was detected in the *miaA* mutants carrying the empty vector (pUCP20-GM) or when additional copies of *hfq* were introduced *in trans* (Table 4.2). These results suggest that even though Hfq has a positive role in phenazine production, the reduced phenazine production by the *miaA* mutant is not the direct result of a polar effect on *hfq* expression.

Table 4. 2. Effect of extra copy of *hfq* on phenazine production in different media

Strain	Phenazine absorbance in medium[a]	
	PPMD	AB-C
WT pUCP20	2.39 ± 0.08 b	2.1 ± 0.07 b
WT pucHfq	3.17 ± 0.28 a	3.08 ± 0.04 a
*Δ*miaA-pUCP20	1.18 ± 0.35 c	0.49 ± 0.08 c
*Δ*miaA-pucHfq	1.15 ± 0.35 c	0.53 ± 0.01 c

[a] Absorbance at 367 nm of phenazine extracted from culture supernatant at OD_{620} = 2.4 and resuspended in 0.1 N NaOH. The values are means ± standard errors based on eight replicates per treatment. Means followed by the same letter are not significantly different by Fisher's Least Significant Differences (LSD) (P > 0.05).

Phenazine production in the miaA mutant was fully rescued by expression of multiple copies of pip, but only partially rescued by rpeB

Previous work demonstrated that the transcriptional regulator Pip regulates both quorum sensing genes as well as the phenazine biosynthetic operon, and that *pip* in turn is transcriptionally controlled by both RpeA/RpeB (two-component signal transduction system) and RpoS (Stationary sigma factor) (Whistler & Pierson, 2003, Girard *et al.*, 2006, Wang *et al.*, 2012a). To characterize the effect of MiaA on the gene expression of *pip*, multiple copies of *pip* behind a constitutive promoter (KT2Pip) were transformed separately into the wild type and the *miaA* mutant, and phenazine production was quantified. As shown in Fig. 4.8C and 4.8D, introduction of the KT2Pip plasmid into both wild type and the *miaA* mutant significantly increased phenazine production compared to same strains carrying an empty vector. These results indicate that reduced

production of phenazine by *miaA* mutant was due to the reduced expression of *pip,* which leads reduction of quorum sensing system *phzI/phzR* activities.

To further characterize the hierarchical control of phenazine, extra copies of the upstream regulators of *pip*, e.g. *rpoS* (pucRpoS) and *rpeB* (pGT2RpeB) were introduced into wild type and *miaA* mutant. Overexpression of *rpoS* in the *miaA* mutant failed to restore the phenazine production, but wild type containing extra copies of *rpoS* exhibited a slightly increase in phenazine production in AB-C medium (Table 4.3). On the other hand, overexpression of *rpeB* in *miaA* mutant partially restored the phenazine production (Fig. 8E and 8F), which indicates that the reduction in phenazine production in the *miaA* mutant was due to partially decreased expression of RpeB.

Table 4. 3. Effect of extra copy of *rpoS* on phenazine production in different media

Strain	Phenazine absorbance in medium[a]	
	PPMD	AB-C
WT pUCP20	2.95 ± 0.06 a	2.14 ± 0.05 b
WT pucRpoS	2.79 ± 0.09 b	2.37 ± 0.05 a
*Δ*miaA-pUCP20	1.23 ± 0.07 c	0.69 ± 0.01 c
*Δ*miaA-pucRpoS	1.17 ± 0.03 c	0.71 ± 0.02 c

[a] Absorbance at 367 nm of phenazine extracted from culture supernatant at OD_{620} = 2.4 and resuspended in 0.1 N NaOH. The values are means ± standard errors based on eight replicates per treatment. Means followed by the same letter are not significantly different by Fisher's Least Significant Differences (LSD) (P > 0.05).

Because the transcript abundance of *rpeB* and *rpoS* in *miaA* mutant was not significantly different than in the wild type, I hypothesized that *miaA* might influence

one or both of these genes at the translational level of RpeB and RpoS. Moreover, if MiaA influence phenazine production by controlling RpeB and RpoS translationally, constitutive expression of *rpeB* and *rpoS* should not completely restore phenazine production in the *miaA* mutant. To test this hypothesis, translational fusions of *rpeB-lacZ* and *rpoS-lacZ* were constructed in pME6015 and introduced separately into wild type and *miaA* mutant. Translational efficiency of RpeB and RpoS was quantified by β-galactosidase activity in the wild type and *miaA* mutant. As shown in Fig. 4.9A and 4.9B, the expression of RpeB and RpoS were significantly lower in the *miaA* mutant compared to wild type (80.79 ± 3.49 and 569.9 ± 18.06 in the *miaA* mutant and 111.4 ± 7.42 and 713.9 ± 18.66 in the wild type). The results indicate that in *P. chlororaphis* 30-84, MiaA positively influences translation of RpeB and RpoS.

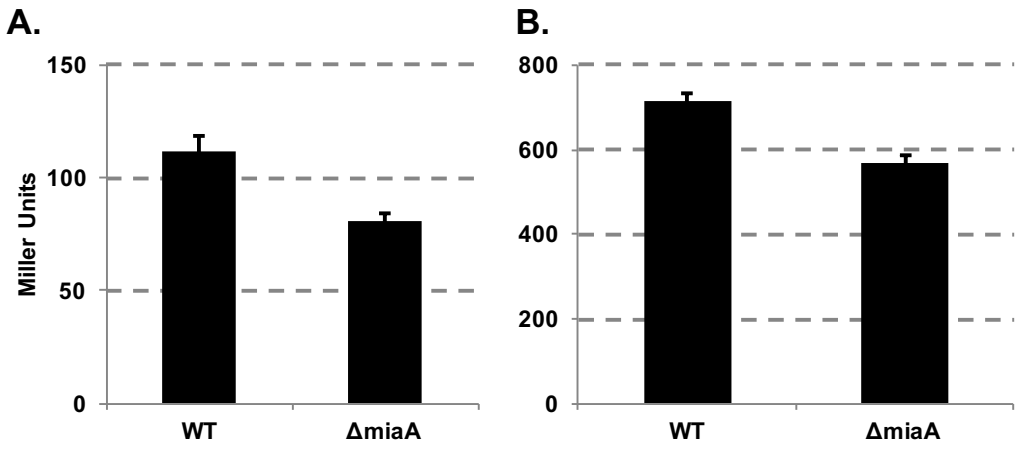


Figure 4. 9 Translational efficiency of RpeB and RpoS in the wild type and *miaA* mutant.
The translational efficiency of the (A) RpeB and (B) RpoS were determined using translational fusion to the *lacZ* reporter. Plasmids pME6015-RpeB and pME6015-RpoS were introduced separately into the wild type and the *miaA* mutant, and the translational level of each gene was measured as β-galactosidase activity. Data are the average of 6 replicates and standard errors are indicated.

Loss of MiaA decreased activities of pathogen inhibition and exoprotease production

The ability of the wild type *P. chlororaphis* 30-84 and the *miaA* mutant to inhibit growth of the plant pathogen *Gaeumannomyces graminis* was determined using an *in vitro* dual culture assay. Consistent with role of phenazines in pathogen inhibition, the *miaA* mutant was diminished in its ability to inhibit *Gaeumannomyces graminis* compared to the wild type (Fig. 4.10A). The mean zones of fungal growth inhibition (nearest distance to mycelia) for *P. chlororaphis* 30-84 and *miaA* mutant were approximately 8.0 and 3.4 mm at 4 dpi, respectively (Fig. 4.10A). In contrast, fungal inhibition ability was restored in the complemented *miaA* mutant (e.g. mean zone of inhibition was 7.6 mm) (Fig. 4.10A). These observations suggest that by controlling phenazine production in *P. chlororaphis* 30-84, MiaA influences fungal inhibition.

