

QUORUM SENSING AND PHENAZINES ARE INVOLVED IN BIOFILM
FORMATION FOR PSEUDOMONAS CHLORORAPHIS STRAIN 30-84.

By

V S R Krishna Maddula

Copyright © V S R Krishna Maddula, 2008.

A Dissertation Submitted to the Faculty of the
DIVISION OF PLANT PATHOLOGY AND MICROBIOLOGY
DEPARTMENT OF PLANT SCINECES
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
WITH A MAJOR IN
PLANT PATHOLOGY
In the Graduate College
THE UNIVERSITY OF ARIZONA

2008

**THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE**

As members of the Dissertation Committee, we certify that we have read the dissertation prepared by V S R Krishna Maddula entitled “QUORUM SENSING AND PHENAZINES ARE INVOLVED IN BIOFILM FORMATION FOR PSEUDOMONAS CHLORORAPHIS STRAIN 30-84” and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

_____ Date: April,1st, 2008

Dr. Leland S. Pierson

_____ Date: April,1st, 2008

Dr. Hans D. VanEtten

_____ Date: April,1st, 2008

Dr. Martha C. Hawes

_____ Date: April,1st, 2008

Dr. Elizabeth A. Pierson

_____ Date: April,1st, 2008

Dr. Joan E. Curry

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College. I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

_____ Date: April,1st, 2008

Dissertation Director: Dr. Leland S. Pierson

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the copyright holder.

SIGNED: V S R Krishna Maddula

ACKNOWLEDGEMENTS

With out the help of many nice people, I might not have been able to finish my work. Unfortunately, due to limitation with space I may not acknowledge everybody that helped me by name, but I sincerely thank each and everyone. I want to sincerely thank my “science parents” Sandy and Betsy. Working with them may be best thing happened in my life. Special thanks to my advisor Dr. Leland (Sandy) Pierson. He is always available and he set me a role model by his actions than words. I highly admire his intellect, humbleness, patience, hard-working nature and his passion to science. I want also to extend my sincere thanks to Dr. Elizabeth (Betsy) Pierson for her help and guidance. She has a great impact on my work. Her teaching/training on preparing talks, scientific writing, and working in lab is invaluable. I profusely thank Dr. Martha Hawes for her guidance in my work as a committee member. I owe a lot for her care and guidance for my future career plans. I am very lucky to have Dr. Hans VanEtten in my committee, his help in executing my project and planning my future career was very valuable. I will always cherish time spent with him on the lunch table discussions. I want to thank Dr. Joan Curry for serving as my dissertation committee member. Her special expertise in bacterial adhesion to surfaces was very useful; it gave me a chance to think about my project from a physical chemistry angle.

I also want to thank Dr. Carl Boswell for his help with confocal microscope. My special thanks to Dr. David Elliott and Dr. Haluk Beyenal for their help in analyzing 3D confocal images.

I wouldn't have reached this stage with out the love and support from my family and friends. My love and hugs to my parents and brothers for their continuous inspiration. My special thanks to Anne Estes, my fellow graduate student. She was always there to talk about my research and lot more. I thank also my friends Sid Schwartz, Gerardo Puopolo, Chrystine Solofoharivelo, Chandra Saripalli, Melania Betts, Rhodesia Celoy, Gerard White, Hema Narra, Rudresh Kottani, Ramesh Alleti, Pradeep Marri, Cathy Wasmann, Mali Guntilaka, and Baomin Wang. I also owe lots of treats to my special buddy Ian Pierson for his recommendations to grant me PhD. My love and hugs to Chris Pierson.

Lastly I want to thank the God almighty for giving me a great life.

DEDICATION

This Dissertation is dedicated to my parents M. M. Rao and M. Sridevi, and to my two elder brothers and their families for their love, support, and encouragement throughout my life.

This Dissertation is also dedicated to the hard working scientific community who continuously strive to make our lives better.

TABLE OF CONTENTS

ABSTRACT	12
I. INTRODUCTION	14
Model System: <i>Pseudomonas chlororaphis</i> 30-84	14
Diversity in phenazine antibiotic production.....	21
Bacterial Biofilms	25
Plant associated bacterial biofilms.....	33
II. PRESENT STUDY.....	35
Importance of the present study.....	35
Introduction and rationale for the present study.....	36
DISSERTATION FORMAT	43
REFERENCES	44
APPENDIX A. Quorum Sensing and Phenazines are Involved in Biofilm Formation by <i>Pseudomonas Chlororaphis (aureofaciens)</i> Strain 30-84.....	60
APPENDIX B. Altering the Ratio of Phenazines in <i>Pseudomonas Chlororaphis</i> <i>(aureofaciens)</i> Strain 30-84: Effects on Biofilm Formation and Pathogen Inhibition.	76
APPENDIX C. Towards Understanding the Mechanisms by which Phenazines Contribute to Biofilm Formation	120
APPENDIX D. Studies on Effects of <i>rpeA/rpeB</i> Two-component Regulators in <i>Pseudomonas</i> spp.	147

TABLE OF CONTENTS - *continued*

APPENDIX E. Standardizing Protocol for pRL-27 Tn5 Mutagenesis for <i>Pseudomonas chlororaphis</i> strain 30-84 and <i>P. cedrella</i> strain PU43.....	155
--	-----

LIST OF TABLES

Table I.1. Genes / Factors that were known to be involved in the initial attachment step of biofilm formation.	28
Table I.2. List of factors known to be involved in bacterial dispersal from biofilms..	32
Table A.1. Bacterial strains and plasmids used in this study.	65
Table A.2. Effect of specific mutations and specific genes <i>in trans</i> on phenazine production, exoprotease activity, and adhesion ability.	66
Table A. 3. Swimming, swarming, and twitching motilities of strain 30-84 and mutants deficient in either or both quorum-sensing systems.	72
Table B. 1. Bacterial strains and plasmids used in this study.	113
Table B. 2. Production of PCA and 2-OH-PCA by strain 30-84 and its altered PZ derivatives.	115
Table B. 3. Percentage of the slide surface area covered by the 30-84 derivatives at early time points.	116
Table B. 4. Biofilm volumetric parameters.	117
Table B. 5. Dispersal rates by 30-84, 30-84PCA and 30-84O* from biofilms. ...	118
Table B. 6. Fungal inhibition assay.	119
Table C. 1. List of genes UP-regulated in the presence of PZs.	142

LIST OF TABLES - *continued*

Table C. 2. List of genes DOWN-regulated in the presence of PZs.	145
Table E. 1. Results summary for <i>Pseudomonas cedrella</i> PU43::Tn5 mutants.	163
Table E. 2. Results summary for <i>Pseudomonas chlororaphis</i> 30-84::Tn5 mutants.	168

LIST OF ILLUSTRATIONS

Illustration I. 1: Structures of the PZ antibiotic compounds produced by <i>Pseudomonas chlororaphis</i> 30-84.	14
Illustration I. 2: Diagram showing the multiple regulators of PZ biosynthesis in <i>P. chlororaphis</i> strain 30-84.	18
Illustration I. 3: Stages of biofilm development.	29
Illustration E. 1. Map of Tn5-pRL27 transposon vector.	156
Illustration E.2. Diagram of Biparental mating.	157
Illustration E. 3. Pictorial depiction of pRL-mutants plasposon generation and one-step cloning.	158
Illustration E.4. Circularized chromosomal fragments with pRL27::Tn5 insertion.	159

LIST OF FIGURES

Figure A.1. Time course of cell adhesion by wild type <i>P. chlororaphis</i> strains 30–84 and its derivatives.	68
Figure A. 2. Effect of AHLs on adhesion by various mutants.	69
Figure A. 3. Effect of genetic complementation of a phenazine structural mutant and addition of phenazine on adhesion by various mutants.	70
Figure A. 4. Flow cell images of biofilm formation by wild type strain 30-84 and various mutants.	71
Figure A. 5. Bacterial populations on seeds and roots.	72
Figure B. 1. (A) Simplified PZ biosynthetic pathway.	110
Figure B. 1. (B) Liquid cultures of the PZ derivative mutants compared to 30-84... ..	110
Figure B. 2. Effect of altered PZ ratio on the initial attachment stage of biofilm formation.	111
Figure B. 3. Effect of altered phenazine ratio on biofilm architecture.	112
Figure C. 1. Effect of exogenous PZ addition on attachment of metabolically inactivated 30-84 wild type bacterial cells.	139
Figure C. 2. Effect of exogenous PZ addition on attachment of metabolically inactivated 30-84Z cells.	140
Figure C. 3. Effect of exogenous PZ addition on attachment of metabolically inactive 30-84Z measured in flow cell assay.	141
Figure D. 1. Cell adhesion by wild type <i>P. chlororaphis</i> strain 30-84 and the PZ-overproducing derivative, 30-84 <i>rpeA</i>	154

ABSTRACT

Pseudomonas chlororaphis (aureofaciens) 30-84 is a biocontrol bacterium effective against take-all disease of wheat. Phenazine (PZ) production by strain 30-84 is the primary mechanism responsible for pathogen inhibition and the rhizosphere persistence of 30-84. The PhzR/PhzI system of strain 30-84 directly regulates PZ production and mutations in this QS system are defective in biofilm formation. Genetic complementation or direct addition of AHL signal restored biofilm formation to a *phzI* mutant. Mutations in PZ biosynthesis were equally defective in biofilm formation. Addition of PZ or genetic complementation of the PZ biosynthetic mutation restored biofilm formation. QS and PZ production also were involved in the establishment of populations on wheat seeds and plant roots. Presence of 10% wild type strain 30-84 in mixtures with QS or PZ mutants restored root colonization. *These data demonstrate that QS and specifically PZ production are essential for biofilm formation by strain 30-84. This is a new role for PZs in the rhizosphere community.*

Strain 30-84 produces primarily phenazine-1-carboxylic acid (PCA) and 2-hydroxy-PCA (2-OH-PCA). We generated derivatives of strain 30-84 that produced the same total amount of PZs as the wild type but produced only PCA, or more efficiently converted PCA to 2-OH-PCA. These derivatives with altered PZ ratios differed from the wild type in initial attachment, biofilm architecture, and dispersal. Increased 2-OH-PCA production increased initial attachment, although both alterations resulted in thicker biofilms and reduced dispersal rates. Loss of 2-OH-PCA production resulted in a

significant reduction in pathogen inhibition. *My findings indicate that alterations in the endogenous ratios of PZs have wide-ranging effects on the biology of strain 30-84.*

I initiated studies to understand the mechanisms by which PZs affect surface attachment and biofilm development. Addition of PZs to metabolically inactivated cells improved adhesion compared to the inactive cells alone, suggesting that PZs may improve initial binding to surfaces. Results from whole genome transcription profiles of wild type strain 30-84 to a PZ mutant indicate that genes potentially involved in biofilm formation were up-regulated in the presence of PZs. *These results provide initial evidence that PZs may modulate cell adhesion and biofilm formation via multiple mechanisms.*

I. INTRODUCTION

1. Model System: *Pseudomonas chlororaphis* 30-84:

Pseudomonas chlororaphis (*aureofaciens*) strain 30-84 was isolated from the rhizosphere of wheat from a field in Kansas. In this field take-all disease of wheat caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (*Ggt*) had been naturally suppressed. Coating strain 30-84 on wheat seeds can protect the growing plants from take-all disease. This biological control bacterium produces several different secondary metabolites. Some examples of secondary metabolites produced by *P. chlororaphis* 30-84 are hydrogen cyanide (HCN); siderophores, exoproteases; and phenazine (PZ) antibiotics. Strain 30-84 produces three PZs: phenazine -1-carboxylic acid (PCA), 2-hydroxy-PCA (2-OH-PCA), and a minor amount of 2-hydroxy-phenazine (2-OH-PZ) (Pierson and Thomashow, 1992).

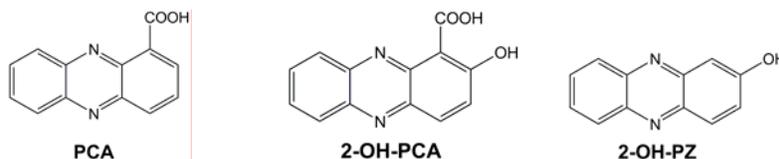


Illustration 1: Structures of the PZ antibiotic compounds produced by *Pseudomonas chlororaphis* strain 30-84.

P. chlororaphis 30-84 influences the rhizosphere community by the production of these secondary metabolites. *P. chlororaphis* 30-84 inhibits a significant number of deleterious rhizosphere bacteria and fungi (Pierson and Thomashow, 1992; Toohy et al., 1965). Thus, secondary metabolite production provides a fitness advantage to *P. chlororaphis* 30-84 in the rhizosphere allowing it to compete and survive. One mechanism for its better survival is by competitive inhibition of the growth of other

rhizosphere microbes (Toohey et al., 1965). In experiments comparing the survival and persistence of wild type strain 30-84 and a PZ structural mutant on wheat roots after repeated cycles of wheat growth in pots in sterile and natural soil, the mutant persisted as well as the wild type in sterile soil but had lower survival compared to wild-type 30-84 in natural soil (Mazzola et al., 1992). Thus, PZ production is a major mechanism for persistence of strain 30-84 in soil in competition with the indigenous microflora. The production of PZs by *P. chlororaphis* 30-84 is additionally the primary mechanism for *Ggt* inhibition. Previous *in vitro* experiments in our lab showed that a PZ mutant could not inhibit the growth of *Ggt* and over-expression of PZs enhanced the strain's ability to inhibit the pathogen *in vitro* (Whistler and Pierson, 2003).

The PZ biosynthetic locus of *P. chlororaphis* 30-84 consists of eight genes in single operon (*phz XYFABCD O*). The regulation of the PZ operon is very complex and under tight control, integrating multiple regulatory mechanisms. These multiple mechanisms include cell density-dependent quorum sensing (PhzI/PhzR), positive two-component global regulation (GacS/GacA), negative two-component regulation (RpeA/RpeB) and multiple forms of post-transcriptional regulation (RsmA/RsmZ and Hfq). PZ gene expression in 30-84 also was found to be influenced by other members of the wheat rhizosphere microbial community. More details for these experiments are given below.

The fact that PZ production is tightly regulated by multiple mechanisms does not support the original hypothesis in the literature that PZs are simple antibiotics that provide a competitive advantage for the bacterium. For example, if PZs are truly

competitive compounds produced to allow the bacterium to compete for colonization of the root, then why are they not produced until the bacterial population size has reached a critical density (i.e. a quorum)? The complex regulation of PZ production is more consistent with the hypothesis that PZs have multiple roles in the bacterial life cycle. This hypothesis is also in agreement with natural selection theory that metabolically expensive antibiotics are more likely to be maintained if they perform multiple functions (Wink, M., 2003).

Quorum sensing regulation of PZs:

Quorum sensing (QS) mechanisms of regulating gene expression are seen in a wide range of bacteria and appear to be important in many cell-cell interactions and cell-host interactions. QS is a cell density or ‘quorum’ dependent mechanism of gene regulation, whereby bacteria sense their population density *via* sensing the amount of low molecular weight signal molecules. The best studied QS signal molecules are cell – membrane diffusible *N*-acyl-homoserine lactone (AHL) molecules in gram-negative bacteria. Typically, a QS regulatory system consists of two genes: an AHL synthase gene and a transcriptional regulatory gene. In the case of PZ biosynthesis, the two QS regulatory genes, *phzI* and *phzR*, encode the AHL synthase and the transcriptional regulator, respectively. PhzI synthesizes hexanoyl-homoserine lactone (HHL), utilizing *S*-adenosyl methionine (SAM) as a precursor. These HHL signal molecules are readily diffusible in and out of the bacterial cell resulting in low intracellular concentrations at lower bacterial densities. As the population density increases, the environmental signal concentration increases resulting in increased intracellular concentrations. When the

intracellular signal concentration reaches a threshold level, the HHL signal molecules bind to their respective transcriptional regulator molecule. In this case the transcriptional regulator is the product of *phzR*. This binding of HHL to PhzR increases the affinity of PhzR to the PZ biosynthetic promoter region resulting in recruitment of RNA polymerase and the initiation of PZ gene expression (Wood and Pierson, 1996). It has been demonstrated for one such regulatory protein, TraR of *Agrobacterium tumefaciens*, that binding to its signal results in dimerization of the protein. Dimerization of regulatory protein results in a conformational shift that makes the protein domains involved in DNA binding accessible (Zhang, R. G. et al., 2002). Originally, this type of regulation was termed “quorum sensing” because it was believed that gene expression was dependent upon reaching a certain population size (e.g. when a quorum had been reached). We now know that activation occurs when the amount of signal reaches a threshold concentration (Pierson et al., 1998b)

A second QS system, CsaR/CsaI, also was characterized in strain 30-84 (Zhang and Pierson, 2001). This system has the QS regulatory homologs CsaR/CsaI, similar to PhzR/PhzI and other QS regulators of pseudomonads. This second QS system has been shown to have little effect on the regulation of PZ production. However, the loss of *csaR* in strain 30-84 showed a clear phenotypic difference from the rough colony morphology of the wild type to a smooth colony phenotype (Zhang and Pierson, 2001).

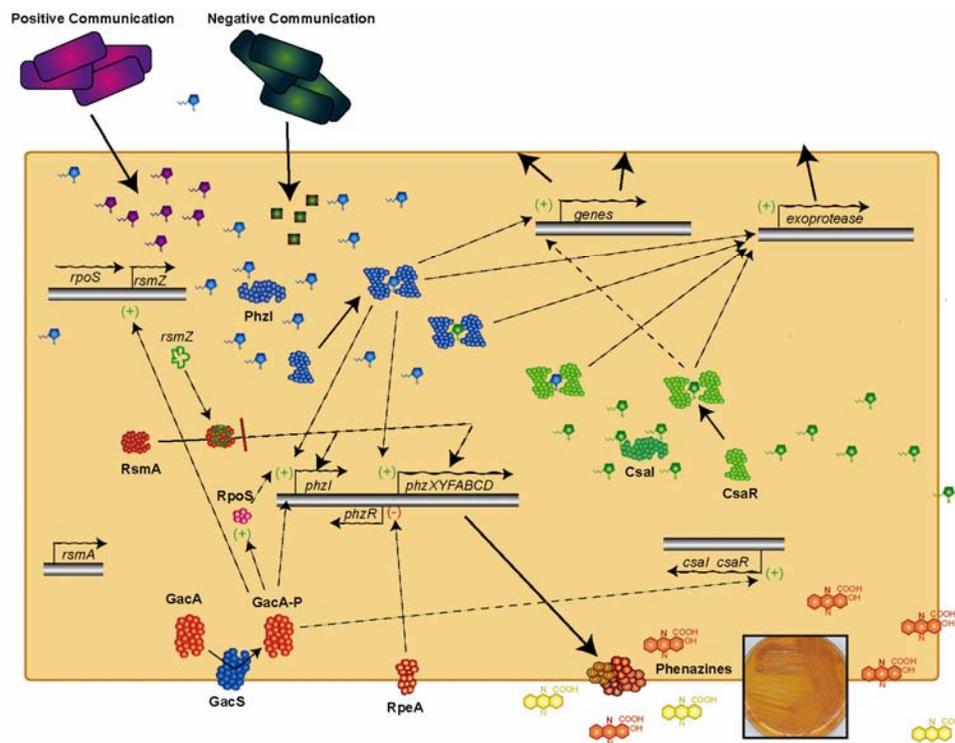


Illustration 2. Diagram showing the multiple regulators of PZ biosynthesis in *P. chlororaphis* strain 30-84 (Source Dr. L. S. Pierson III)

Quorum sensing (QS) is a prevalent mechanism of intercellular signaling among bacteria that has received significant attention in the study of biofilms. Both Gram-negative and Gram-positive bacteria utilize small molecular weight QS signals to regulate the transcription of target genes based on the amount of signal present (reviewed in Miller and Bassler, 2001). For example, in the opportunistic human pathogen *P. aeruginosa*, 6-10% of the genome is estimated to be under QS regulation (Schuster et al., 2003; Wagner et al., 2003). Among plant-associated bacteria, AHL-mediated QS regulation is an integral component of many aspects of bacterial interactions with plants, both pathogenic and beneficial (reviewed in Ramey et al., 2004; Von Bodman et al., 2003; Whitehead et al., 2001). Previous studies using various biosensors that allow detection of AHL

derivatives and their antagonists demonstrated that (i) bacterial populations inhabiting rhizosphere and plant surfaces produce a mixture of diverse AHL signals, (ii) plant associated bacteria are capable of responding to signals produced by different bacterial species and (iii) this type of microbe-microbe communication occurs *in situ* on plants (Cha et al., 1998; McLean et al., 1997; Pierson et al., 1998b). The production of diffusible AHL signals also plays a role in microbe-plant communication (reviewed in Bauer and Mathesius, 2004).

Two -component positive regulation:

GacS and GacA constitute a two-component global regulatory system in strain 30-84 and in other *Pseudomonas* spp. The first component (GacS) is a transmembrane sensor kinase (SK) protein and the second (GacA) is the cytoplasmic regulatory response (RR) protein. Usually the SK protein is a histidine kinase that perceives external stimuli through its N-terminal periplasmic domain. This results in auto-phosphorylation of the SK at a highly conserved histidine residue located in the cellular cytoplasm. Perception of an external stimulus of the SK induces a conformational change that traverses the cell membrane and transfers the phosphate group from the conserved histidine residue of the SK to a highly conserved aspartate residue on the RR protein, thus inducing a conformational change in the RR. The conformational change increases RR binding affinity to specific promoter regions. This improves RNA polymerase recruitment at the promoter and increases the transcription rates of certain genes. Mutations in either of these components result in loss of secondary metabolite production, including PZs, HHL signals, and HCN. Therefore, GacS/GacA serve as global regulators of gene expression

(Chancey et al., 2002). Recent unpublished results in our lab and in other systems demonstrate that GacS/GacA also regulates the RsmA/RsmZ post-transcriptional regulatory system (described below).

Post-transcriptional regulation:

In our lab, we also discovered genes that appear to be involved in post-transcriptional regulation of PZs. We showed via *in trans* studies that a specific gene repressed the *phzB* reporter gene expression 10-fold *in trans*. The predicted product of this gene is similar to RsmA (Chatterjee et al., 2002 and Haas et al, 2003), a protein that targets specific RNA transcripts for degradation. Lee and Pierson (unpublished) cloned *rsmZ*, a small regulatory RNA located adjacent to *rpoS* that counteracts RsmA (Chatterjee et al., 2002 and Haas et al, 2003, Lapouge et al., 2007). In studies of *rsmZ* expression in a GacA mutant, results showed *rsmZ* expression was under GacA control. Expression of *rsmZ* *in trans* from a non GacA-regulated promoter in a GacA mutant resulted in the restoration of PZ production (Lee and Pierson, unpublished).

Negative two-component regulation:

Previous work in our lab identified a gene *rpeA* (repressor of phenazine expression) that is a homologue to the two-component sensor-kinases of many other bacteria. Mutations in *rpeA* allow excess production of PZs in rich media, and also allow PZ production even under low nutrient conditions. In other previous experiments in our lab, *rpeA* mutants were shown to by-pass the QS requirement for PZ production (Whistler and Pierson, 2003). More detailed experiments determining the roles of this negative two-component regulatory system on global gene expression, including characterization of the

rpeA/rpeB homologs of *Pseudomonas aeruginosa* strain PAO1 and strain PA14, are currently under study.

Phenazine production is influenced by the microbial community:

Earlier studies in our lab identified two subpopulations of the wheat rhizosphere community which could influence PZ gene expression. One subset of the population (referred to as positive signalers) was able to synthesize AHL signals that restored PZ production to strain 30-84I (PhzI) on plant roots (Wood et al., 1997; Pierson E A et al., 1998). The second subset (referred to as negative signalers) negatively influenced PZ expression *in vitro* and on roots. These negative signalers inhibited PZ expression in strain 30-84Z (*phzB::lacZ*) by 4-9 fold (Morello et al., 2004). Further studies to identify negative signaling genes in negative signal strains by genetic and biochemical approaches are under way. An approach of creating random Tn5 mutations in strain PU43, one of the most consistent negative signaling strains, is explained in detail in APPENDIX E.

2. Diversity in phenazine antibiotic production.

Phenazines (PZs) are well-known pigmented, nitrogen-containing heterocyclic secondary metabolites produced by a variety of bacteria, including *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium*, *Burkholderia*, *Methanosarcina* and *Erwinia* (Mavrodi et al, 2006, Chin-A-woeng, 2003, Giddens et al, 2002, Laursen and Nielsen, 2004, Turner and Messenger, 1986). More than 6,000 PZ containing compounds and 100 natural derivatives of PZs have been described. PZs have toxic effects on a broad variety of organisms. Natural PZs produced by Eubacteria are produced during late

growth stages and usually are excreted out of bacterial cells by unknown mechanisms, often at high levels (e.g. mg/L to g/L). PZs have been studied longer than many other secondary metabolites (Mavrodi et al., 2006). The majority of the work on PZs has focused on the chemical or biological synthesis, redox properties, antitumor, or biological control activities.

Phenazines as antibiotic biological control compounds:

PZs have been recognized for their antibiotic properties for over 150 years (Mavrodi et al. 2006). PZs effectively control a wide range of plant pathogenic fungi and are a well-characterized mechanism of bacterial plant disease control (Anjaiah et al., 1998; Chin-A-Woeng et al., 2003; Raajmakers et al., 2002; Walsh et al., 2001). Strain 30-84 produces three PZ derivatives PCA, 2-OH-PCA and a small amount of 2-OH-PZ (Pierson and Thomashow, 1992). The well studied opportunistic pathogen of both plants and animals, *P. aeruginosa*, produces the methylated PZ derivative pyocyanin (PYO) and a few *P. aeruginosa* strains also produce PCA (Mahajan et al., 1999). Similarly, another well characterized biological control agent, *P. fluorescens* strain 2-79, produces only PCA (Thomashow and Weller, 1988). *P. chlororaphis* strain PCL1391 produces phenazine-1-carboximide (PCN) and some PCA (Chin-A-Woeng et al., 1998).

The PZs produced by strain 30-84 are responsible for inhibition of *Gaeumannomyces graminis tritici* (Ggt), the take-all fungal pathogen of wheat. PCN was shown to control *Fusarium oxysporum* f. sp. *radicis lycopersici*, the causative agent of tomato foot and root rot. Both PCA and PCN are involved in control of *Pythium myriotylum*, the causative agent of root rot of cocoyam (Tambong and Hofte, 2001). PYO was shown to

inhibit *Septoria tritici* of wheat (Falishman et al., 1990; Kerr et al., 1999). In other studies, PCA and PCN produced by *Pseudomonas* PCL1391 were shown to induce several ABC transporters in *Botrytis cinerea* (Schoonbeek et al., 2002). Antibiotic activity of PZs also was shown against *Rhizoctonia solani*, *Gibberella avenacea*, *Alternaria* spp., and *Drechslera graminea* (Gurusiddaiah et al., 1986; Smirnov and Kiprianova, 1990)

The toxic properties of PZ antibiotics on various rhizosphere bacterial and fungal organisms are not completely understood. One hypothesis is that toxicity results from the generation of reactive oxygen species (ROS) (Chin-A-Woeng et al., 2003). PZs differ in their physical structures based on the various substituents (such as -COOH, -OH, -CH₃) added at different positions to the basic heterocyclic ring, and thus may differ in the antibiotic activity. Among various PZs, 2-OH-PCA from strain 30-84 and PCN from strain *P. aeruginosa* have greater antifungal activity *in vitro* than PCA (Mavrodi et al., 2006).

Additionally, external factors such as pH were shown to affect the biological control activity of PZ compounds (Ownley et al., 1992; Chin-A-Woeng et al., 1998). PCN was shown to have 10-fold more antibiotic activity than PCA for *F. oxysporum* f. sp. *radicis-lycopersici* at pH above 5.7 (Chin-A-Woeng et al., 1998). This might be due to the fact that PCN remains largely protonated at alkaline pH, whereas PCA does not.

Phenazines as virulence factors:

Phenazines also are known to act as virulence factors on mammalian hosts. For example, the production of PYO by *P. aeruginosa* plays an important role for successful

host invasion and disease (Baron and Rowe, 1981; Cox, 1986; Denning et al., 2003; Mahajan et al., 1999; Ran et al., 2003). A higher concentration (27 μg per ml) of PYO was detected in the sputum of patients with infection of *P. aeruginosa* (Wilson et al., 1988). PZ production has multiple effects on host cell processes, including respiration, ciliary beating, epidermal cell growth, calcium homeostasis, prostaglandin release, neutrophil apoptosis, interleukin-2 release, immunoglobulin G secretion, and a protease-antiprotease activity (Hassan and Fridovich, 1980; Laursen and Nielsen, 2004; Ran et al., 2003). Oxidative activity of PZs was required for lung infection by *P. aeruginosa* (Lau et al., 2004).

Phenazines as redox compounds:

PYO may facilitate extracellular electron shuttling, hypothesized to be important in iron acquisition/energy generation in biofilm communities (Abken et al., 1998; Friedheim, 1931; Hernandez and Newman, 2001; Hernandez et al., 2004). Under oxygen limited conditions in the interior of biofilms, PZs may provide energy for growth or help maintain redox homeostasis by acting as electron acceptors for the re-oxidation of accumulating NADH (Price-Whelan et al., 2006). This was supported by the observation that PZ negative mutants of *P. aeruginosa* have higher intracellular NADH/NAD⁺ ratios in stationary phase than wild type cells in planktonic cultures (Price-Whelan et al., 2006).

Studies in biological fuel cells show that PZs constitute good electron transfer mediators to electrodes in fuel cells (Fultz and Durst, 1982). The synthetic PZ neutral red was able to act as an electron transfer mediator in *E. coli* in the reduction of iron oxide (McKinlay and Zeikus, 2004).

Phenazines as iron chelators:

Previous work suggests that PZs might be involved in iron uptake via mineral reduction (Dubiel et al., 2002; Hernandez and Newman, 2001; Hernandez et al., 2004). PYO promoted iron acquisition from transferrin by reducing transferrin-bound Fe (III) resulting in the release of Fe (II) (Cox, 1986). Release of Fe (II) by PYO was blocked in the presence of O₂, suggesting O₂ and Fe (III) may compete as final oxidants of PYO. Experiments showed a relationship between iron, oxygen and PZs produced in *P. chlororaphis* (Korth, 1971). In the presence of iron, O₂ decreased PCA production whereas reduced O₂ stimulated PCA production. This observation is consistent with our hypothesis that PZs may be involved in iron uptake. A recent study showed that PYO added to *P. aeruginosa* cultures repressed the expression of genes involved in ferric iron uptake (Dietrich et al., 2006).

In conclusion, the importance of PZs in various physiological roles is only starting to be realized. An appropriate statement from a review by Laursen and Nielsen, 2004 states, “little is yet known about the physiological function of phenazines in their natural environment.”