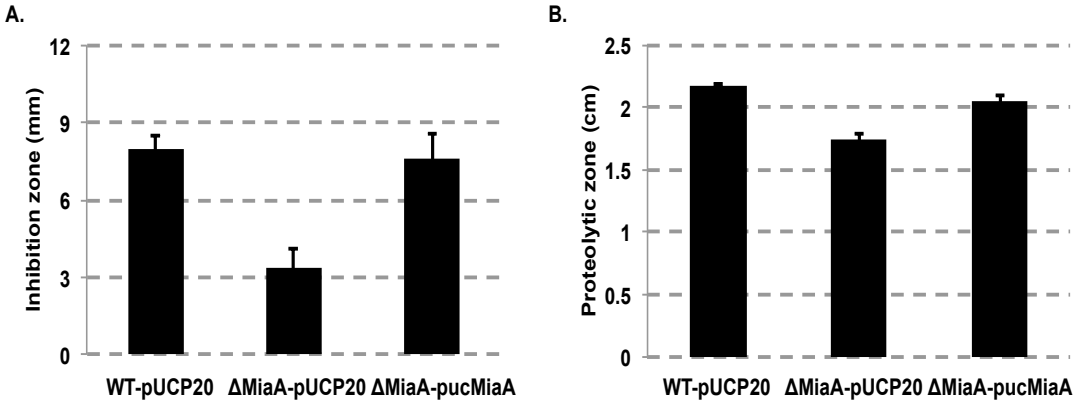


Figure 4. 10 Assays of *in vitro* Fungal inhibition and exoprotease activity.
A. The *in vitro* fungal inhibition assay. Overnight cultures of the wild type (containing the empty vector pUCP20-Gm), the *miaA* mutant (containing pUCP20-Gm), and the *miaA* mutant complemented by plasmid pucMiaA were grown in 3 ml AB-C at 28 C with shaking were adjusted to an OD620 of 0.8. Five microliters of the adjusted cultures were spotted separately onto potato dextrose agar. After 2 days of growth at 28 °C, a 5 mm plug of *G. graminis* was placed in the center of the plates. The inhibition zones were measured at 4 days after inoculation. Data are the average of 6 separate plates and standard errors are indicated. B. Exoprotease activity in the skim-milk agar assay. Bacterial cultures were prepared as describe above for the *in vitro* fungal inhibition assay. Two microliters of cultures were spotted separately onto skim-milk agar. After 2 days of growth, exoprotease activity was determined by measuring clear zone surrounded of each colony. Data are the average of 6 separate plates and standard errors are indicated.

Exoprotease activity was assessed by spotting 2 µl bacterial cultures onto skim-milk agar plates, and determined the size of clear zones surrounding the colonies. As shown in Fig. 4.10B, the *miaA* mutant significantly reduced production of exoprotease compared to the wild type (1.74 ± 0.04 versus 2.175 ± 0.02). As expected, exoprotease activity was able to be fully rescued in the complemented *miaA* mutant (Fig. 4.10B). The results suggest that MiaA also influence production of exoprotease, which is one of the important bacterial secondary metabolites.

MiaA is required for persistence in the soil

Phenazines are important for persistence in *P. chlororaphis* 30-84 in natural soil that contains compete microorganisms (Mazzola *et al.*, 1992). Given the findings of this study that disruption of *miaA* also caused a growth defect in liquid medium, we hypothesized that the persistence of the *miaA* mutant in soil might be impaired even when compared to the phenazine deficient mutant 30-84ZN (*phzB::lacZ* insertion). When inoculated into soil, the bacterial populations remained high for up to 8 weeks (Fig. 4.11). Consistent with previous studies, the bacterial populations of 30-84ZN were similar to wild type for the first 4 weeks, but were significant smaller at 8 weeks. However bacterial populations of the *miaA* mutant were significantly lower than either wild-type or 30-84ZN by 2 weeks and at 8 weeks were 1000 fold less. Moreover, the *miaA* mutant colonies recovered from the soil took longer to grow out on LB plates than both the wild type and 30-84ZN colonies (*miaA* colonies took 30 hours to appear as compared to 24 hours for wild type and 30-84ZN). The results indicate that MiaA plays

an important role in soil persistence, but not exclusively via reduction of phenazine production.

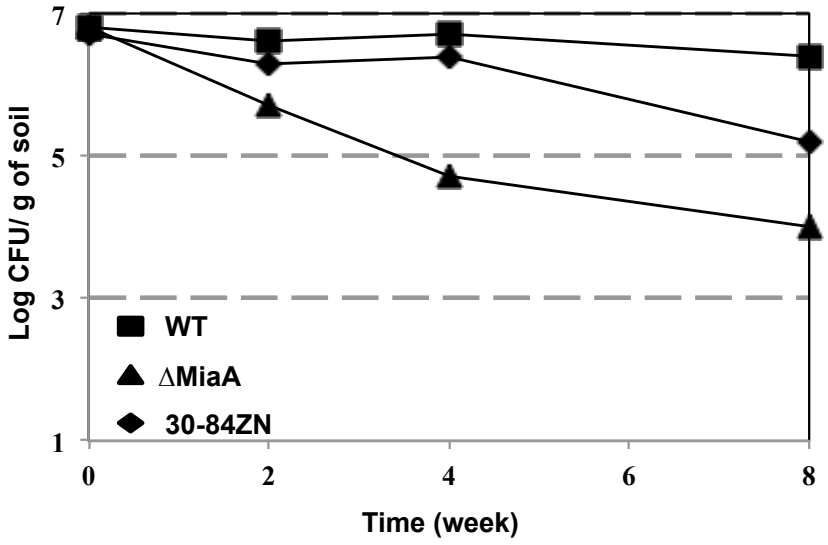


Figure 4. 11 Persistence in soil of the *miaA* mutant.
To prepare the bacterial inoculum, one colony of wild type, *miaA* mutant and 30-84ZN was inoculated into LB broth and incubated at 28 ºC with rapid shake to an OD620 of 1.2. Bacterial cells were washed twice with PBS and then normalized to OD620 = 0.1. Ten grams of pasteurized soil were mixed with 5 ml bacterial inoculum and then transferred into plastic tubes, and incubated at room temperature up to 8 weeks. One gram of soil samples were harvest at 0, 2, 4 and 8 weeks after inoculation, and total populations were determined by serial dilution plating on LB plate with rifampicin. The data expressed as the log10 value of the colony forming units (CFU) per gram of soil. Data are means of four separate replicates and error bars are smaller than the data labels. The experiments were repeated once with similar results.

Discussion

This study provides a functional analysis of MiaA in the phenazine producing biological control agent, *P. chlrororaphis* 30-84. During the screening of an RL27::Tn5Km^R mutagenesis generated *P. chlororaphis* 30-84 library, a light orange mutant was recovered. Sequence analysis of the DNA regions flanking the Tn5 revealed that the insertion occurred in the open reading frame of the gene annotated as *miaA* (Fig.

4.2C). The *miaA* gene encodes a tRNA modification enzyme which specifically functions in the addition of an isopentenyl group from an N-6 atom of adenosine at position 37 (A-37, immediately 3' adjacent to the anticodon sequence) of a tRNA that reads codons starting with UNN (Fig. 4.1) (Eisenberg *et al.*, 1979, Bjork *et al.*, 1987, Connolly & Winkler, 1989, Leung *et al.*, 1997). In *E. coli*, the i^6A-37, which is modified by MiaA is further modified to ms^2i^6A-37 by the action of the MiaB and MiaC enzyme activity that is responsible to methylation, thiolation, and hydroxylation (Esberg & Björk, 1995). In the *miaA* mutant, A-37 will remain unmodified, resulting in multiple defects in translation efficiency, codon context sensitivity, and fidelity (Zhao *et al.*, 2001). As expected mutation of the *miaA* gene has been shown to cause broad pleiotropic phenotypes including decreased growth rate and yield, suppression of tetracycline resistance, increased oxidation of certain amino acids in *E. coli* and *Salmonella enterica*, regulation of tryptophan attenuation in *P. putida*, and reduced expression of *vir* genes in *Agrobacterium tumefaciens* (Ericson & Björk, 1986, Mikkola & Kurland, 1988, Burdett, 1993, Tsui et al., 1994, Taylor et al., 1998, Olekhnovich & Gussin, 2001). However, to the best of our knowledge, this is the first report that shows MiaA, the tRNA modification enzyme, is required for antibiotic production and soil persistence in biocontrol bacterium *P. chlororaphis* 30-84.