3. Bacterial Biofilms

Bacteria in nature typically exist as members of structurally complex, surface-attached communities known as biofilms, and this fact has led to great interest over the last two decades. In nature, biofilms often consist of multiple layers of cells embedded in hydrated matrices and form at almost any solid-liquid or liquid-gas interface. Biofilms

have been studied the most in medically important bacterial pathogens including *Pseudomonas aeruginosa*, *Staphylococcus* spp., and *Enterococcus faecalis*. These pathogens frequently colonize and form biofilms in or on patients and result in very dangerous chronic infections. *P. aeruginosa* is known to exist as biofilms in lungs of cystic fibrosis patients (Parsek and Singh, 2003). Dental plaques constitute biofilms of many species of bacteria existing together and can cause vascular diseases when parts of the biofilm enter the blood stream (Okuda et al., 2004). These same pathogens also may form biofilms on various medical devices leading to serious infection.

Biofilm formation is hypothesized to facilitate the development of a “micro niche” (term first used by Costerton et al., 1994) that protects bacteria against various physical and chemical stresses (Elkins et al., 1999; Mah et al., 2003) and confers resistance to deleterious agents such as antibiotics and detergents (Davies et al., 2003). Bacteria in biofilms also were shown to avoid phagocytosis (Leid et al., 2002). The biofilm matrix helps to slow the diffusion of small molecules in and out of the biofilm, which provides good environment for metabolic exchange (Kierek-Pearson and Karatan, 2005). In nature, biofilms may consist of multiple species or strains coexisting together. The presence of bacteria very close to each other in biofilms may increase chance for lateral gene transfer (Dahlberg et al., 1997 and Roberts et al., 1999). Biofilm communities differ dramatically from surrounding conditions and enable cells to coordinately carry out functions not typically undertaken outside of the biofilm (Morris and Monier, 2003).

Genetic analyses of medically important microbes have identified a diversity of genes involved in biofilm formation and demonstrated that the gene expression patterns by bacteria within biofilms differ substantially from their expression patterns within planktonic cultures (reviewed in Beloin and Ghigo, 2005; Lazazzera, 2005). Laboratory biofilm studies indicate that the genetic factors and pathways involved in biofilm formation vary depending on the bacterial species and the growth conditions.

Stages of biofilm development

Biofilm development is a well organized phenomenon and follows defined steps. Previous studies demonstrated that these steps include: reversible attachment, irreversible attachment, microcolony formation, maturation into 3-dimensional mushroom-like structures with open channels, and dispersal (Kierek-Pearson and Karatan, 2005; Sauer et al., 2007). Although the molecular mechanisms regulating these stages may differ from species to species, common themes can be derived from various studies (Sauer et al., 2007).

Attachment: Initially, planktonic bacteria make weak and transient connections, referred to as reversible attachment, when they encounter surfaces. This initial reversible attachment step may involve either physical forces including sedimentation, liquid flow, and gravity, or may involve the active swimming of bacteria. Several genes / factors have been shown to play roles in the initial stages of biofilm formation in different bacterial systems. The list of genes / factors that are involved in the initial attachment steps are shown in the table 1.

Table 1. Genes / Factors that were known to be involved in the initial attachment step of biofilm formation.

Model System	Gene / Factor in attachment	Reference
<i>Escherichia coli</i> K-12	Flagella	Pratt and Kolter, 1998
<i>Pseudomonas fluorescens</i>		O`Toole and Kolter, 1998b
<i>Pseudomonas aeruginosa</i>	cup fimbriae	Vallet et al., 2001
<i>Vibrio cholerae</i>	Type IV pili	Watnick and Kolter, 1999
<i>Vibrio cholerae</i>	exopolysaccharides	Moorthy and Watnick, 2004
<i>Pseudomonas fluorescens</i>	Clp proteases	O`Toole and Kolter, 1998b
<i>Pseudomonas aeruginosa</i>	<i>sad</i> genes	Meritt et al., 2007
<i>Pseudomonas fluorescens</i>	Adhesins such as LapA, LapD	Hinsa and O`Toole, 2006
<i>Rhizobiaceae</i>	Adhesins such as rhicadhesin and Raps	Danhorn and Fuqua, 2007
<i>Staphylococcus epidermidis</i>	Autolysin AtlE	Heilmann et al., 1997
<i>Staphylococcus aureus</i>	<i>dtlA</i> gene	Gross et al., 2001
<i>Pseudomonas chlororaphis</i>	2-OH-PCA	This study

Microcolony formation: Once bacteria irreversibly attach to the surface, they form microcolonies. Often, this step involves clonal growth and cell-cell interactions (Rieser et al., 2003). Quorum sensing signal molecules such as *N*-acyl homoserine lactones play a

critical role in sensing the population density of bacterial cells in a particular niche and hence aid in microcolony formation (Sauer et al., 2007). In *P. aeruginosa*, type IV pili were shown to be involved in microcolony formation (O'Toole and Kolter, 1998a). In Gram-positive bacteria *S. aureus* and *S. epidermidis*, PIA adhesin, a polysaccharide intercellular adhesin, was shown to mediate the microcolony formation (Kierrek-Pearson and Karatan, 2005).

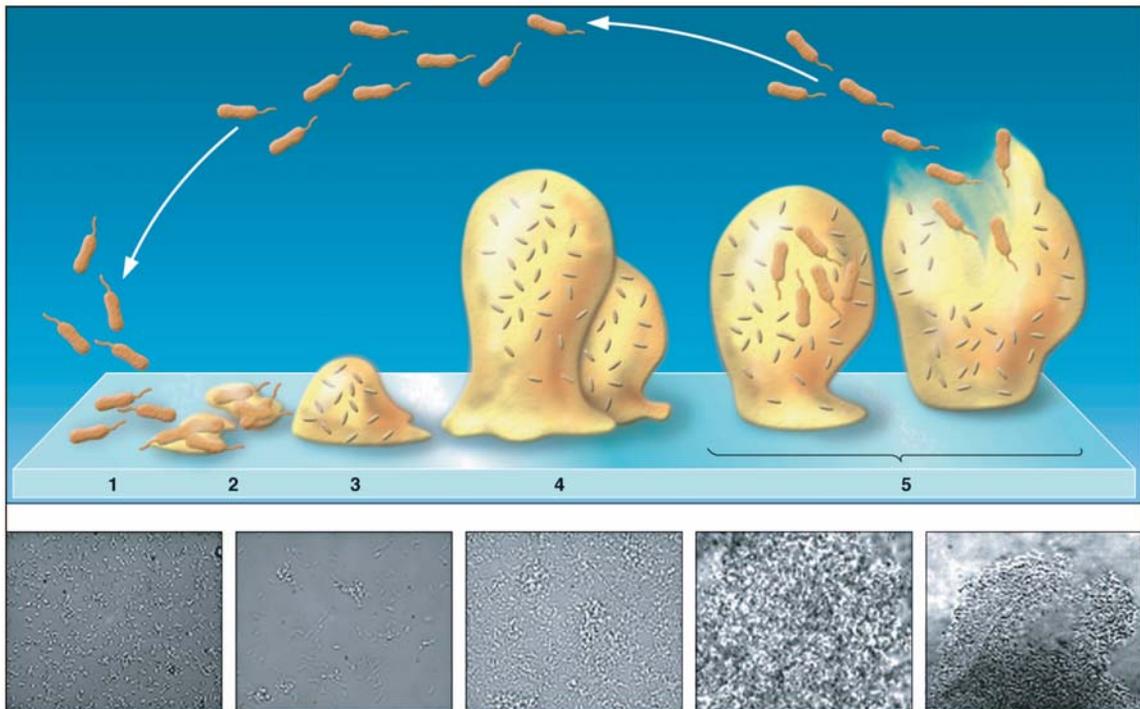


Illustration 3: Stages of biofilm development. (1) reversible attachment, (2) irreversible attachment, (3) maturation-1, (4) maturation-2, and (5) dispersion (From: Sauer et al., 2007).

Biofilm maturation: Bacterial cells go through significant changes during the transition from initial attachment to maturation. These changes result in the formation of a

complex, three-dimensional architecture with mushroom-like cell cluster structures having channels and pores for water, nutrient and gas exchange. Bacterial cells within the biofilms are held together by an extracellular matrix composed of various polysaccharides, proteins, extracellular DNA and water. Lawrence et al., 1991 utilized Scanning Confocal Laser Microscopy (SCLM) and flow cells to visualize, for the first time, the complex biofilm architecture of *P. aeruginosa*, *P. fluorescens* and *V. parahaemolyticus*. They found that the complex biofilms of *P. aeruginosa*, *P. fluorescens* and *V. parahaemolyticus* differed in structure and morphology.

Different factors affect biofilm structure and architecture, including: nutrient availability, hydrodynamic conditions, quorum sensing, rhamnolipid and lipopeptide surfactants, and production of exopolysaccharides (EPS) like alginate, colanic acid, PZ secondary metabolites (Kierek-Pearson and Karatan, 2005; Maddula et al., 2006). Sheikh et al., 2001 found that plasmid-encoded aggregative adherence fimbriae enhance biofilm formation in *E. coli*. Conjugative pili expressed from plasmids act as adhesion factors and promote biofilm formation on glass (Ghigo et al., 2001). Alternatively, production of these factors is in turn regulated by other genes. For example, EPS is dependent on other factors including availability of nutrients (carbon, nitrogen, potassium and phosphates) and growth rates (Sutherland, 2001).

Microarray studies have demonstrated that the transcriptional patterns of mature biofilms and exponential growth phase cultures differed significantly (Beloin et al., 2004; Beloin and Ghigo, 2005). Beloin et al., 2004 found within the biofilms, 250 genes (5.8%) were induced and 188 genes (4.4%) were repressed as compared to the planktonic

cultures of *E. coli* K-12. The induced genes included envelope stress response, cell envelope biogenesis, energy and carbohydrate metabolism and other genes whose functions were not known (Beloin et al., 2004). In *P. aeruginosa* microarrays analysis, only 73 genes (in total) were differentially expressed in mature biofilm compared to planktonic growth (Whiteley et al., 2001).

Dietrich et al. 2006 demonstrated that addition of high levels of the PZ secondary metabolite PYO to a pyocyanin-defective mutant of *P. aeruginosa* strain PAO1 induced global changes in gene expression patterns, suggesting that PYO may be serving as a regulatory signal in mature biofilms.

Dispersal: Biofilms present a very dynamic environment in that bacterial cells continuously grow and detach. The detached cells are planktonic and usually land on surfaces to initiate new rounds of biofilms growth. Thus, dispersal of bacterial cells from the biofilms constitutes an important stage of the biofilm life cycle (Kierek-Pearson and Karatan, 2005). The molecular mechanisms for dispersal of sessile biofilm bacterial cells to free living planktonic cells are not well understood. A list of factors that may be involved in the dispersal of the biofilms is presented in table 2.

Quorum sensing and biofilms: QS has been demonstrated to influence various aspects of biofilm development and maturation (reviewed in Davies et al., 1998; O'Toole et al., 2000; Rice et al., 2005; Yoshida et al., 2005; Parsek and Greenberg, 2005; Arevalo-Ferro et al., 2005). In laboratory studies, specific QS-regulated factors shown to play critical roles in surface attachment, aggregation, and dispersal include structural components of flagella, type IV pili, polysaccharide biosynthesis, protease (reviewed in Kierek-Pearson

and Karatan, 2005) and rhamnolipids (Davey et al., 2003). Several of these same factors also were expressed differentially in response to attachment of the plant saprophyte *P. putida* to plant surfaces (Sauer and Camper, 2001). Although QS is a global regulatory system affecting the expression of multiple genes, the exact role of QS in biofilm development is unclear (Parsek and Greenberg, 2005). Thus, specific factors under QS control need to be identified and their precise role in biofilm formation characterized. My work explained in this dissertation mainly focuses on this aspect. Of particular interest is the potential role intercellular signaling pathways may play in coordinating gene expression among biofilm constituents and the identification of specific genes under signaling control that function in the development of bacterial biofilms on plants.

Table 2. List of factors known to be involved in bacterial dispersal from biofilms

Model system	Factor or processes in dispersal	Reference
<i>Pseudomonas aeruginosa</i>	Rhamnolipid biosurfactants	Boles et al., 2005
<i>P. aeruginosa</i>	Chelators like EDTA	Banin et al., 2005
<i>P. aeruginosa</i>	'BdIA' Chemotaxis regulator	Morgan et al., 2006
<i>P. aeruginosa</i>	Nitric oxide (NO)	Barraud et al., 2006
<i>P. aeruginosa</i>	Oxygen availability	Barraud et al., 2006

4. Plant- associated bacterial biofilms

Bacteria associated with plants may have different interactions with plants and are present as pathogens, commensals, and mutualists. On plants, bacteria may form aggregate communities on leaves, roots, and other plant surfaces and within intercellular spaces of plant tissues (reviewed in Morris and Monier, 2003; Ramey et al, 2004; Danhorn and Fuqua, 2007). Similar to clinical bacterial biofilms, biofilms on plant surfaces can benefit plant associated bacteria. Biofilms can provide resistance to various environmental stresses such as dessication, UV, and antibiotics, and also may aid in gene transfer. Formation of biofilms may involve different mechanisms depending on the bacterial species. Various models of biofilm formation on plants have been proposed from studies of single species biofilms *in vitro*, however little is known about the relative importance of these models of biofilm development outside the laboratory. It also is not clear whether factors implicated in biofilm development by clinical species are important for biofilm formation by plant-associated microbes.

The rhizosphere represents a relatively nutrient rich environment due to plant exudation (Watt et al., 2006). This study showed that bacterial biofilms were highly heterogeneous, covering about 40% of the roots. Biofilms extended from 2-30 μm and were often associated with root hairs, axial groves, and soil particles. The elongation zone of roots showed the lowest bacterial density (Watt et al., 2006). *P. fluorescens* was shown to colonize crevices between the epithelial cells of roots (Normander et al., 1999). The Gram-positive biological control agent, *Bacillus cereus*, developed dense surface-associated populations, and its biological control properties were linked with biofilm

formation (Bais et al., 2004). *P. putida* was shown to colonize and form stable populations on corn roots using chemotaxis and microcolony formation in as short as 2 days (Espinosa-Urgel et al., 2002). The nitrogen fixing bacteria, *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium meliloti*, attach to the root surfaces and also form biofilms (Fujishige et al., 2006).

Similar to plant beneficial bacteria, pathogenic bacteria also form biofilms to enhance their virulence and protection against plant defense responses. The opportunistic pathogen *P. aeruginosa* forms biofilms on sweet basil and this biofilm confers resistance against rosmarinic acid, an antimicrobial compound from sweet basil (Walker et al., 2004). Among the pathogenic *Agrobacterium tumefaciens* biovars, non- motile and non-chemotactic mutants were restricted in biofilm formation and virulence (Danhorn and Fuqua, 2007).

Although the literature on plant-associated biofilms is not as extensive as for the human pathogenic biofilms, it is clear that biofilms serve similar functions regardless of the host or surface colonized.

II. PRESENT STUDY

1. Importance of the present study

My dissertation focuses on understanding biofilm formation in *Pseudomonas chlororaphis* strain 30-84. Specifically, I focused on the role of QS and PZs in biofilm formation by this important biological control strain. The following points summarize the importance of the present study.