The *miaA* gene is the last gene composed of a complex gene operon that consists of Pchl3084_0549, Pchl3084_0550, Pchl3084_0551 and DNA mismatch repair protein *mutL.* It is located directly upstream of the *hfq* gene that encodes RNA-chaperon protein (Loper *et al.*, 2012). In *E. coli* K-12, *miaA* is located within a superoperon (*yjeF, tsaE,*

amiB, mutL, miaA, hfq, hflX, hflX, hflK, hflC), and expression of *miaA* directly influences the expression of *hfq* because there are two functional *hfq* promoters within the open reading frame of *miaA* (Tsui et al., 1994, Tsui et al., 1996, Thompson & Gottesman, 2014). The regulatory role of Hfq has been extensively studied in various Gram negative bacteria, where it is known as a global posttranscriptional regulator by either directly binding to the target mRNA or enabling to pair between sRNAs and its target mRNAs (Brennan & Link, 2007). An *hfq* mutant of *P. aeruginosa* PAO1 is severely altered the production of elastase, catalase, and blue pigmented phenazine, pyocyanin along with concomitant with a significant reduction of its virulence (Sonnleitner *et al.*, 2003). Furthermore, in order biocontrol agent *Pseudomonas* species Hfq positively regulates production of antibiotics including 2,4-diacetylphloroglucinol (DAPG), PCA and pyoluteorin (Plt) (Wu *et al.*, 2010, Wang *et al.*, 2012b). In this study, transcriptomic analysis of *miaA* mutant revealed that the expression of *hfq* is slightly reduced (Fig. 4.6), however introduction of additional copies of *hfq* in a high-copy-number vector into a *miaA* mutant indicated that reduced phenazine production is not due solely to a polar effect on *hfq* expression (Table 4.2). Moreover, complementation of the *miaA* mutant with extra copies of *miaA* completely restored phenazine production to the wild type level, confirming that impaired phenazine production in the *miaA* mutant is only caused by disruption of *miaA* (Fig. 4.2A, 4.2B and Fig. 4.4A).

This study also showed mutation in *miaA* resulted in a reduced growth rate in AB-C and PPMD media, and the largest reduction was observed in relatively rich PPMD medium (Fig. 4.5). Decreased growth rate and yield are one of the pleiotrophic

phenotype of the *miaA* mutant in *E. coli* K-12 and *Salmonella typhimurium* LT2 (Ericson & Björk, 1986, Mikkola & Kurland, 1988, Connolly & Winkler, 1989). The *miaA* mutant in *S. typhimurium* LT2 showed 20 to 50 % reduction in growth rate in all growth media, but especially in more nutritious medium that supported faster growth of the wild type (Ericson & Björk, 1986). One hypothesis for the impaired growth is that MiaA influences the regulation of several amino acid biosynthetic operons. For example, in *S. typhimurium,* mutation of *miaA* makes the cell more sensitive to some of amino acid analogs, which suggests MiaA is required for synthesis of those amino acids (Ericson & Björk, 1986), and similar examples of influence on amino acid synthesis can be found in *E. coli* and *P. putida.* For example, $tRNA^{Trp}$ is one of the tRNA species that required MiaA-mediated modification and is required for attenuation in the tryptophan operon. Absence of functional MiaA in *P. putida* revealed that up to 30-fold higher expression of *trpE* and *trpGCD* under the condition of with or without exogenously supplied tryptophan (Olekhnovich & Gussin, 2001). In *E. coli,* mutation of *miaA* prevents attenuation of the *trp* operon but stimulates the attenuation of the *tna* operon, which encodes trytophanase an enzyme that hydrolyses tryptophan to create indole, pyruvate and ammonia (Newton *et al.*, 1965, Yanofsky & Soll, 1977, Gollnick & Yanofsky, 1990, Yanofsky *et al.*, 1991). In *P. chlororaphis* 30-84, whole transcriptomic analysis revealed that genes involved in aromatic amino acid metabolism were significantly up-regulated in a *miaA* mutant, however expression of genes involved in histidine and leucine uptake and degradation were significantly down-regulated (Pierson et al., unpublished data). These results suggest that MiaA has regulatory roles in the

expression of genes involved in bacterial amino acid utilization and metabolism, which in turn significantly influence of the cell growth.

In *P. chlororaphis* 30-84, phenazine production is positively regulated by the PhzI/PhzR quorum sensing system, which directly activation the phenazine biosynthetic operon *phzXYFABCD* (Pierson et al., 1995, Wood & Pierson, 1996). Therefore, we questioned whether reduced phenazine production, which is one of the key phenotypes of the *miaA* mutant, is related decreased AHL production or AHL-mediated activation. A comparison of total AHL levels produced by the *miaA* mutant compared to the wild type indicated that AHL production was greatly reduced in *miaA* mutant in all media type we tested was gene expression of *phzI* in *miaA* mutant determined via transcriptional fusions and qRT-PCR (Fig. 4.6 and 4.7). Moreover, overexpression of *phzR* under a constitutive promoter restored phenazine production (Fig 4.8A and 4.8B). These data are consistent with the hypothesis that MiaA influences phenazine production via regulation of quorum sensing.

Previous work established a hierarchical network of genes that control the phenazine biosynthetic genes as well as regulate the PhzR/PhzI quorum sensing system (Wang *et al.*, 2012a, Wang *et al.*, 2013). This includes regulation by Gac/Rsm, the stationary sigma factor (RpoS), the two-component signal transduction system RpeA/RpeB, and the transcriptional regulator Pip (Schuster *et al.*, 2004, Girard *et al.*, 2006, Selin *et al.*, 2012, Wang *et al.*, 2012a, Wang *et al.*, 2013). The transcriptional regulator Pip (phenazine inducing protein) was previously identified as an important positive regulator of phenazine production and quorum sensing in *P. chlororaphis*

PCL1391 and 30-84 (Girard *et al.*, 2006, Wang *et al.*, 2012a). In these studies, *pip* is regulated in a hierarchical way by RpoS and/or RpeA/RepB, but not by the Gac/Rsm network (Girard *et al.*, 2006, Wang *et al.*, 2012a). In the present study, characterization of the *miaA* mutant focused on identifying how MiaA might regulate this network. Data presented as part of this study suggested *miaA* may regulate translation of one or both of these *pip* regulators.

Previous work demonstrated that RpeA (repressor of phenazine expression) is a putative two component system sensor kinase that interacts with RpeB a response regulator that has a positive role in phenazine production (Wang *et al.*, 2012a). These products are encoded by genes that are co-transcribed as a single operon. Previous work suggested RpeA may function as a sensor of the metabolic state or stress condition of the cell. RpeA has a major impact on *phzB* gene expression under conditions when phenazine production is not favored (e.g. under nutrient or stress) (Whistler & Pierson, 2003). RpeB likely functions as a response regulator that is controlled by RpeA or other small phospho-donors. Phosphorylated RpeB positively regulates the transcription of *pip,* which in turns enhances phenazine production via activate quorum sensing system (Wang *et al.*, 2012a). Interestingly, RpeA/RpeB together positively regulate phenazine production, which is confirmed by a *rpeAB* double mutant having reduced phenazine production and lower amounts of AHL signals (Wang *et al.*, 2012a).

The observed reduction in *pip, phzI, phzR* and *phzX* expression in the *miaA* mutant but not in the genes that regulate *pip* may be explained if MiaA directly effects the translation of these genes. Our results demonstrating that introducing extra copies

rpeB into the *miaA* mutant only partially restored phenazine production support this hypothesis. The observation that the expression of translational fusions e.g. *rpoS-lacZ* and *rpeB-lacZ* fusions were significantly reduced in the *miaA* background as compared to the wild type (Fig. 4.9A and 4.9B) supports the hypothesis that these genes are affected by *miaA* mutation at the translational level.

Translational fidelity is important for regulating proper gene expression particularly genes that are part of integrated regulatory networks. MiaA modifies tRNA species that begins with uridine including Trp (UGG), Cys (UGU and UGC), Tyr (UAU and UAC), Phe (UUU and UUC), Ser (UCU, UCC, UCA and UCG) and Leu (UUA and UUG) (Leung *et al.*, 1997, Thompson & Gottesman, 2014). Mutation of *miaA* in *E. coli* and *S. enterica* resulted in multiple defects in translation efficiency and fidelity (Li *et al.*, 1997, Zhao *et al.*, 2001). A recent study showed that MiaA greatly impacts translation of stationary phase/general stress response sigma factor, RpoS in *E. coli* (Thompson & Gottesman, 2014). The *rpoS* coding sequence is significantly enriched for Leu codons, which use tRNA species that requires MiaA modification. Thus a significant impairment in the translational level of RpoS in the absence of MiaA would be predicted. In *Shigella flexneri,* a human pathogenic Gram-negative bacterium, mutation of *miaA* resulted in a 10- fold reduction of VirF level (Durand *et al.*, 1997). Analysis of codon usage revealed that *virF* contains a large fraction of the UUX Leu codon (Thompson & Gottesman, 2014). Consistently, analysis of the codon usage of RpeB and RpoS showed that both genes have 10.1% and 13% of uridine (UXX) codon usage supporting the hypothesis

that tRNA modification affects the translation of global transcriptional regulators such as RpeB and RpoS.

In *P. chlororaphis* 30-84, phenazines are essential for bacterial rhizosphere persistence (Mazzola *et al.*, 1992). The results from the present are consistent with previous reports that the populations of 30-84ZN are significantly lower in the soil than wild type. Because the *miaA* mutant produces less phenazines than the wild type, but more than 30-84ZN, if phenazines are the sole determinant of soil persistence, the survival rate of the *miaA* mutant should be intermediate to the survival of wild type and 30-84ZN. Surprisingly, its ability to persist in soil is weaker than that of 30-84ZN (Fig. 4.11). These results are consistent with the growth defect observed for the *miaA* mutant in liquid culture. Significantly higher populations of microorganisms can be found in rhizosphere soil than bulk soil because of root exudation of organic compounds, modification of pH, and release metal ions and minerals by roots (Wang & Zabowski, 1998). Therefore, soil without the living host plant may be a harsh condition due to the poor nutrition. For that reason, it is possible that alteration of its ability to utilize nutrient source for cell growth in *miaA* mutant may cause the reduction in soil persistence.

P. chlororaphis 30-84 produces three phenazine antibiotics, which are primarily responsible for the inhibition of fungal growth and control of take-all disease of wheat as a biological control agent. As expected, decreased phenazine production results in the reduction in the ability of the *miaA* mutant to inhibit *G. graminis* in *in vitro* plate experiments (Fig. 4.10A). Interestingly, *miaA* mutant also displayed significantly less exoprotease activity by comparison to the wild type (Fig. 4.10B). The expression of

exoprotease has previously been linked to phenazine expression (Wang *et al.*, 2016), so the effect of MiaA on exoprotease may be related to reduced phenazine production. Taken together these data suggest that a functional MiaA is required for many functions related to its biological control capabilities in wheat rhizosphere.

In summary, this study is the first functional analysis demonstrating MiaA control of antibiotic production, pathogen inhibition, bacterial growth and soil persistence in biological control agent *Pseudomonas chlororaphis* 30-84. Future studies will determine the role of MiaA in bacterial metabolomics changes using LC-MS analyses and will connect to the whole transcriptomic data for identifying gene expression patterns related to the function of MiaA.

Materials and Methods

Bacteria strains and growth conditions

Bacterial strains and plasmids are listed in Table 4.1 and oligonucliotides and primer sets are listed in Table 4.2. A spontaneous rifampicin-resistant derivative of *P. chlororaphis* 30-84 was used in all experiments (Pierson & Thomashow, 1992). *P. chlororaphis* 30-84 and its derivatives were grown at 28°C in various media including Luria-Bertani (LB) medium containing 5 g of NaCl per liter, M9 minimal medium, pigment production medium-D (PPMD), Skim milk agar (Difco, Franklin Lakes, NJ) and AB minimal medium supplemented with 2% casamino acids (AB-C) (Difco). *Escherichia coli* strains were grown at 37 °C in LB with appropriate antibiotics. Potato dextrose agar (PDA) was used for fungal inhibition assays. For *P. chlororaphis*, when

necessary, the following antibiotics were added to the medium: gentamycin (Gm) 50 μg/ml, kanamycin (Km) 50 μg/ml, rifampicin (Rif) 100 μg/ml and tetracycline (Tc) 50 μg/ml; for *E. coli*: ampicillin (Ap) 100 μg/ml, gentamycin 15 μg/ml, kanamycin 50 μg/ml and tetracycline 25 μg/ml.

DNA manipulation and sequence analysis

Plasmid DNA isolation, cloning, restriction enzyme digestion and T4 DNA ligation were performed using standard procedures (Sambrook et al., 2001). Polymerase chain reaction (PCR) was carried out using FideliTaq DNA polymerase (Affymetrix, Santa Clara, CA) at 95 °C for 2 min, followed by 25 cycles of 95 °C for 30 sec, 55 °C (or at the recommended Tm for the primers) for 30 sec and 72 °C for 90 sec, and a final elongation step of 72°C for 10 min. DNA sequencing was performed by the Laboratory for Genome Technology within Institute for Plant Genomics and Biotechnology, Texas A&M University using an ABI 3130xl Genetic Analyzer. Nucleotide and amino acid homology searches were conducted using the BLAST programs at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST).

Triparental matings and electroporation

Triparental matings were performed as described previously (Pierson & Thomashow, 1992). Briefly, overnight cultures of *E. coli* DH5α (as the donor), HB101 containing pRK2013 (as the helper), and *P. chlororaphis* 30-84 (as the recipient) were spotted onto pieces of sterile nitrocellulose membrane on an LB agar plate and incubated at 28 °C for 24 h. Transconjugants were selected on LB plates supplemented with appropriate antibiotics. For electroporation, the electro-competent cells were prepared

from strains of *P. chlororaphis* 30-84 as described previously (Wang *et al.*, 2012a). Briefly, *P. chlororaphis* 30-84 and its derivatives were grown in 3 ml LB at 28 °C with rapid shake. Subsequently, overnight cultures were re-inoculated in 1:100 dilutions to 10 ml LB broth and grown to exponential phase (OD_{620} = 0.8). Cells were initially chilled on ice for 30 min, and then washed 3 times with ice-cold 300 mM sucrose. Total 300 – 500 ng of plasmid DNA was mixed with 100 μl of electro-competent cells and electroporation was performed according to standard protocol. Transformants were selected on LB plates supplemented with appropriate antibiotics.

Isolation of the miaA mutant

P. chlororaphis 30-84 was mutagenized using the pRL27::Tn5Km^R plasposon, as described previously (Larsen *et al.*, 2002). Briefly, overnight cultures of the *E. coli* donor containing pRL27::Tn5KM^R and recipient 30-84 were grown to mid-logarithm phase. Cells were collected by centrifugation and spotted onto pieces of sterile nitrocellulose membrane on an LB agar plate and incubated at 28 °C for 24 h. Putative transconjugants were selected on LB plates supplemented with Km and Rif. One colony (ID: 30-84::19-30) was chosen visually for reduced phenazine production (light orange color) as compared with the wild type (bright orange color). Primer pair tpnRL17-tpnRL13, which was designed to target from inside of Tn5-pRL to outward flanking sequence, were used to amplify the mutational region in the 30-84::19-30 strain using its genomic DNA. The PCR product was sequenced to position the Tn5 insertion. Sequencing analyses revealed Tn5 was inserted into coding sequence of *miaA*. To verify the exact location of insertion in *miaA,* primer pair miaAseqF-miaAseqR was used to

amplify the flanking region of Tn5 insertion. Sequence analyses of the PCR product verified that the Tn5 was inserted 785-bp downstream of the start codon and 188-bp upstream of the stop codon of *miaA*.

Cloning and in trans expression of the miaA, hfq, pip, rpoS, phzR and rpeB genes

The coding sequences for *miaA, hfq, pip and rpoS* and flanking sequences were PCR amplified using the primer pairs miaA1-miaA2, hfq1-hfq2, pip1-pip2 and rpos1-rpos2, respectively, each possessing appropriate restriction enzyme sites for cloning (Table 4.2). The PCR fragments *miaA* (1106 bp)*, hfq* (299 bp) and *rpoS* (1405 bp) were purified and cloned into the appropriate restriction enzyme sites within the multiple cloning site of pUCP20-Gm (Chiang & Burrows, 2003). For *pip*, the purified PCR fragment (784 bp) was cloned into pKT2pTac*lacZ*Tc^R (Yu et al, unpublished). The final plasmids were designated pucMiaA, pucHfq, pucRpoS and KT2Pip, respectively (Table 4.1), and each construct was confirmed by both enzyme digestion analysis and sequencing. For overexpression of *phzR* and *rpeB,* pGT2PhzR and pGT2RpeB were used from previous studies (Wang *et al.*, 2012a, Wang *et al.*, 2013), which containing coding regions of *phzR* or *rpeB* and driven by either hybrid constitutive promoter pTac (pGT2PhzR) or its own promoter (pGT2RpeB). These plasmids were then transformed into *P. chlororaphis* 30-84 wild type and 30-84ΔMiaA by either electroporation or triparental conjugation. Transformants were selected on LB plates supplemented with appropriate antibiotics.