1. Bacteria often exist as complex multicellular communities termed, biofilms, in nature. Bacteria can survive and form biofilm communities on different plant surfaces including roots, leaves, seeds and vascular tissues.
2. Plant health depends on the plant's interactions with the environment including microorganisms living on or inside the plant. These interactions result either in a beneficial, neutral, detrimental, and pathogenic associations.
3. Biofilms constitute a unique control point where these important interactions occur. *To improve plant health, it is important to understand how biofilm formation is regulated.*
4. Bacterial biofilms offer a unique microniche, which differs dramatically from planktonic counterparts. Successful biofilm communities are capable of optimizing nutrient utilization, redox environment, and energy generation.
5. Rhizosphere associated *Pseudomonas* species are great rhizosphere colonizers and can form biofilms. Many *Pseudomonas* spp. are well studied beneficial agents for their growth promoting or pathogen inhibition properties.

6. Success of a root-associated biological control agent depends primarily on its ability to colonize the roots or to form biofilms. Thus, the biological control property of an agent depends upon its ability to form biofilms on plants.
7. Biofilm formation also may aid in the efficacy of the biological control agent. Biofilms are enclosed with in exopolysaccharide (EPS) membranes. EPS can aid in slow release of the antibiotic compounds for prolonged time periods improving efficacy of the biological control agent.
8. Production of PZ secondary metabolites is under complex gene regulation. Their production is metabolically expensive but on the contrary they are evolutionarily conserved. Although very little is known about the physiological function of PZs in their natural environment, recent work is identifying some of their roles.
9. *Pseudomonas chlororaphis* strain 30-84 is a great model system to study biofilm formation. Strain 30-84 colonizes the wheat rhizosphere providing protection against take-all fungal pathogen by competition and production of antibiotics.

2. Introduction and rationale for the present study

APPENDIX A:

Quorum sensing (QS) or cell-to-cell signaling has been recognized to be important in biofilm formation in a variety of microorganisms. The first logical question to address in the study of *Pseudomonas chlororaphis* strain 30-84 biofilms was: *Does QS regulate biofilm formation in strain 30-84?* In order to answer this question, I first needed to

develop standardized protocols for biofilm assays in our model system. So, my initial studies were concentrated on developing assays and protocols including:

- (a) A microtitre plate assay for rapidly screening the adhesion ability of various strains and mutants,
 - (b) A flow cell assay for in vivo visualization of biofilm structures,
 - (c) A modified flow cell assay for measuring initial attachment and dispersal rates,
- and
- (d) Adopting ISA software protocols for quantifying differences in biofilm architecture.

Utilizing the technologies I developed, my first experiments focused on testing the QS mutants of strain 30-84 for biofilm formation. My first adhesion plate experiments showed that derivatives having single mutations in either QS systems, produced significantly less biofilm than the parental strain even after 84 h. Genetic and biochemical complementation of QS mutants by *in trans* expression of wild-type alleles and the addition of specific AHLs to the growth media restored the biofilm formation by all mutants. These data provide evidence that both quorum-sensing systems are involved in biofilm development by *P. chlororaphis* strain 30-84 and that the PhzR/I QS system plays a greater role.

Surprisingly, I observed a PZ structural mutant of strain 30-84 was equally impaired as QS mutants in my initial biofilm assays. The fact that PZs were involved in biofilm formation was unexpected. However, this initial observation is supported by a long-standing hypothesis of this lab group, that PZs can have multiple functions in the biology

of the bacterium. Additionally, we know that PZ antibiotics are regulated in a complex and multiple mechanisms (explained above). This hypothesis also is in agreement with the natural selection theory that metabolically expensive antibiotics may have chances of being selected AGAINST, and that more chances of being selected FOR exist if genes serve multiple functions.

Based on this hypothesis, my next experiments focused on confirming that *PZs are involved in the biofilm formation of strain 30-84*. Genetic complementation of PZs in a PZ structural mutant with cosmids containing the phenazine biosynthetic genes *in trans* restored biofilm formation to wild-type levels in both microtitre plate assay and flow cell assays. Biochemical complementation of PZ non-producer with crude PZ extracts restored biofilm formation to wild types levels in microtitre plate assays. These experiments provided the first published evidence for the role of PZ secondary metabolites in biofilm formation of strain 30-84. These results also demonstrate that in addition to their role in pathogen inhibition, PZs play a secondary role in the biology of the organism; they are involved in biofilm formation.

APPENDIX B:

Pseudomonas species are known to synthesize various PZ compounds often contributing to control of different pathogenic agents. *P. chlororaphis* 30-84 synthesizes several secondary metabolites including the phenazine (PZ) antibiotics phenazine -1-carboxylic acid (PCA), 2-hydroxy-PCA (2-OH-PCA) and a minor amount of 2-hydroxy-phenazine (2-OH-PZ). *P. chlororaphis* 30-84 produces primarily the yellow PCA (~80-90%), and low amounts of the orange 2-OH-PCA (~10-20%). *P. fluorescens* strain 2-79

produces only PCA, whereas *P. chlororaphis* strain PCL1391 produces phenazine-1-carboximide (PCN) and some PCA. The opportunistic pathogen *P. aeruginosa* produce the methylated PZ derivative pyocyanin (PYO). These observations raise the fundamental questions regarding PZs as secondary metabolites including: *Why do most strains produce more than one PZ? What are the various roles of PZs for the producing organism?* We hypothesize that the types of PZs produced and the ratios in which they are produced play important roles in the competitive survival and persistence of the producing organism. In order to test this hypothesis, my next experiments were concentrated on studying the effects of altering ratios of PZs produced in strain 30-84 on biofilm formation. I constructed mutants of strain 30-84 which make only PCA (30-84PCA) or which makes more 2-OH-PCA (30-84O*). Using flow cell assays, I tested PZ altered mutants of strain 30-84 for differences in biofilm parameters including: initial attachment, mature biofilm architecture, and dispersal rates. I found that these PZ-altered derivatives of strain 30-84 differed from the wild type in initial attachment, biofilm architecture, and dispersal from biofilms. The PZ-altered derivatives of strain 30-84 also differed in their ability to inhibit the fungal pathogen *Ggt*. Results from these studies indicate that alterations in the ratios of PZs produced may have wide-ranging effects including antibiosis and bioiflm formation in strain 30-84.

APPENDIX C:

In my previous studies (APPENDIX A, B), I provided evidence that PZ secondary metabolites are involved in biofilm formation by strain 30-84. This raises the important question: *How do PZs influence biofilm formation in strain 30-84—what is the*

mechanism? Several lines of evidence suggest that PZs may alter cell surface properties, may serve as signals that regulate other genes, or can function via multiple mechanisms. In this section, I tested two hypotheses regarding mechanisms by which PZs might affect biofilm development. The first hypothesis tested was that PZs alter the surface properties of the cell reducing the repulsive forces. I tested this hypothesis by adding various concentrations of PZs to metabolically-inactivated bacteria and measuring cell adhesion. Results from these studies indicate that PZs improved the attachment of metabolically inactive cells to abiotic surfaces. This suggests that PZs interact physically with the cell and may alter cell surface properties of bacteria. The second hypothesis tested was that PZs act as signal molecules regulating the expression of PZ regulons involved in biofilm development. This hypothesis was tested utilizing *P. aeruginosa* Affymetrix GeneChips. We compared gene expression patterns of 30-84WT (PZ producer) and 30-84Z (PZ non-producer) to identify genes regulated by PZs. For these experiments, I standardized protocols for purification of high quality RNA from strain 30-84. My preliminary results from microarrays comparing wild type strain 30-84 and the PZ strain 340-84ZN indicate that in the presence of PZs, genes involved in biofilm formation including fimbriae, pilli, exopolysaccharides were up-regulated. Results from these studies suggest that PZs may regulate biofilm formation via multiple mechanisms in strain 30-84.

APPENDIX D:

Work by others in the lab had identified mutations in a two-component histidine sensor kinase, *rpeA* that de-repressed PZ production in *P. chlororaphis* strain 30-84. This strain was interesting to me because the mutant overproduces PZs and I was interested in

knowing what effect this might have on biofilm production. In order to determine the effect of excess PZ production, I used this previously characterized mutant, 30-84*rpeA*, in different biofilm assays (e.g. microtiter plate and chamber slide assays). My results indicate that excess PZ improves the biofilm formation in strain 30-84. In a related project, I also was involved in developing protocols for construction of *rpeA/rpeB* mutants in *P. aeruginosa* strains PAO1, and PA14. Mutants were either procured or constructed by Dr. E. A. Pierson. As part of the mutant characterization, I was involved in developing the methodology to extract and quantify pyocyanins. This work was part of a NSF funded project to characterize the RpeA/RpeB two-component regulators in *P. chlororaphis* strain 30-84 and *P. aeruginosa* strains PAO1 and PA14. I am presently carrying out microarray experiments to characterize the *rpeA/rpeB* regulons in strains PAO1 and PA14. My contributions to this project include development of the methodology to extract high quality RNA, working with the microarray lab on the experiments, and analysis of the microarray data.

APPENDIX E:

In a project completely unrelated to any of the previously mentioned projects, I assisted in the development of a standardized protocol pRL-27 Tn5 mutagenesis. This unique pRL-27 Tn5 plasmid system offers an easy method of creating non-specific, stable, non-leaky mutations and allows one-step cloning and sequencing for identification of the mutated gene. In APPENDIX E, an explanation for standardizing protocol for pRL-27 Tn5 mutagenesis and one-step cloning to find the mutated gene was included. I also was instrumental in adopting this technique to microbial genetics laboratory class

428L / 528L. I utilized this technique for *Pseudomonas cedrella* strain PU43 with a goal for creation and identification of negative signaling genes. *P. cedrella* PU43 is a reliable negative signaling strain, which synthesizes an unknown negative signal that inhibits PZ biosynthesis in strain 30-84. I also used pRL-27 Tn5 mutagenesis to create and identify mutants that were altered in PZ biosynthesis in *P. chlororaphis* strain 30-84. A list of identifications of various interesting mutants of strains PU43 and strain 30-84 also are included. This project has wide applicability in determination of specific gene effects on the phenotype of the producing bacteria.

DISSERTATION FORMAT

The work presented in this dissertation has been published (APPENDIX A, B), or will shortly be submitted for publication (APPENDIX C, D and E). Due to this reason, sections were presented as appendices, what would usually be chapters in a dissertation according to the University of Arizona manual for dissertations format. The appendices' were written in formats are according to the journal requirements. My research contributions to the work presented was explained above in the Introduction and rationale to the present study section.

REFERENCES

- Abken, H. J., M. Tietze, J. Brodersen, S. Baumer, U. Beifuss, and U. Deppenmeier.** 1998. Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Gol. J. Bacteriol. **180**:2027-2032.
- Anjaiah, V., N. Koedam, B. Nowak-Thompson, J. E. Loper, M. Höfte, J. T. Tambong, and P. Cornelis.** 1998. Involvement of Phenazines and Anthranilate in the Antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 Derivatives Toward *Fusarium* spp. and *Pythium* spp. Mol. Plant-Microbe Interact. **11**:847-854.
- Arevalo-Ferro, C., G. Reil, A. Gorg, L. Eberl, and K. Riedel.** 2005. Biofilm formation of *Pseudomonas putida* IsoF: the role of quorum sensing as assessed by proteomics. Syst Appl Microbiol **28**: 87-114.
- Bais, H. P., Fall, R. and Vivanco, J. M.** 2004. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production, *Plant Physiol* **134**: 307–319.
- Banin, E., K. M. Brady, and E. P. Greenberg.** 2005. Chelator-Induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. Appl. Environ. Microbiol. **72**: 2064–2069.
- Bakker, P. A., D. C. Glandorf, M. Viebahn, T. W. Ouwens, E. Smit, P. Leeflang, K. Wernars, L. S. Thomashow, J. E. Thomas-Oates, and L. C. van Loon.** 2002. Effects of *Pseudomonas putida* modified to produce phenazine-1-carboxylic acid and 2, 4-

diacetylphloroglucinol on the microflora of field grown wheat. *Antonie Van*

Leeuwenhoek. **81**:617-624.

Baron, S. S., and J. J. Rowe. 1981. Antibiotic action of pyocyanin. *Antimicrobial Ag Chemother*. **20**:814-820.

Barraud, N., D. J. Hassett, S. H. Hwang, S. A. Rice, S. Kjelleberg, and J. S. Webb. 2006. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J. Bacteriol*. **188**:7344-7353.

Bauer, W. D., and U. Mathesius. 2004. Plant responses to bacterial quorum sensing signals. *Curr Opin Plant Biol* **7**:429-433.

Beloin, C., J. Valle, P. Latour-Lambert, P. Faure, M. Kzreminski, D. Balestrino, J. A. Haagensen, S. Molin, G. Prensier, B. Arbeille, and J. M. Ghigo. 2004. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Mol Microbiol*. **51**:659-674.

Beloin, C., and J. M. Ghigo. 2005. Finding gene-expression patterns in bacterial biofilms. *Trends in Microbiol* **13**:16-19.

Boles, B. R., M. Thoendel, and P. K. Singh. 2005. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Mol. Microbiol*. **57**:1210-1223.

Cha, C., P. Gao, Y. C. Chen, P. D. Shaw, and S. K. Farrand. 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol Plant Microbe Interact* **11**:1119-1129.

- Chancey, S. T., D. W. Wood, E. A. Pierson, and L. S. Pierson. III.** 2002. Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *Appl. Environ. Microbiol.* **68**:3308-3314.
- Chatterjee, A., Y. Cui, and A. K. Chatterjee.** 2002. RsmA and the quorum-sensing signal, N-[3-oxohexanoyl]-L-homoserine lactone, control the levels of *rsmB* RNA in *Erwinia carotovora* subsp. *carotovora* by affecting its stability. *J Bacteriol.* **184**:4089-4095.
- Chin-A-Woeng, T. F. C., G. V. Bloemberg, A. J. van der Bij, K. M. G. F. van der Drift, J. Schripsema, B. Kroon, R. J. Scheffer, C. Keel, P. A. H. M. Bakker, H. V. Tichy, F. J. de Bruijn, J. E. Thomas-Oates, and B. J. J. Lugtenberg.** 1998. Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Molec. Plant Microbe Interact.* **11**:1069-1077.
- Chin-A-Woeng, T. F. C., J. E. Thomas-Oates, B. J. J. Lugtenberg, and G. V. Bloemberg.** 2001. Introduction of the *phzH* gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. *Mol. Plant-Microbe Interact.* **14**:1006-1015.
- Chin-A-Woeng, T. F. C., G. V. Bloemberg, and B. J. J. Lugtenberg.** 2003. Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytologist* **157**:503-523.
- Costerton, J. W., Z. Lewandowski, D. DeBeer, D. Caldwell, D. Korber, and G. James.** 1994. Biofilms, the customized microniche. *J. Bacteriol.* **176**: 2137-2142.

Cox, C. D. 1986. Role of pyocyanin in the acquisition of iron from transferrin. *Infect. Immun.* **52**:263-270.

Dahlberg, C., C. Linberg, V. L. Torsvik, and M. Hermansson. 1997. Conjugative plasmids isolated from bacteria in marine environments show various degrees of homology to each other and are not closely related to well-characterized plasmids. *Appl. Environ. Microbiol.* **63**: 4692-4697.

Danhorn, T., and C. Fuqua. 2007. Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* **61**:401-422.

Davey, M. E., N. C. Caiazza, and G. A. O'Toole. 2003. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185**:1027-1036.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**:295-298.

Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Disc.* **2**:114-122.

Denning, G. M., S. S. Iyer, K. J. Reszka, Y. O'Malley, G. T. Rasmussen, and B. E. Britigan. 2003. Phenazine-1-carboxylic acid, a secondary metabolite of *Pseudomonas aeruginosa*, alters expression of immunomodulatory proteins by human airway epithelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* **285**:584-592.

- Dietrich, L. E., A. Price-Whelan, A. Petersen, M. Whiteley, and D. K. Newman.** 2006. The phenazine pyocyanin is a terminal signaling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **61**:1308-1321.
- Dubiel, M., C. H. Hsu, C. C. Chien, F. Mansfeld, and D. K. Newman.** 2002. Microbial iron respiration can protect steel from corrosion. *Appl. Environ. Microbiol.* **68**:1440-1445.
- Elkins, J. G., D. J. Hassett, P. S. Stewart, H. P. Schweizer, and T. R. McDermott.** 1999. Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. *Appl. Environ. Microbiol.* **65**: 4594-4600.
- Espinosa-Urgel, M., R. Kolter, and J-L. Ramos.** 2002. Root colonization by *Pseudomonas putida*: love at first site. *Microbiology* **148**:341–43.
- Flaishman, M., Z. Eyal, C. Voisard, and D. Haas.** 1990. Suppression of *Septoria tritici* by Phenazine- or Siderophore-deficient mutants of *Pseudomonas*. *Curr. Microbiol.* **20**:121-124.
- Friedheim, E. A. H.** 1931. Pyocyanine, an accessory respiratory pigment. *J. Expt. Med.* **54**:207-221.
- Fujishige, N. A., N. N. Kapadia, P. L. De Hoff, and A. M. Hirsch.** 2006. Investigations of *Rhizobium* biofilm formation. *FEMS Microbiol. Ecol.* **56**:195–206.
- Fultz, M. L., and R. A. Durst.** 1982. Mediator compounds for the electrochemical study of biological redox systems: a compilation. *Anal. Chim. Acta.* **140**:1-18.
- Ghigo, J. M.** 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature.* **412**:442-5.

- Giddens, S. R., Y. Feng, and H. K. Mahanty.** 2002. Characterization of a novel phenazine antibiotic gene cluster in *Erwinia herbicola* Eh1087. *Molec. Microbiol.* **45**:769-783.
- Gross, M., S. E. Cramton, F. Götz, and A. Peschel.** 2001. Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. *Infect Immun.* **69**:3423-6.
- Gurusiddaiah, S., D. M. Weller, A. Sarkar, and R. J. Cook.** 1986. Characterization of an antibiotic produced by a strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp. *Antimicrob. Agents Chemother.* **29**:488-495.
- Haas, D., and C. Keel.** 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Ann. Rev. Phytopathol.* **41**:117-153.
- Hassan, H. M., and I. Fridovich.** 1980. Mechanism of action of pyocyanin. *J. Bacteriol.* **141**:156-163.
- Heilmann, C., M. Hussain, G. Peters, and F. Götz.** 1997. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. *Mol. Microbiol.* **20**: 1083-1091.
- Hernandez, M. E., A. Kappler, and D. K. Newman.** 2004. Phenazines and other redox-active antibiotics promote microbial mineral reduction. *Appl. Environ. Microbiol.* **70**:921-928.

- Hernandez, M. E., and D. K. Newman.** 2001. Extracellular electron transfer. Cellular and Molec. Life Sci. **58**:1562-1571.
- Hinsa, S. M., and G. A. O'Toole.** 2006. Biofilm formation by *Pseudomonas fluorescens* WCS365: a role for LapD. Microbiol. **152**: 1375-1383.
- Kerr, J. R., G. W. Taylor, A. Rutman, N. Hoiby, P. J. Cole, and R. Wilson.** 1999. *Pseudomonas aeruginosa* pyocyanin & 1-hydroxyphenazine inhibit fungal growth. Clin Pathol. **52**:385-7.
- Kierrek-Pearson, K., and E. Karatan.** 2005. Biofilm development in bacteria. Adv. Appl. Microbiol. **57**: 79–111.
- Klausen, M., M. Gjermansen, J. U. Kreft, and T. Tolker-Nielsen.** 2006. Dynamics of development and dispersal in sessile microbial communities: examples from *Pseudomonas aeruginosa* and *Pseudomonas putida* model biofilms. FEMS Microbiol. Lett. **261**:1-11.
- Korth, H.** 1971. Effect of iron and oxygen on the formation of pigments of some *Pseudomonas* species. Arch. Mikrobiol. **77**:59-64.
- Lapouge, K., M. Schubert, F. H. Allain, and D. Haas.** 2007. Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behavior. Mol. Microbiol. **67**:241-253.
- Lau, G. W., D. J. Hassett, H. Ran, and F. Kong.** 2004. The role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends Mol. Med. **10**:599-606.
- Laursen, J. B., and J. Nielsen.** 2004. Phenazine natural products: Biosynthesis, synthetic analogues, and biological activity. Chem. Rev. **104**:1663-1685.

- Lawrence, J. R., D. R. Korber, B. D. Hoyle, J. W. Costerton, and D. E. Caldwell.** 1991. Optical sectioning of microbial biofilms. *J Bacteriol.* **173**:6558-67.
- Lazazzera, B. A.** 2005. Lessons from DNA microarray analysis: the gene expression profile of biofilms. *Curr Opin in Microbiol* **2**: 222-227.
- Lee, S., and L. S. Pierson, III.** Expression of *rsmZ* from bypasses the requirement for a functional GacS/GacA two component regulatory system in *Pseudomonas aureofaciens* strain 30-84. (in prep).
- Leid, J. G., M. E. Shirtliff, J. W. Costerton, and A. P. Stoodley.** 2002. Human leukocytes adhere to penetrate, and respond to *Staphylococcus aureus* biofilms. *Infect. Immun.* **70**: 6339-6345.
- Maddula, V. S. R. K., Z. Zhang, E. A. Pierson, and L. S. Pierson III.** 2006. Quorum sensing and phenazines are involved in biofilm formation by *Pseudomonas chlororaphis* (*aureofaciens*) strain 30-84. *Microb. Ecol.* **52**:289-301.
- Mah, T. F., B. Pitts, B. Pellock, G. C. Walker, P. S. Stewart, and G. A. O'Toole.** 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* **426**: 306-310.
- Mahajan, M. S., M. W. Tan, L. G. Rahme, and F. M. Ausubel.** 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa*-*Caenorhabditis elegans* pathogenesis model. *Cell* **96**:47-56.
- Mazzola, M., R. J. Cook, L. S. Thomashow, D. M. Weller, and L. S. Pierson III.** 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. *Appl. Environ. Microbiol.* **58**:2616-2624.

- McKinlay, J. B., and J. G. Zeikus.** 2004. Extracellular iron reduction is mediated in part by neutral red and hydrogenase in *Escherichia coli*. *Appl. Environ. Microbiol.* **70**:3467-3474.
- McLean, R. J., M. Whiteley, D. J. Stickler, and W. C. Fuqua.** 1997. Evidence of autoinducer activity in naturally occurring biofilms. *FEMS Microbiol Lett* **154**:259-263.
- Merritt, J.H., K. M. Brothers, S. L. Kuchma, and G. A. O'Toole.** 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *J. Bacteriol.* **189**:8154-8164.
- Miller, M. B., and B. L. Bassler.** 2001. Quorum sensing in bacteria. *Annu Rev Microbiol* **55**:165-199.
- Moorthy, S., and P. I. Watnick.** 2004. Genetic evidence that the *Vibrio cholerae* monolayer is a distinct stage in biofilm development. *Mol Microbiol.* **52**:573-87.
- Morgan, R., S. Kohn, S. Hwang, D. J. Hassett, and K. Sauer.** 2006. BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. *J. Bacteriol.* **188**:7335-7343.
- Morello, J. E., E. A. Pierson, and L. S. Pierson III.** 2004. Negative cross-communication among wheat rhizosphere bacteria: effect on antibiotic production by the biological control bacterium *Pseudomonas aureofaciens* 30-84. *Appl. Environ. Microbiol.* **70**:3103-3109.
- Morris, C. A., and J. M. Monier.** 2003. The ecological significance of biofilm formation by plant associated bacteria. *Annu Rev Phytopathol* **41**:429-53.

- Normander, B., N. B. Hendriksen, and O. Nybroe.** 1999. Green fluorescent protein-marked *Pseudomonas fluorescens*: localization, viability, and activity in the natural barley rhizosphere. *Appl. Environ. Microbiol.* **65**:4646–51.
- O`Toole, G. A., and R. Kolter.** 1998a. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol.* **30**:295-304.
- O`Toole, G. A., and R. Kolter.** 1998b. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol. Microbiol.* **28**: 449-461.
- O`Toole G. A., H. B. Kaplan, and R. Kolter.** 2000. Biofilm formation as microbial development. *Annu Rev Microbiol* **54**:49-79.
- Okuda, K., T. Kato, and K. Ishihara.** 2004. Involvement of periodontopathic biofilm in vascular diseases. *Oral Dis.* **10**: 5-12.
- Ownley BH, Weller DM, Thomashow LS.** 1992. Influence of in situ and in vitro pH on suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* 2-79. *Phytopathology* **82**:178–84.
- Parsek, M. R., and P. K. Singh.** 2003. Bacterial biofilms: An emerging link to disease and pathogenesis. *Annu. Rev. Microbiol.* **57**: 677-701.
- Parsek, M. R., and E. P. Greenberg.** 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* **13**: 27-33.

- Pierson, L. S., III, and L. S. Thomashow.** 1992. Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. *Mol. Plant Microbe Interact.* **5**:330-339.
- Pierson, E. A., D. W. Wood, J. A. Cannon, F. M. Blachere, and L. S. Pierson, III.** 1998a. Interpopulation signaling via *N*-acyl-homoserine lactones among bacteria in the wheat rhizosphere. *Molec. Plant-Microbe Interact.* **11**:1078-1084.
- Pierson, L. S., III, D. W. Wood, E. A. Pierson, and S. T. Chancey.** 1998b. Homoserine lactone-mediated gene regulation in plant-associated bacteria. *Annu Rev Phytopathol* **36**:207-225.
- Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol. Microbiol.* **30**:285-293.
- Price-Whelan, A., L. E. Dietrich, and D. K. Newman.** 2006. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat. Chem. Biol.* **2**:71-78.
- Raaijmakers, JM, Vlami, M, de Souza, JT.** 2002. Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* **81**:537-547.
- Ramey, B. E., M. Koutsoudis, S. B. von Bodman, and C. Fuqua.** 2004. Biofilm formation in plant-microbe associations. *Curr Opin Microbiol* **7**: 602-609.
- Ran H, Hassett DJ, Lau GW.** 2003. Human targets of *Pseudomonas aeruginosa* pyocyanin. *Proc Natl Acad Sci USA* **100**:14315-20.

- Reisner, A., J. A. Haagensen, M. A. Schembri, E. L. Zechner, and S. Molin.** 2003. Development and maturation of *Escherichia coli* K-12 biofilms. *Mol Microbiol.* **48**:933-46.
- Rice, S. A., K. S. Koh, S. Y. Queck, M. Labbate, K. W. Lam, and S. Kjellberg.** 2005. Biofilm formation and Sloughing in *Serratia marcescens* are controlled by Quorum sensing and Nutrient cues. *J Bacteriol* **187**:3477-3485.
- Roberts, A. P., J. Pratten, M. Wilson, and P. Mullany.** 1999. Transfer of a conjugative transposon, Tn5397 in a model oral biofilm. *FEMS Microbiol. Lett.* **177**: 63-66.
- Sauer, K., and A. K. Camper.** 2001. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J Bacteriol* **183**:6579-6589.
- Sauer, K., A. H. Rickard, and D. G. Davies.** 2007. Biofilms and Biocomplexity. *Microbe* **2**:347-353.
- Schoonbeek, HJ, Raaijmakers, JM, De Waard, MA.** 2002. Fungal ABC transporters and microbial interactions in natural environments. *Mol. Plant-Microbe Interact.* **15**:1165-1172.
- Schuster, M., C. P. Lostroh, T. Ogi, E. P. Greenberg.** 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* **185**:2066-2079.
- Sheikh, J., S. Hicks, M. Dall`Agnol, A. D. Phillips, and J. P. Nataro.** 2001. Roles for Fis and YafK in biofilm formation by enteroaggregative *Escherichia coli*. *Mol.*

- Microbiol. **41**: 983-997. **Smirnov V, Kiprianova E.** 1990. *Bacteria of Pseudomonas genus*. Kiev: Naukova Dumka. 264 pp. (In Russian).
- Sutherland, I.** 2001. Biofilm expolysaccharides: A strong and sticky framework. *Microbiology* **147**: 3-9.
- Tambong, JT, and Hofte, M.** 2001. Phenazines are involved in biocontrol of *Pythium myriotylum* on cocoyam by *Pseudomonas aeruginosa* PNA1. *Eur. J. Plant Pathol.* **107**:511-521.
- Thomashow, LS, and Weller, DM.** 1988. Role of phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaumannomyces graminis* var. *tritici*. *J. Bacteriol.* **170**:3499-3508.
- Timms-Wilson, T. M., R. J. Ellis, A. Renwick, D. J. Rhodes, D. V. Mavrodi, D. M. Weller, L. S. Thomashow, and M. J. Bailey.** 2000. Chromosomal insertion of phenazine-1-carboxylic acid biosynthetic pathway enhances efficacy of damping-off disease control by *Pseudomonas fluorescens*. *Mol. Plant-Microbe Interact.* **13**:1293-1300.
- Toohy, J. I., C. D. Nelson, and G. Krotkov.** 1965. Toxicity of phenazine carboxylic acid to some bacteria, algae, higher plants, and animals. *Can. J. Bot.* **43**:1151-1155.
- Turner, JM, and Messenger, AJ.** 1986. Occurrence, biochemistry and physiology of phenazine pigment production. *Adv. Microb. Physiol.* **27**:211-275.
- Vallet, I., J. W. Olson, S. Lory, A. Lazdunski, and A. Filloux.** 2001. The chaperone /usher pathways of *Pseudomonas aeruginosa*: Identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation. *Proc Natl Acad Sci U S A.* **98**: 6911–6916.

Von Bodman, S. B., W. D. Bauer, and D. L. Coplin. 2003. Quorum sensing in plant-pathogenic bacteria. *Ann Rev Phytopathol* 41:455-482.

Wagner, V. E., D. Bushnell, L. Passador, A. I. Brooks, and B. H. Iglewski. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* 185:2080-2095.

Watnick, P. I., and R. Kolter. 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol Microbiol* 34:586-595

Walker, T. S., H. P. Bais, E. Deziel, H. P. Schweizer, and L. G. Rahme. 2004. *Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, biofilm formation, and root exudation. *Plant Physiol.* 134:320–331.

Walsh, U. F., J. P. Morrissey, and F. O'Gara. 2001. *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Curr Opin Biotechnol.* 12:289-295.

Watt, M., P. Hugenholtz, R. White, and K. Vinall. 2006. Numbers and locations of native bacteria on field-grown wheat roots quantified by fluorescence in situ hybridization (FISH). *Environ. Microbiol.* 8:871–84.

Weller, D. M. 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathol.* 97:250-256.

Whistler, C. A., and L. S. Pierson III. 2003. Repression of phenazine antibiotic production in *Pseudomonas aureofaciens* strain 30-84 by RpeA. *J. Bacteriol.* 185:3718-3725.