Table 4. 4. Oligonucleotides used for gene cloning and qPCR

Oligonucleotide[a]	Sequence (5'-3')
tpnRL17	AACAAGCCAGGGATGTAACG
tpnRL 13	CAGCAACACCTTCTTCACGA
miaAseqF	ATACTGTCGCGAACTTGGCCA
miaAseqR	CCCAAGGATAGACGGCAATTGC
miaA1	TGGAAAAGGATCCGCGCAGCGGTCAAT (*Bam*HI)
miaA2	CCCACCAAGCTTGATAGACGGCAATTGC (*Hin*dIII)
rpos1	CGCGAATTCGTCAAAGTCGGACAGGCAAT (*Eco*RI)
rpos2	CGCAAGCTTATGCGCCTGCATATTTCATC (*Hin*dIII)
pip1	CGGGATCCGCCGTCCTGCTTTCCTTC (*Bam*HI)
pip2	CGAAGCTTTCGACAGCGGGGTTCA (*Hin*dIII)
hfq1	TATTGAGGATCCAAGGAGTGCGGCACATGT (*Bam*HI)
hfq2	AGGAGAAAGCTTTCAGGCGTTACCTGG (*Hin*dIII)
phzITXF	CGCGAATTCCATTCTCTACGACTACCTGG (*Eco*RI)
phzITXR	CGCGGATCCTGTGTGCTCTTCCATGTGC (*Bam*HI)
rpoSTLF	CCGCGAATTCTCCATTACCGCCT (*Eco*RI)
rpoSTLR	TTTGACGGATCCACCAGGTGGCATAGGTCG (*Bam*H I)
rpeBTLF	CGATGCGAATTCTCAGTTCGCTGCTGATCGACA (*Eco*RI)
rpeBTLR	TATCACGGATCCCAACAGTACGCGCGGCTCGCAAGGCTTTGT (*Bam*HI)
miaART1	CAGGTACTGCATGAGCGTATT
miaART2	CACTTCTAGAACGCAGAGCTAC
rpeART1	GTCGACGACGAACTGGAATC
rpeART2	CTGGATACGCTTTTCGCAAT
rpeBRT1	CATCCTTCTGGTCGAAGACG
rpeBRT2	AGGTCGAGAATCACCAGGTC
gacART1	GAGATCCAGATTGCGCTGAT
gacART2	CAGCAACGTCAGTTCGACAT
gacSRT1	AAACCTTCGTCGTGATCCAG
gacSRT2	ACTACCTGACCAAGCCCATC
phzRRT1	CGCAAGGATAATCCCATCAG
phzRRT2	CACATTCCCTACCGCTGAAC
phzIRT1	CTACCTCCTGGCGTTCAATG
phzIRT2	GAAGCGAGTCATTTCCCAGA
phzXRT1	AACCACTTCTGGGTGGAAAG
phzXRT2	ATCTTGCCGTCATCCAGTTC
pipRT1	AAAAGACCCGCGAGAACATT
pipRT2	ACGTACAGCTGCTCCTTGCT

Table 4. 4. Continued

Oligonucleotide[a]	Sequence (5'-3')
rpoSRT1	ATCAGTGGCTTTCCGAATTG
rpoSRT2	GACCTTCGACCTGGATCTGA
hfqRT1	GTCAAAAGGGCATTCGCTAC
hfqRT2	CTGACGGTGTTCTTCAGCAG
16RT1	ACGTCCTACGGGAGAAAGC
16RT2	CGTGTCTCAGTTCCAGTGTGA
rpoDRT1	ACGTCCTGAGCGGTTACATC
rpoDRT2	CTTTCGGCTTCTTCTTCGTC

[a] Underlined nucleotides are restriction sites added and the restriction enzymes are indicated at the end of primers.

Growth Curves

Growth rates of wild type, 30-84ΔMiaA, and 30-84ΔMia complemented by the expression of *miaA in trans* were determined in rich and minimal media. Triplicate of single colonies of each strain were inoculated into 3 ml PPMD and AB-C broth and were grown overnight at 28°C. The cell densities were normalized to OD_{620} = 0.8, and 200 μl of each culture was added to 20 ml of the same medium. The cultures were grown at 28°C with rapid aeration and were sampled once every 2 -3 h. The measurements at 24 - 28 h confirmed that the culture had entered stationary phase. Absorbance at OD_{620} nm was determined and plotted versus time. This assay repeated twice.

Quantification of phenazine production

To quantify phenazine production by the wild type, 30-84ΔMiaA and 30-84ΔMiaAcomplemented strain, 3 single colonies were selected from different plates and grown overnight in PPMD, LB, or AB-C broth at 28 °C. After adjusting OD_{620} = 0.8, 5 μl of the adjusted culture was re-inoculated into 5 ml of each fresh medium, and grown

at 28 °C with rapid aeration. When cultures reached OD_{620} = 2.4, cells were harvested by centrifugation, and the supernatants were acidified to ca. pH 2 with concentrated HCl. Phenazines were extracted with an equal volume of benzene, and the separated benzene layer was collected. Following evaporation of the benzene under air, dried phenazines were resuspended in 0.1 N NaOH, and quantified via serial dilutions at absorbance of 367 nm. The assays were repeated at twice.

AHL extraction and quantification using AHL-specific reporter 30-84 I/Z

Total AHLs were extracted and quantified from cell-free supernatants of the wild type and 30-84ΔMiaA as described previously (Whistler & Pierson, 2003). Briefly, triplicates of single colonies were inoculated at 5 ml PPMD broth and grown at 28°C with shaking up to an OD_{600} of 1.6. The supernatants were collected by centrifugation and mixed with an equal volume of acidified ethyl acetate. After one-hour of fast shaking, the ethyl acetate phase was transferred to 15-ml tubes and evaporated in the hood. The dried extracts containing AHLs were resuspended into different media (AB-C, LB and PPMD) with a volume equal to the original culture and then filter-sterilized. AHL production was quantified by inoculating the filter sterile cultures containing the AHLs with the AHL-specific reporter 30- 84I/Z (*phzI*¯, *phzB*::*lacZ*). The β-galactosidase of 30-84I/Zwas determined according to Miller (1972) after 24 h growth with shaking at 28°C. The assays were repeated at least three times.

Construction of the phzI-lacZ, phzR-lacZ, and phzX-lacZ transcriptional reporters

A *phzI* transcriptional reporter was constructed to compare *phzI* expression in wild type and 30-84ΔMiaA. The PCR fragment containing the *phzI* promoter (-35, *phz*

box, and -10 sequence features) and flanking sequence from 209 bp upstream and 18 bp downstream of *phzI* translation start site was amplified using primer set phzITXF-phzITXR having a unique *Eco*RI and *Bam*HI to facilitate cloning (Table 4.4). This 230-bp PCR fragment containing the *phzI* promoter initially was ligated into the TOPO vector (Invitrogen) by TA cloning, and then ligated into the promoter trap vector pGT2*lacZ* to make the *phzI* transcriptional fusion, pGT*phzI-lacZ* (Table 4.1). To measure the transcriptional expression of *phzX* and *phzR,* previously constructed reporter vectors of pGT2*phzX-lacZ* and pGT2*phzR-lacZ* were used (Table 4.1, Yu et al., unpublished). These reporter plasmids are containing the putative promoter region, 5' untranslated region and 19 bp and 20 bp of partial sequence of each gene's coding region. These reporters were introduced separately into the wild type and 30-84ΔMiaA by triparental mating.

RNA preparation and transcriptomic analysis

Single colonies of *P. chlororaphis* 30-84 wild type and 30-84ΔMiaA from three separate AB-C plates were chosen and grown with rapid aeration at 28^{o} C in 3ml of AB-C to an OD_{600} of 1.2. Prior to RNA extraction, the RNA was stabilized by the addition of 2 ml Qiagen RNA Protect reagent to 1 ml samples. Cells were harvested by centrifugation for 10 min at 4000 × *g* and total RNA was extracted using a Qiagen RNeasy Protect Bacteria Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Genomic DNA was removed using Qiagen RNase-Free DNase Kit (Qiagen) on-column digestion for 30 min. RNA concentration and purity (A_{260}/A_{280} ratio, > 1.9) was determined with a GE Nanovue spectrophotometer (GE Healthcare).

Five micrograms of total RNA were reverse-transcribed using random primers (Invitrogen Life technologies, Carlsbad, CA) and Superscript III (Invitrogen) at 50 °C for 1 h and inactivated at 75 °C for 15 min. For the negative control, the same reaction was performed using sterilized water instead of reverse transcriptase.

Quantitative PCR (qPCR) assay was performed to measure the expression levels of the target genes as previously described (Wang *et al.*, 2012a). Synthesized cDNA (2 ng/reaction) or a negative control was used for qPCR reactions with Fast SYBR Green PCR Master Mix (Applied Biosystems) and gene specific primers (500 nM final concentration). Primer pairs miaART1-miaART2, gacART1-gacART2, gacSRT1-gacSRT2, rpeBRT1-rpeBRT2, rpeART1-rpeART2, pipRT1-pipRT2, phzRRT1-phzRRT2, phzIRT1-phzIRT2, hfqRT1-hfqRT2, rpoSRT1-rpoSRT2, 16RT1-16RT2 and rpoDRT1-rpoDRT2 were used to detect the expression of *hfq*, *gacA*, *gacS*, *rpeB*, *rpeA*, *pip*, *phzR*, *phzI*, *hfq*, *rpoS*, 16S rDNA and *rpoD* genes, respectively, in the wild type and 30-84ΔMiaA (Table 4.4). qPCR amplifications were carried out at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, and a final dissociation curve analysis step from 65°C to 95°C. Three technical replicate experiments were performed for each triplicate biological sample. Amplification specificity for each reaction was confirmed by the dissociation curve analysis. The relative expression of the target gene was determined based on the mean cycle threshold (Ct) values of triplicate, and ΔΔCt analysis performed to normalize the results using the 16S rDNA as the reference genes.

Construction of the RpoS and RpeB translational reporters

Translational reporter was constructed to compare the expression of RpoS and RpeB in the wild type and 30-84ΔMiaA. The putative promoter, the 5' untranslated region and the partial coding sequences of *rpoS* (155th codon from the first codon) and *rpeB* (110th codon from the first codon) were amplified using primer sets RpoSTLF-RpoSTLR and RpeBTLF-RpeBTLR, having a unique *Eco*RI and *Bam*HI to facilitate cloning (Table 4.4). The PCR fragments were purified, digested with *Eco*RI and *Bam*HI and inserted into in-frame with the *lacZ* gene in the translational fusion vector pME6015 (Heeb *et al.*, 2002, Kulkarni *et al.*, 2014). This vector has been deleted of promoter and first eight codons of the *E. coli lacZ* gene, to facilitate translational expression study (Kulkarni *et al.*, 2014). The translational fusion vectors of pME6015-RpoSTL and pME6015-RpeBTL were introduced into the wild type and the 30-84ΔMiaA by electroporation, and translational efficiency was measured by β-galactosidase assay.

Pathogen inhibition and exoprotease activity

To measure the ability of strains to inhibit the pathogen *G. graminis* var. *graminis*, bacterial cultures of wild type, *miaA* mutant, and *miaA* complemented strain were grown in LB medium at 28 ºC with rapid shaking. Overnight cultures were adjusted OD of 620 at 0.8, and 5 µl of each culture was spotted onto potato dextrose agar (PDA, Difco). A Five-day-old plug of *G. graminis* var. *graminis* was placed in the center of each of the plates. After 2 days, triplicated 5 µl of overnight bacterial cultures were spotted onto at 3 cm apart from center of fungal plug inoculated. Zones of inhibition were measured after 5 days by calculating the distance between the edge of the bacterial colony and the fungal mycelium.

To measure the ability of strains to produce exoprotease, wild type, 30-84ΔMiaA and *miaA* complemented strain were grown in 3 ml LB medium at 28 ºC with shaking for overnight. Cultures were adjusted to OD_{620} = 0.8 with fresh LB medium and 2 µl of each culture was spotted onto skim milk agar plates (Maddula *et al.*, 2006). The proteolytic activity was determined after 48 h by measuring diameter of the clearing zone.

Soil persistence assay

Bacterial soil persistence assay was performed as described (Mazzola *et al.*, 1992) with few modifications. Soils used in this study were obtained from the Texas A&M University Horticultural Research Farm, College Station, Texas. Clay soil was collected from the upper 20 cm of the soil profile and passed through a 0.5-cm-mesh screen prior to use. Soil was pasteurized by treating moist soil with a steam-air mixture at 95°C for 90 min. Bacterial cultures of wild type, 30-84ΔMiaA and 30-84ZN were grown overnight at 28°C with shaking in LB broth to an OD_{600} of 1.2. Bacterial cells were collected by centrifugation and washed twice with PBS and then normalized to OD_{600} = 0.1. Ten gram of pasteurized natural soil were mixed with 5 ml bacterial suspension and then transferred into 25 × 200 mm sterile plastic tubes. The tubes were arranged in a random design and incubated at room temperature. Samples were collected at 0, 2, 4 and 8 weeks, and total bacterial population was determined by serial dilution plating on LB plate with rifampicin. The assays were repeated once.

Statistical Analysis

All data presented in this study are mean ± SEM from multiple determinations. Differences between strains were analyzed statistically using ANOVA and Fisher's protected Least Significant Difference (LSD) test ($P<0.05$).

CHAPTER V

SUMMARY AND RECOMMENDATION

A biological control approach is an alternative way to reduce the use of synthetic chemicals to manage plant pathogens because it is safer, more ecologically-friendly and more sustainable. Fluorescent pseudomonads are abundantly present in natural soils and are considered to be agriculturally important due to their ability to promote plant growth and their capability to suppress plant diseases. Many of these pseudomonads are able to colonize the rhizosphere, utilize root exudates for their survival, and produce phytohormones, siderophores, antibiotics, extracellular enzymes and fungicidal compounds, which are the essential traits of potent biological control agents.

The biological control strain studied in this dissertation is *Pseudomonas chlororaphis* 30-84, which was selected for its ability to suppress take-all disease of wheat caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Pierson & Thomashow, 1992). Phenazine production of *P. chlororaphis* 30-84 is crucial for fungal pathogen inhibition and wheat rhizosphere competence (Mazzola *et al.*, 1992, Pierson & Thomashow, 1992). Phenazine-1-carboxylic acid (PCA) is a phenazine that is produced by most phenazine producing *Pseudomonas* species including *P. chlororaphis* 30-84 and the biosynthetic operon for this phenazine is highly conserved (Mavrodi *et al.*, 2001, Mavrodi *et al.*, 2006, Pierson & Pierson, 2010). In addition to the biosynthetic operon, phenazine producing strains often contain one or more extra genes that encode phenazine modifying enzymes. These enzymes are responsible for the conversion of

PCA to other derivatives (Mavrodi *et al.,* 2006). For instance, *P. chlororaphis* 30-84 contains the gene *phzO,* which encodes a bacterial aromatic monooxygenase that converts PCA into 2OHPCA (Delaney *et al.*, 2001). Additionally, the opportunistic human pathogen, *P. aeruginosa* is able to produce several phenazine derivatives due to the possession of multiple phenazine modifying genes including *phzH*, *phzM*, and *phzS*, which encode enzymes that act independently or together to convert PCA into other phenazine derivatives (e.g. PCN, 1OHPCA, 5MPCA and PYO, (Mavrodi *et al.*, 2001).

In Chapter II, I studied the ecological roles of individual phenazine derivatives in bacterial fitness. I constructed isogenic derivatives of *P. chlororaphis* 30-84 that produced phenazines made by other *Pseudomonas*. This was accomplished by introducing the phenazine modifying genes (e.g. *phzH, phzM, phzS* and *phzM* + *phzS* together of *P. aeruginosa*) *in trans* into strain 30-84PCA, a mutant of *P. chlororaphis* 30-84 that produces only PCA (*phzO*::Tn5) (Maddula *et al.*, 2008). The results of colometric, semi-qPCR and HPLC-MS analysis showed that the introduced genes were successfully expressed and the enzymes produced were functionally capable of converting some of the PCA to the appropriate phenazine derivatives (Fig. 2.1. 2.2 and 2.3). Since phenazines are known to be strong antibiotic bacterial secondary metabolites and play a major role in pathogen inhibition, I determined their antifungal activity using the altered phenazine producers. Interestingly, the production of at least one additional phenazine by the altered phenazine producing derivatives generally resulted in greater fungal inhibition than observed for 30-84PCA, suggesting a benefit to converting some PCA to another type of phenazine (Table 2.3). The results also indicated that altering the

type of phenazines produced by *P. chlororaphis* 30-84 affected the specificity of fungal pathogen inhibition. Of particular interest was the result that production of 2OHPCA and PYO generally resulted in greater fungal inhibition including a broader spectrum of pathogens inhibited. Therefore, it will be interesting to determine whether the antifungal activity of a derivative that is able to produce both 2OHPCA and PYO together (i.e. 30-84 wild-type harboring pGT2*phzMS*) is greater than that of the wild-type. However since *in vitro* antifungal activity does not always correlate with disease suppression *in situ,* follow-up studies will be required to determine the ability of the altered phenazine producers to suppress plant disease against various pathogens. Disease suppression is the most important measure of effectiveness for biological control agents. My investigation of this important consideration was greatly hampered by our not being able to obtain a virulent isolate of Ggt until the last month of my graduate career.