- Whitehead, N. A., A. M. Barnard, H. Slater, N. J. Simpson, and G. P. Salmond.** 2001. Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* **25**:365-404.
- Whiteley, M., M. G. Banger, R. E. Bumgarner, M. R. Parsek, G. M. Teitzel, S. Lory, and E. P. Greenberg.** 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature*. **413**:860-864.
- Wilson, R., D. A. Sykes, D. Watson, A. Rutman, G. W. Taylor, and P. J. Cole.** 1988. Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. *Infect. Immun.* **56**:2515-2517.
- Wink, M.** 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* **64**:3-19.
- Wood, D. W., and L. S. Pierson, III.** 1996. The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**:49-53.
- Wood, D. W., F. Gong, M. M. Daykin, P. Williams, and L. S. Pierson, III.** 1997. *N*-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *J. Bacteriol.* **179**:7663-7670.
- Yoshida, A., T. Ansai, T. Takehara, and H. K. Kuramitsu.** 2005. LuxS-based signaling affects *Streptococcus mutans* biofilm formation. *Appl Environ Microbiol* **71**: 2372-2380.
- Zhang, R. G., T. Pappas, J. L. Brace, P. C. Miller, T. Oulmassov, J. M. Molyneux, J. C. Anderson, J. K. Bashkin, S. C. Winans, and A. Joachimiak.** 2002. A. Structure of

a bacterial quorum-sensing transcription factor complexed with pheromone and DNA.

Nature **417**: 917-974.

Zhang, Z., and L. S. Pierson III. 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*.

Appl. Environ. Microbiol. **67**:4305-4315.

APPENDIX A

Quorum Sensing and Phenazines are Involved in Biofilm Formation by *Pseudomonas Chlororaphis (aureofaciens) Strain 30-84*

SPRINGER LICENSE TERMS AND CONDITIONS

This is a License Agreement between V. S. R. K. Maddula ("You") and Springer ("Springer"). Please note that you are liable to account for Value Added Tax (VAT). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

License Number	1863770103283
License date	Jan 07, 2008
Licensed content publisher	Springer
Licensed content publication	Microbial Ecology
Licensed content title	Quorum Sensing and Phenazines are Involved in Biofilm Formation by <i>Pseudomonas chlororaphis (aureofaciens) Strain 30-84</i>
Licensed content author	V. S. R. K. Maddula
Licensed content date	Aug 3, 2006
Volume number	52
Issue number	2
Pages	289 - 301
Type of Use	Thesis / Dissertation
Details of use	University intranet
Portion of the article	Full text
Title of your thesis / dissertation	Quorum Sensing and Phenazines are Involved in Biofilm Formation by <i>Pseudomonas chlororaphis (aureofaciens) Strain 30-84</i>
Expected completion date	Apr 2008
Total	0.00 USD
Terms and Conditions	

Introduction

The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and

Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

Limited License

With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry.

Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided it is password protected or on the university's intranet, destined to microfilming by UMI and University repository. For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

The material can only be used for the purpose of defending your thesis, and with a maximum of 100 extra copies in paper.

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, provided permission is also obtained from the (co) author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well). Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted

However figures and illustrations may be altered minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights

Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

Copyright Notice: Disclaimer

You must include the following copyright and permission notice in connection with any

reproduction of the licensed material:

"Springer and the original publisher /journal title, volume, year of publication, page, chapter/article title, name(s) of author(s), figure number(s), original copyright notice) is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media"

Warranties: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Indemnity

You hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License

This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing

This license may not be amended except in a writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms

Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction

All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by the country's law in which the work was originally published.

v1.1

Quorum Sensing and Phenazines are Involved in Biofilm Formation by *Pseudomonas chlororaphis* (*aureofaciens*) Strain 30-84

V.S.R.K. Maddula, Z. Zhang, E.A. Pierson and L.S. Pierson III

Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA

Received: 27 December 2005 / Accepted: 27 December 2005 / Online publication: 4 August 2006

Abstract

The biological control bacterium *Pseudomonas chlororaphis* (*aureofaciens*) strain 30-84 employs two quorum sensing (QS) systems: PhzR/PhzI regulates the production of the antibiotics phenazine-1-carboxylic acid, 2-hydroxy-phenazine-1-carboxylic acid, and 2-hydroxy-phenazine, whereas CsaR/CsaI regulates currently unknown aspects of the cell surface. Previously characterized derivatives of strain 30-84 with mutations in each QS system and in the phenazine biosynthetic genes were screened for their ability to form surface-attached biofilm populations *in vitro*, using microtiter plate and flow cell biofilm assays, and on seeds and roots. Results from *in vitro*, seed, and root studies demonstrated that the PhzR/PhzI and the CsaR/CsaI QS regulatory systems contribute to the establishment of biofilms, with mutations in PhzR/PhzI having a significantly greater effect than mutations in CsaR/CsaI. Interestingly, phenazine antibiotic production was necessary for biofilm formation to the same extent as the PhzR/PhzI QS system, suggesting the loss of phenazines was responsible for the majority of the biofilm defect in these mutants. *In vitro* analysis indicated that genetic complementation or AHL addition to the growth medium restored the ability of the AHL synthase *phzI* mutant to form biofilms. However, only phenazine addition or genetic complementation of the phenazine biosynthetic mutation *in trans* restored biofilm formation by mutants defective in the transcriptional activator *phzR* or the *phzB* structural mutant. QS and phenazine production were also involved in the establishment of surface-attached populations on wheat seeds and plant roots, and, as observed *in vitro*, the addition of AHL extracts restored

the ability of *phzI* mutants, but not *phzR* mutants, to form surface attached populations on seeds. Similarly, the presence of the wild type in mixtures with the mutants restored the ability of the mutants to colonize wheat roots, demonstrating that AHL and/or phenazine production by the wild-type population could complement the AHL- and phenazine-deficient mutants *in situ*. Together, these data demonstrate that both QS systems are involved in the formation of surface-attached populations required for biofilm formation by *P. chlororaphis* strain 30-84, and indicate a new role for phenazine antibiotics in rhizosphere community development beyond inhibition of other plant-associated microorganisms.

Introduction

Bacteria in nature typically exist as members of structurally complex, surface-attached communities known as biofilms. On plants, bacteria may form aggregate communities on leaves, roots, and other plant surfaces, and within intercellular spaces of plant tissues (reviewed in [25, 34]). Biofilm formation is hypothesized to facilitate the development of a microniche that protects bacteria against various physical and chemical stresses. Genetic analyses of medically important microbes have identified a diversity of genes involved in biofilm formation and demonstrated that the gene expression patterns by bacteria within biofilms differ substantially from their expression patterns within planktonic cultures (reviewed in [4, 20]). Laboratory biofilm studies indicate that the genetic factors and pathways involved in biofilm formation vary depending on the bacterial species and the growth conditions. However, little is known about the relative importance of these factors and pathways in biofilm development outside the laboratory, and whether factors implicated in biofilm development by clinical

Present address of Z. Zhang: Department of Biology, University of California, San Diego, CA 92093, USA
Correspondence to: L.S. Pierson III; E-mail: lsp@u.arizona.edu

species are also important for biofilm formation by plant-associated microbes. Of particular interest is the potential role intercellular signaling pathways may play in coordinating gene expression among biofilm constituents, and the identification of specific genes under signaling control that function in the development of bacterial biofilms *on plants*.

Quorum sensing (QS) is a prevalent mechanism of intercellular signaling among bacteria that has received significant attention in the study of biofilms. Both Gram-negative and Gram-positive bacteria utilize small molecular weight QS signals to regulate the transcription of target genes based on the amount of signal present (reviewed in [24]). This type of communication is referred to as "quorum sensing" because signal availability is related to the density or "quorum" of signal producers present. In Gram-negative bacteria, the best-studied examples of QS utilize *N*-acyl-homoserine lactone signals (AHLs) (reviewed in [12, 24, 42, 45]). In the opportunistic human pathogen *P. aeruginosa*, 6–10% of the genome is estimated to be under QS regulation [38, 43]. Among plant-associated bacteria, AHL-mediated QS regulation is an integral component of many aspects of bacterial interactions with plants, both pathogenic and beneficial (reviewed in [34, 42, 45]). Previous studies using various biosensors that allow detection of AHL derivatives and their antagonists demonstrated that (1) bacterial populations inhabiting rhizosphere and plant surfaces produce a mixture of diverse AHL signals, (2) plant associated bacteria are capable of responding to signals produced by different bacterial species, and (3) this type of microbe–microbe communication occurs *in situ* on plants [6, 23, 32]. The production of diffusible AHL signals also plays a role in microbe–plant communication (reviewed in [3]).

QS influences various aspects of biofilm development and maturation [2, 10, 26, 29, 35, 48]. In laboratory studies, specific QS-regulated factors shown to play critical roles in surface attachment, aggregation, and dispersal include structural components of flagella, type IV pili, polysaccharide biosynthesis, protease (reviewed in [18, 41]), and rhamnolipids [9]. Several of these factors were also differentially expressed in response to attachment of the plant saprophyte *P. putida* to plant surfaces [37]. However, as QS is a global regulatory system affecting the expression of multiple genes, the exact nature of the role of QS in biofilm development is unclear [29]. Thus, specific factors under QS control need to be identified and their precise role in biofilm formation characterized.

QS regulation of phenazine production has been well characterized in the root-colonizing bacterium *Pseudomonas chlororaphis* (*aureofaciens*) strain 30-84 [30, 47]. This strain is capable of suppressing take-all disease of wheat caused by the ascomycete *Gaeumannomyces graminis* var. *tritici* [31]. The production of phenazine

antibiotics by strain 30-84 is the primary mechanism responsible for fungal pathogen inhibition and persistence on wheat roots [22, 31]. The PhzR/PhzI QS system regulates phenazine production via *phzR*, which encodes a transcriptional regulator of the phenazine operon, and *phzI*, which encodes an AHL synthase that directs the synthesis of hexanoyl homoserine lactone (HHL) [30, 47]. A second QS regulatory system (*CsaR/CsaI*) in strain 30-84 has been identified [49]. Inactivation of this system had no effect on phenazine production, but did result in alterations in the bacterial cell surface.

In this study, we examine whether QS, in general, and more specifically phenazine biosynthesis, which is under QS control, are involved in biofilm development in laboratory assays and *on plants*. We used static microtiter plate assays and single-pass flow cell analyses to screen previously characterized mutant derivatives of strain 30-84 for their ability to form surface-attached populations. The microtiter plate assay is used extensively to quantify biofilm formation by measuring crystal violet dye retention by surface attached bacterial cells [14, 17, 21, 27, 28, 33, 44]. The flow cell apparatus also has been extensively employed to visualize the structure of bacterial biofilms microscopically [7, 16]. We also examine the role of QS and phenazines on the establishment of biofilm populations on seeds and roots.

Methods

Bacteria, Plasmids, and Media. Bacterial strains and plasmids are described in Table 1. A spontaneous rifampicin-resistant derivative of *P. chlororaphis* strain 30-84 was used in all studies, and all mutants were derived from this parental strain [31]. Triparental matings into strain 30-84 or its derivatives were performed as previously described [30]. Unless otherwise indicated, all media formulations are as previously described [49]. Strain 30-84 and its derivatives were grown at 28°C in Luria-Bertani medium (LB) containing 5 g NaCl per liter, King's B medium (KMB), M9 minimal media, AB minimal broth amended with 2% casamino acids (AB+CAA), skim milk (SM) water agar, or pigment production medium (PPM-D). Where applicable, antibiotics were used at the following concentrations (µg/µL) for *Escherichia coli*: ampicillin (100), gentamycin (25), kanamycin (50), and tetracycline (25); for *P. chlororaphis*: gentamycin (50), kanamycin (50), rifampicin (100), and tetracycline (50).

Microtiter Plate Assay. We screened wild-type strain 30-84 and mutant derivatives (Table 1) for their ability to form biofilms in polystyrene microtiter plates by using a modified version of the procedure described by Genavaux *et al.* [13] and O'Toole and Kolter [28]. Assays were initially conducted in 96-well microtiter plates for 48–84 h. For subsequent work (AHL and phenazine

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
<i>P. chlororaphis</i> 30-84	Wild type, Rif ^r	W. Bockus, personal communication
30-84I	<i>phzI</i> : <i>kan</i> genomic fusion, Km ^r , Rif ^r	[46]
30-84R	<i>phzR</i> : <i>Tn5lacZ</i> genomic fusion, Km ^r , Rif ^r	[30]
30-84I2	<i>csaI</i> : <i>uidA-Gm</i> genomic fusion, Gm ^r , Rif ^r	[49]
30-84R2	<i>csaR</i> : <i>uidA-Gm</i> genomic fusion, Gm ^r , Rif ^r	[49]
30-84I1/I2	<i>phzI</i> : <i>kan</i> and <i>csaI</i> : <i>uidA-Gm</i> genomic fusion, Km ^r , Gm ^r , Rif ^r	[49]
30-84R1/R2	<i>phzR</i> : <i>Tn5lacZ</i> and <i>csaR</i> : <i>uidA-Gm</i> genomic fusion, Km ^r , Gm ^r , Rif ^r	[49]
30-84Z	<i>phzB</i> : <i>lacZ</i> genomic fusion, Rif ^r	[30]
30-84Ice	<i>phzB</i> : <i>inaZ</i> genomic fusion, Rif ^r	[47]
Plasmids		
pLAFR3	IncP1, Tc ^r	[39]
pLAF- <i>phzI</i>	pLAFR3 carrying <i>phzI</i>	[46]
pLAF- <i>phzR</i>	pLAFR3 carrying <i>phzR</i>	[30]
pLAF- <i>csaI</i>	pLAFR3 carrying <i>csaI-csaI</i>	[49]
pLAF- <i>csaR</i>	pLAFR3 carrying <i>csaR</i>	[49]
pUC18- <i>csaI</i>	pUC18 carrying <i>csaI</i> gene under the control of the vector <i>lacZ</i> promoter	[49]
pLSP259	pLAFR3 carrying contiguous 11.2 kb and 9.2 kb <i>EcoR1</i> fragments of 30-84 chromosomal DNA with <i>phzR</i> , <i>phzI</i> , and the phenazine biosynthetic region	[31]
pLSP10-30F	pLAFR3 carrying the 9.2 kb <i>EcoR1</i> fragment of 30-84 chromosomal DNA with the phenazine biosynthetic region	[30]
pLSP2.7	pLAFR3 carrying the 2.7 kb <i>PstI</i> fragment of 30-84 chromosomal DNA with <i>phzR</i> and <i>phzI</i>	[26]

^aAp^r, Km^r, Tc^r, and Gm^r denote resistance to ampicillin, kanamycin, tetracycline, and gentamycin, respectively.

addition experiments), we used 24-well plates for 32–38 h because differences were readily visible in the larger wells over a shorter time-span. For the 96-well assays, strains were grown on solid medium, inoculated into LB for 16 h, and resuspended in LB to a final cell density of OD₆₂₀ = 0.8. Each strain (1.2 μL) was inoculated into 120 μL AB + CAA in triplicate wells of 96-well polystyrene plates. Additional triplicate wells were not inoculated or inoculated with strain 30-84 as negative and positive controls, respectively. Plates were incubated at 28°C for 48–84 h without shaking and final cell densities determined (OD₆₂₀). Unattached cells were removed by inversion of the plate, followed by vigorous tapping on absorbent paper. The remaining adherent bacteria were fixed to the plates for 20 min at 50°C and then stained for 1 min with 150 μL/well of crystal violet (0.1%). Excess stain was removed by inversion of the plate followed by two washings (each 250 μL/well) with distilled water. Adherent cells were decolorized with a 20% acetone/80% ethanol solution (200 μL/well) for 5 min to release the dye into solution. A sample (100 μL) of each well was transferred to another 96-well plate and the amount of dye (proportional to the density of adherent cells) was quantified (OD₅₄₀). For the

time-course experiment, multiple replicates of each treatment were simultaneously inoculated into multiple microtiter plates, with each plate containing triplicate wells of each test strain. Cell density and biofilm formation were determined at time zero and at 4-h intervals until 84 h after inoculation. All experiments were repeated at least once.