Previous studies showed that phenazine derivatives produced by *P. chlororaphis* 30-84 have distinct roles in biofilm formation (e.g. cell attachment and architecture) (Maddula *et al.*, 2008). Moreover, altering the ratio of phenazines produced by *P. chlororaphis* significantly affected the ability to promote extracellular DNA release and structured biofilm matrix formation (Wang *et al.*, 2016). The results in Chapter II showed that altered phenazine-producing strains differed in their ability to promote eDNA release, which resulted in different levels of biofilm formation (Fig. 2.4). Several studies showed that eDNA has a critical role in structural components of biofilm architecture (Allesen-Holm *et al.*, 2006, Das *et al.*, 2010, Gloag *et al.*, 2013, Wang *et al.*, 2016), however embedded mechanisms of eDNA release are still unresolved. Recently,

Wang *et al*. (2016) postulated that eDNA release may be related in part to the activity of a pyocin, which is induced by ROS that generated by phenazine's redox property and reactivity. I am particularly interested in knowing whether different phenazines can alter the ability to promote pyocin release. To evaluate this idea, pyocin quantification from biofilm matrix produced by each altered phenazine producer is required accompanied with transcriptomic analysis of pyocin related genes. Together, the results from Chapter II provide important insights on how phenazines differently affect several characteristics related to biological control efficacy and will help guide strategies to improve biological control based on selection for the production of the most useful secondary metabolites.

In *P. chlororaphis* 30-84, phenazine production is dependent on the PhzR/PhzI quorum sensing system located immediately upstream of phenazine biosynthetic operon (Pierson *et al.*, 1994, Wood & Pierson, 1996). The gene *phzI* encodes an AHL synthase responsible for the production of the signal molecule *N*-Acyl-homoserine lactone (AHL) and PhzR is a transcriptional factor. Once the concentration of AHL signals is sufficient to bind to PhzR, the PhzR-AHL complex binds to the phenazine operon promoter and activates expression of phenazine biosynthetic operon and it also binds to the *phzI* promoter to enhance the *phzI* gene (Pierson *et al.*, 1994, Wood & Pierson, 1996, Khan *et al.*, 2005, Khan *et al.*, 2007). I am particularly interested in the regulation of the phenazine biosynthetic genes, because this affects the quantity and timing of phenazine production. In Chapter III, my work focused on DNA sequences in the promoter region of the biosynthetic operon of the *P. chlororaphis* 30-84. Interestingly, bioinformatic analysis revealed that the DNA sequences downstream of the phenazine promoter

contained features, which are predicted to form significant secondary structures (e.g. stem loop) (Fig. 3.2 and 3.5). To attempt to delineate the role of these features, subclones of phenazine promoter-*lacZ* fusion were created by deletions of various sequences or site-specific alterations (Table 3.1, Fig. 3.2 and 3.6). Of significance, deletion of a 185 bp region including the 5' untranslated region (5'UTR) and the translation start site of *phzX* (the first gene of phenazine operon) resulted in 5-fold higher *lacZ* expression as compared to the wild-type promoter (Fig. 3.3). This suggests that the sequence features capable of forming secondary structures likely function as a negative regulatory element.

To better understand the role of the promoter negative regulatory element, we generated a mutant, 30-84 Enh that contains a 90 bp deletion designed to disrupt the potential stemloop structures. As we expected, 30-84 Enh resulted up to 6-fold higher phenazine production, but expression remained under quorum sensing control. Interestingly, qPCR analysis also revealed that 30-84 Enh exhibited 1.7- and 1.3-fold higher expression of *phzR* and *phzI* compared to 30-84 wild-type, indicating that the stemloop region negatively regulates phenazine production at least in partially through *phzR* and *phzI*.

DNA sequence comparison of the promoter region of the 30-84 phenazine biosynthetic operon to the promoter region of the phenazine biosynthetic operons of other phenazine producing pseudomonads revealed that these sequence features are unique to 2OHPCA producing strains (i.e. *P. chlororaphis* strain O6 and strain PA23). It is logical to speculate that the phenazine molecule (especially 2OHPCA) might be

involved in the regulation of its own gene expression acting on this region in a manner similar function to riboswitch governed gene regulation system. However, endo- or exogenously supplied phenazine derivatives (PCA and 2-OH-PCA) did not alter *phzX* expression (Fig. 3.10). To determine whether an unidentified protein is interacting with the 90 bp region, biotinylated DNA probes of phenazine promoter regions from wild-type and 30-84 Enh were constructed and binding assay conducted using total protein isolated from crude extracts from the wild-type (Fig. 3.12A). The results indicated that 90 bp promoter regulatory region may interact with an unidentified repressor protein at lower cell density, whereas this protein has reduced affinity for the enhanced promoter at higher cell density (Fig. 3.12B). Future work needs to focus on identifying this protein, by extraction of this complex (e.g. protein bound DNA probes), followed by protein sequence analysis to identify the protein. Gel-shift protein binding assay (i.e. electrophoretic mobility shift assay) with isolated protein and DNA probes of the phenazine promoter region will be needed to elucidate the function of the protein.

Phenazine production by *P. chlororaphis* 30-84 contributes in multiple ways to its ecological fitness and biological control capabilities (e.g. pathogen inhibition, biofilm formation, root colonization and rhizosphere persistence) (Mazzola *et al.*, 1992, Pierson & Thomashow, 1992, Whistler & Pierson, 2003, Maddula *et al.*, 2006, Wang *et al.*, 2016). Consistent with my hypothesis that enhanced phenazine production should result in enhanced biological control capability, compared to the wild type, 30-84 Enh was enhanced in its ability to form biofilms, inhibit target fungi, and promote wheat root growth. Previously, it was reported that overproduction of key antifungal metabolites by

genetically modified biological control strains does not always correlate with enhanced biological control capability in greenhouse assays (Delany *et al.*, 2001, Haas & Keel, 2003, Huang *et al.*, 2004). Secondary metabolite biosynthesis is tightly regulated to avoid excessive energy consumption. Therefore, overexpression of regulatory genes by introduction of high expression vectors might reduce the efficacy of biological control capacity. An advantage of using 30-84 Enh is that phenazine production is significantly increased by modification of the innate regulation of phenazine production in 30-84. This means 30-84 Enh produces phenazine only upon recognition of natural stimuli, which is partly controlled by quorum sensing. Thus, the use of 30-84 Enh for enhanced biocontrol activity is promising, because this strain may not be limited by excessive energy consumption (e.g. phenazine biosynthesis is still tightly controlled, but overall phenazine production is significantly higher). Future studies with the enhanced biological control strain will consider whether these capabilities result in better disease suppression. Together, the results from Chapter III will provide greater insight into the characterization of the quorum sensing controlled promoter as well as potential applications for improved biological control by this phenazine-producing strain.

In Chapter IV, I showed that a Tn5 plasposon-generated mutant that disrupted the gene *miaA* significantly reduced phenazine (Fig. 4.1A and 4.1B). The *miaA* gene encodes a tRNA prenyltransferase, with the specific function of the addition of isopentenyl group of adenosine at position 37 of tRNA (Esberg & Björk, 1995, Esberg *et al.*, 1999). The objectives of Chapter IV were to characterize functional roles of MiaA in phenazine biosynthesis as well as capabilities related to bacterial fitness and biological

control activity including, bacterial growth, antifungal activity, exoenzyme production and soil persistence. I observed that compared to wild type phenazine production and promoter activity of phenazine operon in the miaA mutant was significantly reduced. However phenazine production was restored by introducing *miaA in trans* in high expression vector (Fig. 4.4), indicating that functional MiaA is required for appropriate phenazine production. Transcriptomic analysis revealed that expression of quorum sensing system *phzR*/*phzI* and *pip* (the transcriptional regulator previously shown to regulate quorum sensing) were significantly reduced in the *miaA* mutant (Fig. 4.6). These findings were confirmed by transcriptional fusions to the promoters of *phzI* and *phzR*, as well as AHL quantification (Fig. 4.7). Additionally, over expression of the *phzR* and *pip* in high expression vectors *in trans* in the *miaA* mutant restored phenazine production indicating that reduced phenazine production in *miaA* mutant was due to the reduced expression of *pip* through quorum sensing system (Fig. 4.8).

It has been reported previously that *miaA* mutation caused major defects in translation efficiency and fidelity (Li *et al.*, 1997). Transcriptional analysis and over expression of other key regulatory genes previously shown to regulate *pip* including RpoS (stationary sigma factor) and RpeB (two component response regulator) indicated that these were not transcriptionally regulated by MiaA. Therefore it was logical to speculate that MiaA may influence the translational efficiency of RpoS and/or RpeB. Of significance, the results indicated that MiaA affects phenazine production via a reduction in the translational efficiency of both of these two important phenazine regulatory genes (Fig. 4.9).

Mutation in *miaA* causes broad pleiotropic phenotypes, likely because translational efficiency and fidelity are important for proper synthesizing the functional proteins. As expected, the *miaA* mutant of *P. chlororaphis* 30-84 showed multiple pleiotropic phenotypes including reduced bacterial growth, survival rate in the soil, exoprotease production and antifungal activity. Overall the results presented in Chapter IV suggest that MiaA influences the production of secondary metabolites and other traits involved niche adaptation in *P. chlororaphis* 30-84 via its tight control of the translation of key global regulatory genes.

Previous studies suggested that MiaA influences the regulation of several amino acid biosynthetic operons (Bjork, 1984, Ericson & Björk, 1986). Similarly, in *P. chlororaphis* 30-84, whole transcriptomic analysis revealed that genes involved in aromatic amino acid metabolism were differentially regulated compared to wild-type (Pierson et al., unpublished data). These results suggest that MiaA has regulatory roles in the expression of genes involved in bacterial amino acid utilization and metabolism. Further studies would require to analysis of bacterial metabolomic changes using LC-MS analyses.

Overall Summary of Each Chapter (Fig. 5.1):

1. In Chapter II: The types of phenazine derivatives produced by *P. chlororaphis* 30-84 influences on key biological control properties including pathogen inhibition, specificity of the antagonism against different pathogens, eDNA release and biofilm formation. However, types of phenazine derivative produced do not significantly influence root colonization under the condition that I tested.

2. In Chapter III: In phenazine promoter of *P. chlororaphis* 30-84 contains negative regulatory elements, which controlling gene expression by forming secondary structures. Chromosomal deletion of negative regulatory region in the phenazine promoter resulted in significantly higher phenazine production, but still under quorum sensing system control. Additionally, the generation of a phenazine enhanced mutant improved *in vitro* fungal inhibition capacity, biofilm formation and promotion of wheat root growth.
3. In Chapter IV: MiaA, tRNA prenyltransferase influences the production of bacterial secondary metabolites (i.e. phenazine and exoprotease) and other ecological traits involved in environmental adapataion via control of key regulatory proteins in *P. chlororaphis* 30-84. Specifically, MiaA influences phenazine biosynthesis via controlling translation of RpeB and RpoS, which in turns regulate transcription of *phzR/phzI* quorum sensing system through transcriptional regulator *pip.* In addition, MiaA also contributes to bacterial proper growth and soil persistence.

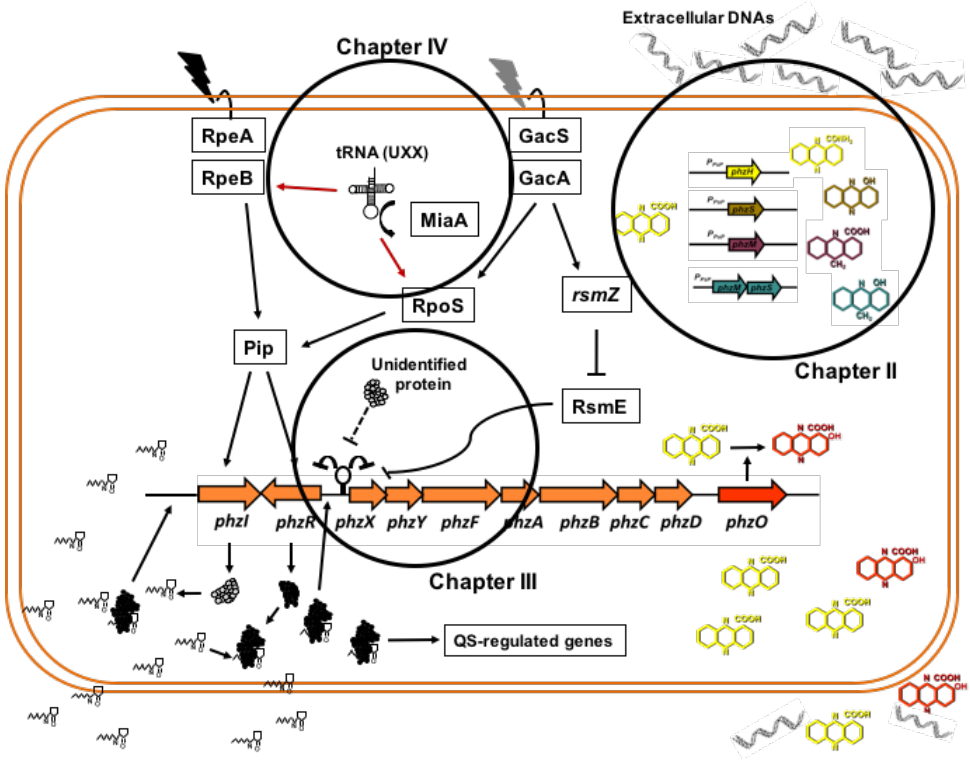


Figure 5. 1. Proposesed model for the regulaton of phenazine biosynthesis in *P. chlororaphis* 30-84 with summary of how results from my work advance the current model.

Recommendation of Specific Future Works

Chapter II:

- In this chapter, altering the type of phenazines produced by *P. chlororaphis* 30-84 affected the specificity of fungal pathogen inhibition at *in vitro* dual culture condition. To better understand the role of phenazines as an antibiotic, *in situ* disease suppression assays will be required because disease suppression is the most important trait for the biological control agents.
- The results in Chapter II also showed that modification of the type of phenazine produced by *P. chlororaphis* 30-84 affected eDNA release, and the amount of

eDNA release is correlated with the capability of biofilm formation. To delineate the mechanism of eDNA release, pyocin quantification from biofilm matrix produced by each altered phenazine producer is required.

Chapter III:

- The results in Chapter III indicated that 90 bp promoter negative regulatory region might interact with an unidentified protein that has a repressor function. To identify this protein, protein-DNA probe complex should be isolated by gel extraction and sequencing its amino acid. To elucidate the function of the protein, gel-shift binding assay with purified protein and DNA probes of the phenazine promoter region will be required.
- Enhanced phenazine producing strain 30-84Enh showed improved ecological traits involved in biological control capabilities including greater biofilm formation, imcreased antifungal activity and promotion of root growth. To verify 30-84Enh is a potentially better candidate as a biological control agent, *in situ* disease suppression assays will be required.

Chapter IV:

- MiaA influence translational expression of RpeA and RpoS because those two proteins contain 10.1 % and 13 % of uridin codon (UXX). Codon usage has been proposed as one of the post-transcriptional regulatory mechanisms and influence bacterial protein expression. To better understand the phenazine biosynthetic regulatory mechanism in *P. chlororaphis* 30-84, biased codon usage analysis will be required for all phenazine regulatory systems.

- The results in Chapter IV suggested that MiaA influences the regulation of bacterial secondary metobalites. Also whole transcriptomic analysis of *miaA* revealed that genes involved in aromatic amino acid metabolism were differentially regulated. Future studies would be required to anaylsis of bacterial metabolomics changes using LC-MS analyses.

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