The 24-well plate assays were performed as described above, except that 10 μL of each culture was inoculated into 1 mL of AB + CAA in triplicate wells of 24-well polystyrene plates, and treated similarly except that proportionally larger volumes (1.25 mL/well of stain, wash, and decolorization solution) and shorter time intervals (32–38 h) were used. All experiments were repeated at least once.

For all AHL and phenazine addition assays, bacterial treatments were inoculated into fresh medium containing no addition (negative control), AHL, or phenazine, and no subsequent additions were made.

Flow Cell Assay. Flow cell assays as described by Christensen *et al.* [7] and Heydorn *et al.* [16] with slight modifications were used to visually compare biofilm formation by strains 30-84, 30-84R (*phzR*), 30-84Z

(*phzB::lacZ*), and strain 30-84Z complemented for phenazine production via *in trans* expression of specific wild-type allele (see phenazine complementation below). Bacterial biofilms were grown in flow cells (Stovall Life Sciences, Inc., Greensboro, NC, USA) consisting of three chambers each measuring 4 × 40 mm and 1 mm with a coverslip for direct, nondestructive visualization of biofilms. Inoculum for each treatment was prepared from overnight cultures grown in LB broth and subsequently diluted (OD₆₂₀ = 0.4) with AB + CAA. A 300-μL aliquot of the dilute culture was then inoculated into separate flow chambers and maintained under static conditions for 1.5 h to allow the cells to attach to the glass cover slip. Biofilms were grown under continuous flow conditions in AB + CAA. An Ismatec IP high precision multichannel pump (Model 78023-22; Cole Parmer Instrument Company, Chicago, IL, USA) was used to generate a continuous flow rate of 160 μL/min through each chamber. Biofilms were visualized using an Olympus light microscope (BX60, Olympus America, Inc., Lake Success, NY, USA) at 400 and 1000× magnification. Images were taken at multiple time intervals up to 6 days after inoculation. At each time interval, 10 images were taken and representative images for each treatment are presented. Each experiment was repeated twice.

QS Complementation Assays. QS mutants were complemented by the introduction of specific alleles (Tables 1 and 2) *in trans* via triparental mating [30]. Respective strains containing pLAFR3 *in trans* were

included as controls. Cell adherence was determined after 48 h by the retention of crystal violet in 96-well microtiter plates (OD₅₄₀ ± standard error). Data for each treatment were standardized to the value for 30-84 (pLAFR3). Exoprotease activity was assessed by spotting 2-μL cell suspensions (OD₆₂₀ = 0.8) onto SM agar. The presence and the size (diameter) of the clear zones surrounding the colonies were determined after 48 h. Phenazine antibiotic production was determined qualitatively on PPM-D agar plates and quantified at OD₃₆₇ after extraction from cell-free supernatants as described previously [31].

AHL Addition Assays. AHL extracts containing either total AHLs (both the PhzI and CsaI AHLs) or the CsaI AHL alone were generated from cell-free supernatants of overnight cultures of strain 30-84Ice or *E. coli* DH5α (pUC18-*csaI*), respectively, as described previously [47]. Briefly, cell-free supernatants were extracted with an equal volume of ethyl acetate (acidified with 0.1 mL of glacial acetic acid per liter) for 2 h. The ethyl acetate phase was collected after centrifugation (3,000 × g, 5 min), rotary evaporated, and subsequently dried under nitrogen gas. The AHL extracts were resuspended in either AB + CAA for the biofilm assay as described above or M9 for the seed adherence assay (described below). To determine the effect of AHL dosage on cell adherence, the dried extracts were resuspended in 0.5, 1.0, or 2.0 times the original volume of the culture from which they were extracted. A control lacking AHLs was prepared similarly by extracting

Table 2. Effect of specific mutations and specific genes *in trans* on phenazine production, exoprotease activity, and adhesion ability

Strain	Phenazine*	Exoprotease*	Relative Adhesion**
30-84 (pLAFR3)	+	+	1.00 ± 0.05 ^{***}
30-84I (pLAFR3)	-	+	0.43 ± 0.05 ^c
30-84I (pLAF- <i>phzI</i>)	+	+	0.99 ± 0.08 ^a
30-84R (pLAFR3)	-	+	0.42 ± 0.03 ^{cd}
30-84R (pLAF- <i>phzR</i>)	+	+	1.01 ± 0.08 ^a
30-84I2 (pLAFR3)	+	+	0.75 ± 0.05 ^b
30-84I2 (pLAF- <i>csaRI</i>) ^ψ	+	+	1.03 ± 0.06 ^a
30-84R2 (pLAFR3)	+	+	0.71 ± 0.07 ^a
30-84R2 (pLAF- <i>csaR</i>)	+	+	1.06 ± 0.12 ^a
30-84I/I2 (pLAFR3)	-	-	0.21 ± 0.02 ^e
30-84I/I2 (pLAF- <i>phzI</i>)	+	-	0.32 ± 0.02 ^{cd}
30-84I/I2 (pLAF- <i>csaRI</i>) ^ψ	±	-	0.28 ± 0.03 ^{cd}
30-84R/R2 (pLAFR3)	-	-	0.17 ± 0.01 ^d
30-84R/R2 (pLAF- <i>phzR</i>)	+	-	0.26 ± 0.02 ^d
30-84R/R2 (pLAF- <i>csaR</i>)	±	-	0.19 ± 0.03 ^e

Phenazine production and exoprotease activity were determined qualitatively by the presence or absence of orange pigmentation indicative of phenazine production on PPM-D and a zone of clearing surrounding the colony on skim milk agar plates, respectively. Adhesion ability was determined after 48 h by the retention of crystal violet (OD₅₄₀ ± standard error). Data for each treatment were then standardized to the value for strain 30-84 (pLAFR3). Respective strains containing the cloning vector pLAFR3 *in trans* were included as a control. Data are the means of three replicates (± standard error) from one representative experiment.

*The symbols + and - denotes the presence/absence of phenazine production or exoprotease activity, respectively. The symbol ± indicates that phenazine was detected, but at a significantly lower level than produced by the wild type.

**Relative adhesion computed from retention of crystal violet (OD₅₄₀) standardized to the value for strain 30-84 (pLAFR3).

***Values with different superscript letters are significantly different.

^ψ*csaI* complementation via the introduction of the *csaI/csaR* region because we could not isolate the *csaI* gene.

a culture of strain 30-84I/12, a mutant unable to produce either the PhzI, or CsaI AHL, or phenazines, as described above. Purified PhzI AHL (HHL), synthesized as described previously [47], was added to AB + CAA at concentrations of 1, 10, 50, or 100 μ M.

Phenazine Complementation/Addition Assays. Cosmids containing *phzR*, *phzI* and the phenazine biosynthetic region, the phenazine biosynthetic region alone, or *phzR*, *phzI* alone (Table 1) were introduced into strain 30-84Z by triparental mating [30]. Phenazine extracts were prepared from cell-free supernatants of strain 30-84 as previously described [31]. Dried phenazine extracts were resuspended in AB + CAA minimal media in 0.5 or 1.0 times the original volume of the culture from which they were extracted, to determine the effect of dosage on cell adherence. Purified phenazine ($C_{12}H_8N_2$, Sigma Aldrich) consisting of the core phenazine ring structure but lacking carboxyl or hydroxyl substituents was added to AB + CAA at concentrations of 2.5, 12.5, or 25 μ g/mL.

Seed and Root Adherence Assays. Wheat seeds (cultivar Penawawa) were surface-disinfested and coated with various bacterial strains as previously described [47]. Briefly, bacterial strains were grown for 24 h, resuspended in M9 ($OD_{620}=0.5$), diluted 1/50 in M9 or M9 containing AHLs extracted from 30-84Ice, and placed in sterile beakers containing eight seeds. After 96 h at room temperature, the seeds were washed three times (3 mL each) with sterile water to remove nonadhering bacteria. After the removal of water, the seeds were resuspended in PBS (2 mL) and bacteria were isolated by vortexing (10 s) and sonication (10 s) three times. Bacterial populations were determined by serial dilution plating on KMB agar plus rifampicin.

For root assays, wheat seeds were surface-disinfested and pregerminated on water agar for 3 days to ensure sterility before planting. Overnight bacterial cultures grown on LB agar were suspended in PBS and then normalized to $OD_{620} = 0.8$. The seedlings were mixed with the bacterial cell suspensions for 10 min. Bacterial strains used for root bioassays included strain 30-84 and single and double QS mutants. Bacterial mixtures (wild type/mutant ratios of 1:1 or 1:9) composed of the wild type and mutant 30-84I/12 (*phzI/csaI*), or 30-84R/R2 (*phzR/csaR*) were also tested. Initial ratios for each bacterial mixture were confirmed via serial dilution plating on LB agar with and without kanamycin. Inoculated seedlings were sown in 25 \times 200 mm glass tubes (1 seedling/tube) that contained 20 cm of planting medium (equal volumes of pasteurized natural soil, vermiculite, and sand) prewetted with 15 mL sterile 1/3 Hoaglands solution (macronutrients only) as previously described [48]. The tubes were arranged in a randomized design in a Conviron™ growth chamber (20/15°C, 75%

relative humidity, 12-h light/dark cycle). Plants were harvested after 30 days and entire root systems were rigorously washed to remove nonadhering bacteria. Roots were blotted dry on filter paper, weighed, and cut into 1-cm sections. Bacteria from root sections were removed from the roots in 5 mL PBS by vortexing (10 s) and sonication (10 s) three times. Total bacterial populations were determined by serial dilution plating on KMB agar with rifampicin. For treatments involving strain mixtures, total populations were determined as described above and mutant populations were determined by also plating on KMB agar with rifampicin and kanamycin. The populations of strain 30-84 were calculated by subtracting the mutant population from the total population. Bacterial population data from both seed and root bioassay are reported as the means of three replicates and each experiment was repeated once.

Motility Assays. These properties were determined qualitatively as described by Deziel *et al.* [11]. Bacterial cells grown overnight on LB agar served as inoculum for all motility experiments. (1) Swimming. Tryptone swim plates (1% tryptone, 0.5% NaCl, and 0.3% agarose) were inoculated using toothpicks, incubated 16 h at 30°C, and flagellar motility assessed by the size of the circular zone. (2) Swarming. Swarm plates [nutrient broth (8 g/L), dextrose (5 g/L), and 0.5% agar or agarose] were dried overnight, inoculated with 1.5- μ L cell suspensions ($OD_{620}=1.0$), and the sizes of the swarming zones were measured after 16 and 24 h. (3) Twitching. Cells were stab inoculated to the bottom of swarming plates, incubated 48 h at 30°C, and swarming measured as the zone of growth between the agar and plate bottom.

Statistical Analyses. In all microtiter plate experiments, in order to control for variation in the retention of crystal violet between replicate plates, data were standardized to the value of the strain 30-84 control in each plate (e.g., values are expressed as biofilm formation relative to the untreated control). All microtiter, seed, and root experiments were statistically analyzed. Means were compared among treatments ($p < 0.05$) by using analysis of variance (ANOVA) and protected least significant difference (LSD) multiple comparison tests (SAS Version 8.2, SAS Institute Inc., Cary, NC, USA).

Results

Role of QS in Biofilm Initiation and Secondary Metabolite Production. Wild-type strain 30-84 and derivatives with mutations in genes involved in QS and phenazine production were analyzed for biofilm formation by using the microtiter biofilm assay. The amount of biofilm formed by strain 30-84 became measurable after 36 h (after cell density had reached an $OD_{620} > 0.8$), increased with

time, and reached peak levels after 68–72 h (Fig. 1). In contrast, derivatives having single mutations in either QS system, 30-84R (*phzR*), 30-84I (*phzI*), 30-84R2 (*csaR*), or 30-84I2 (*csaI*) produced significantly less biofilm than the parental strain even after 84 h (data after 72 h not shown), demonstrating that both QS systems are involved in cell adhesion. Interestingly, mutants deficient in phenazine production, strain 30-84R, strain 30-84I, and the phenazine structural mutant 30-84Z, were significantly more impaired in biofilm formation than the cell surface alteration mutants, strains 30-84R2 and 30-84I2. Double mutants in which both AHL synthases (*phzI*, *csaI*) or both transcriptional activators (*csaR*,

phzR) were inactivated produced negligible biofilms even after 84 h, indicating that loss of both QS systems dramatically impairs biofilm formation (Fig. 1A). There were no differences between the wild type and mutants in doubling times indicating the decrease in biofilm formation by the mutants was not a result of impaired growth (Fig. 1B).

We also investigated the effect of complementation of the QS mutants on biofilm formation by both genetic and biochemical means using *in trans* expression of specific wild-type alleles and the addition of specific AHLs to the growth media. Complementation of the specific single mutants 30-84I (*phzI*), 30-84R (*phzR*), 30-84I2 (*csaI*), and 30-84R2 (*csaR*) with their corresponding wild-type alleles *in trans* restored the wild-type phenotypes, including phenazine production to the *phzI* and *phzR*-deficient mutants and biofilm formation by all mutants (Table 2). As expected, the presence of multiple copies of the AHL synthase *phzI* alone significantly increased phenazine production by double synthase mutant 30-84I/2, whereas introduction of multiple copies of the *csaI* synthase resulted in minimal phenazine production by 30-84I/2. Similarly, multiple copies of the transcriptional activator *phzR* alone significantly increased phenazine production in the double mutant 30-84R/2, but introduction of *csaR* alone resulted in minimal phenazine production. Interestingly, the presence of multiple copies of either AHL synthase alone in the double synthase mutant 30-84I/2 improved, but did not restore, biofilm formation to wild-type levels. However, only the *phzR* transcriptional activator improved (but did not restore to wild-type levels) biofilm formation by the double mutant 30-84R/2. These data further support the hypothesis that both quorum-sensing systems are involved in biofilm development by *P. chlororaphis* strain 30-84 and that the PhzR/I system plays a greater role.

The effect of the addition of AHL signals prepared from cell-free supernatants of either strain 30-84Icc (*phzI*⁺, *csaI*⁺, *phzB*:*inaZ*) containing both AHLs or *E. coli* DH5 α (pUC18-*csaI*) containing only the CsaI product on biofilm formation by strain 30-84 and various mutants was examined by using three dosage levels (Fig. 2A, B). Dosage levels were prepared from culture extracts that were 0.5, 1.0, or 2 times their original concentration. Addition of both AHLs (Fig. 2A) at the most dilute concentration (0.5 \times) nearly restored biofilm formation by the *phzI* synthase mutants 30-84I and 30-84I/2, but was unable to restore biofilm formation by the *phzR* transcriptional activator mutant 30-84R or the phenazine structural mutant 30-84Z. These results demonstrate that the exogenous AHLs specifically complemented the AHL-deficient mutants. Mutants deficient in the *csaR/I* system (but with an intact *phzR/I* system) were only slightly reduced in cell adherence compared to

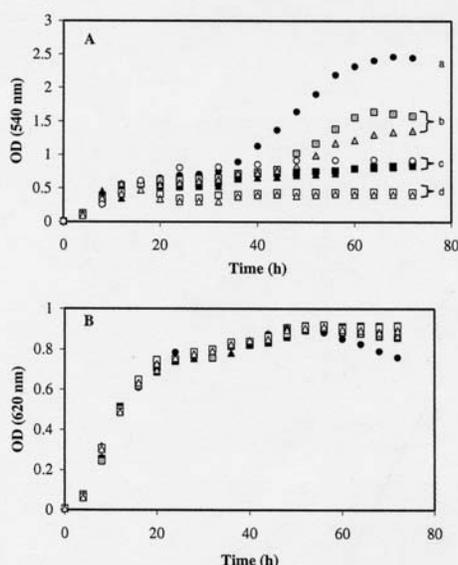


Figure 1. Time course of cell adhesion by wild type *P. chlororaphis* strains 30-84 and its derivatives. (A) Cell adhesion was measured based on the optical density (540 nm) of crystal violet-stained cells adhering to the polystyrene after washing. (B) Cell density of the culture medium was measured by optical density (620 nm). Replicate cultures of each treatment were inoculated at the same time, and at 4-h intervals, three replicates of each treatment were assayed for cell adhesion. Strains tested included wild type 30-84 (●), regulatory mutants 30-84I (*phzI*) (●), 30-84R (*phzR*) (▲), 30-84I2 (*csaI*) (■), 30-84R2 (*csaR*) (▲), double QS mutants 30-84I/2 (*phzI*/*csaI*) (□), and 30-84R/2 (*phzR*/*csaR*) (△), and a phenazine structural mutant 30-84Z (*phzB*:*lacZ*) (○). Data are the means of three replicates, and standard errors were less than 0.03 and 0.01 for data in A and B, respectively. Data from one representative experiment are given. Superscript letters denote groups of treatments that are significantly different at 72 h.

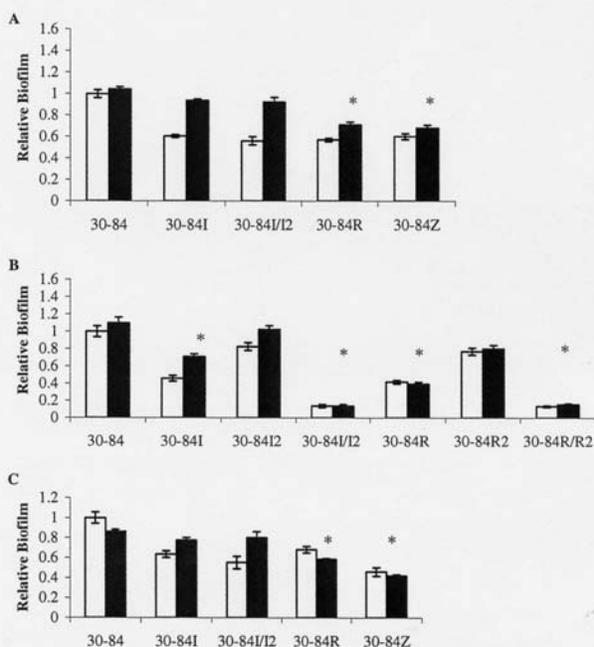


Figure 2. Effect of AHLs on adhesion by various mutants. Mutants include 30-84I (*phzI*), 30-84R (*phzR*), 30-84I2 (*csaI*), 30-84R2 (*csaR*), double QS mutants 30-84I/I2 (*phzI/csaI*) and 30-84R/R2 (*phzR/csaR*), and the phenazine structural mutants 30-84Z (*phzB::lacZ*). Treatments include “no addition” (white) and AHL addition (black). (A) Addition of an AHL extract from 30-84Ice containing both the PhzI and CsaI AHL signals (0.5× concentration). (B) Addition of an AHL extract from *E. coli* DH5α (pUC18-*csaI*) containing only the CsaI product (2.0× concentration). (C) Addition of purified HHL (50 μM). The optical density of crystal violet-stained cells (after 32–48 h) adhering to the polystyrene after washing was measured at OD₅₄₀. Data for each treatment were then standardized to the value for the untreated wild type (e.g., relative biofilm). Data are the means of eight, six, and six replicates (A, B, and C, respectively) from one representative experiment (±standard errors). *Mutants that produce very little or no phenazine with the addition of the AHL used in the experiment.

wild type, and produced significantly more biofilm when both AHLs were added (data not shown). Addition of both AHLs at a 2× concentration still failed to restore biofilm formation by the transcriptional activator mutants 30-84R or the phenazine structural mutant 30-84Z (data not shown). As expected, addition of the CsaI product alone (Fig. 2B) improved biofilm formation by single AHL synthase mutants, but was unable, even at the highest dosage (2×), to restore biofilm formation by the *phzI* mutant (30-84I) to wild-type levels. The CsaI product alone also failed to improve biofilm formation by the double AHL synthase mutant 30-84I/I2 or the transcriptional activator mutants 30-84R, 30-84R2, or 30-84R/R2. These findings further demonstrate that the exogenous AHL specifically complemented AHL-deficient mutants, and further support the hypothesis that

although some cross-communication between QS systems may occur, an intact *phzR/I* system is required for wild-type biofilm formation.

To specifically test the role of the PhzI product on bacterial adhesion, purified HHL was added to the culture medium to final concentrations of 1, 10, 50, or 100 μM. Addition of the purified HHL product at 1 and 10 μM concentrations had no significant affect on biofilm formation (data not shown). Addition of purified HHL at 50 μM (Fig. 2C) and 100 μM (data not shown) concentrations restored biofilm formation to all QS mutants having a functional *phzR* gene except the phenazine structural mutant, demonstrating that the addition of extra *phzI* product alone cannot restore biofilm formation to mutants that are unable to produce phenazines.

Role of Phenazines in Biofilm Formation. Strains 30-84R, 30-84R/R2, and 30-84Z unable to produce phenazine because of either a *phzR* mutation or a phenazine structural mutation were significantly impaired in their ability to form biofilms compared to the wild-type strain in both microtiter plate and flow cell assays (Figs. 3 and 4). The effect of complementation of the phenazine structural mutant strain 30-84Z on biofilm formation by both genetic and biochemical means using *in trans* expression of specific wild-type alleles and the addition of specific phenazines to the growth media was investigated. Complementation of the *phzB* mutant strain 30-84Z with cosmids containing *phzR*, *phzI* and the phenazine biosynthetic genes (pLSP259) or the phenazine

biosynthetic regions alone (pLSP10-30F) *in trans* restored biofilm formation to wild-type levels, whereas introduction of the *phzR*, *phzI* alone (pLSP2.7) had no effect (Figs. 3A and 4). The effect of the addition of bulk phenazines prepared from cell-free supernatants of strain 30-84 on biofilm formation by strain 30-84 and various mutants was examined by using two dosage levels (Fig. 3B). Dosage levels were prepared from culture extracts 0.5 or 1.0 times their original concentration. Addition of bulk phenazines at a 0.5 \times concentration restored biofilm formation by *phzR* mutant 30-84R and phenazine structural mutant 30-84Z to wild-type levels, and improved biofilm formation by the double QS mutant 30-84R/R2 (Fig. 3B). These results are consistent with the hypothesis

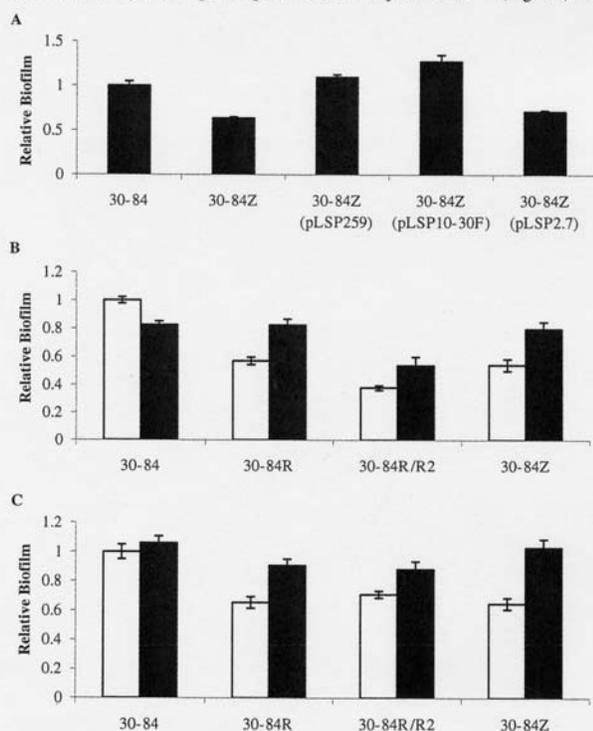


Figure 3. Effect of genetic complementation of a phenazine structural mutant and addition of phenazine on adhesion by various mutants. (A) Genetic complementation of the phenazine structural mutant 30-84Z (*phzB*:*lacZ*) by cosmids containing *phzR*, *phzI* and the phenazine biosynthetic genes (pLSP259), the phenazine biosynthetic regions alone (pLSP10-30F), or *phzR*, *phzI* alone (pLSP2.7). (B) Addition of a phenazine extract from a cell free supernatant of strain 30-84 (0.5 \times concentration) or (C) 12.5 μ g/mL purified phenazine. Mutants include 30-84R (*phzR*), double QS mutant 30-84R/R2 (*phzR/csaR*), and the phenazine structural mutant 30-84Z (*phzB*:*lacZ*). Treatments include "no addition" (white) or a phenazine addition (black). The optical density of crystal violet-stained cells adhering (after 32 h) to the polystyrene after washing was measured at OD₅₄₀. Data for each treatment were then standardized to the value for the untreated wild type (e.g., relative biofilm). Data for A, B, and C are the means (\pm standard error) of nine, six, and six replicates, respectively, from one representative experiment.

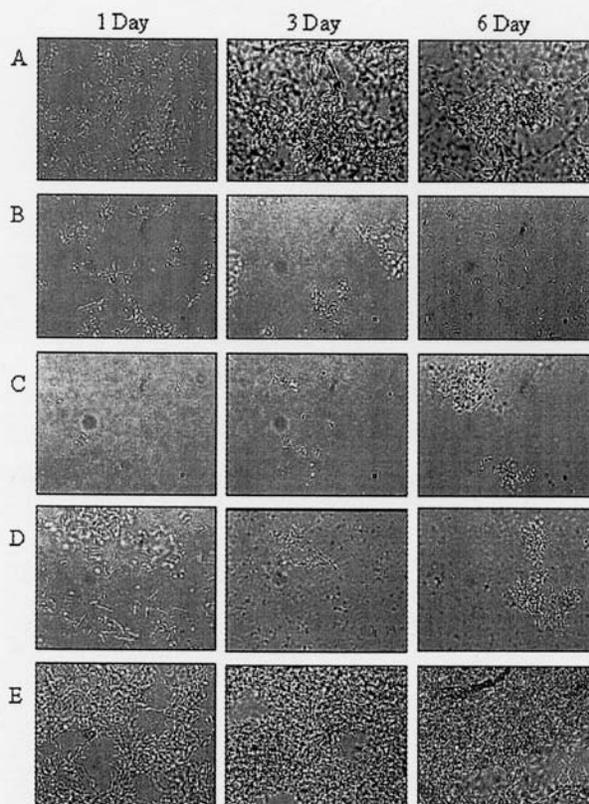


Figure 4. Flow cell images of biofilm formation by wild type strain 30-84 and various mutants. Treatments include (A) wild type strain 30-84, (B) mutant 30-84R (*phzR*), (C) the phenazine structural mutant 30-84Z (*phzB::lacZ*), and (D) genetic complementation of strain 30-84Z *in trans* by cosmids containing either *phzR*, *phzI* (pLSP2.7) (E) or the phenazine biosynthetic genes (pLSP10-30F). Ten images for each treatment were taken 1, 3, and 6 days after inoculation and representative images (1000 \times) of average biofilm production for each treatment are shown.

that phenazines play a role in bacterial attachment, but that other traits under QS control are involved in biofilm formation. There was no significant improvement in cell adhesion when phenazines were added at a higher dosage (1.0 \times , data not shown). To further verify that phenazines were responsible for the effect, commercial phenazine was added to the medium at concentrations of 2.5, 12.5, and 25 $\mu\text{g}/\text{mL}$. This commercial derivative comprised the core phenazine ring structure, but lacks side-chain modifications such as carboxyl and hydroxyl groups. Additions of 12.5 $\mu\text{g}/\text{mL}$ (Fig. 3C) and 25 $\mu\text{g}/\text{mL}$ phenazine (data not shown) were sufficient to improve biofilm formation by 30-84R and 30-84R/R2 QS mutants and restore biofilm formation by phenazine structural mutant 30-84Z to

wild-type levels. There was no significant effect on biofilm formation compared to the untreated control at the lowest (2.5 $\mu\text{g}/\text{mL}$) dosage (data not shown).

Biofilm Formation on Wheat Seeds. Various quorum-sensing mutants were compared with the parental strain for establishment of surface-attached populations on wheat seeds (Fig. 5A). After thorough washing, we recovered smaller surface-adherent populations of all single quorum-sensing mutants 30-84I (*phzI*), 30-84R (*phzR*), 30-84I2 (*csaI*), and 30-84R2 (*csaR*) than wild-type populations from seed surfaces, indicating that the mutants were impaired in establishing biofilm populations on seeds. However, in the culture supernatant,

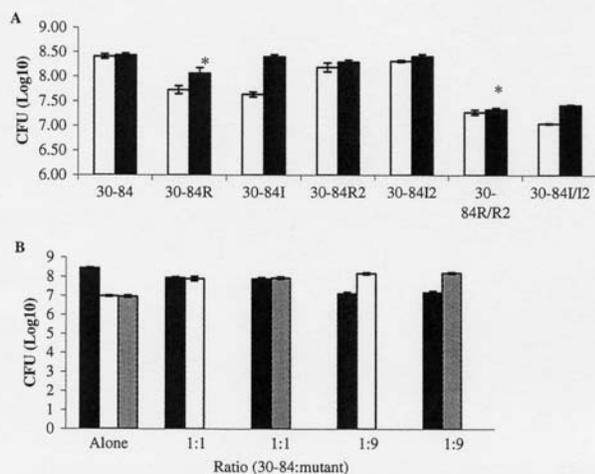


Figure 5. Bacterial populations on seeds and roots. (A) Seeds were treated with bacterial suspensions containing either no AHLs (white) or AHLs extracted from 30-84Ice containing both the PhzI and CsaI AHL signals (black). (B) Roots were initially treated with either strain 30-84 (black) or double QS mutants 30-84I/12 (*phzI/csaI*) (white) or 30-84R/R2 (*phzR/csaR*) (gray), either alone or mixtures with 1:1 or 1:9 ratios of the wild type and mutants, respectively. Populations are expressed as the log₁₀ value of the colony forming units (CFU) per seed or per gram of fresh roots (\pm standard error). In the root experiment, total populations were determined as described in Methods. Seed and root data are the means of data from three separate seeds or four separate plants with three replicate plate counts each. Data from only one representative experiment are given. *Mutants that produce very little or no phenazine with the addition of the AHL used in the experiment.

bacterial populations of mutants and the wild type were not significantly different (data not shown), indicating that the survival of the mutants in the liquid culture surrounding the seeds was not impaired. Compared to the *csaR* and *csaI* mutants, the *phzR* and *phzI* QS mutants formed significantly lower populations on seeds. Consistent with the results observed in the microtiter plate assay, both double mutants 30-84I/12 and 30-84R/R2

were dramatically impaired in adherence on seeds, with their populations on the seeds being 14- and 23-fold lower than that of the wild type (Fig. 5A).

To determine the effects of the addition of AHL on biofilm formation on seeds, AHL extracts prepared from cell-free supernatants of 30-84Ice were added to the tubes containing M9 minimal broth and bacterial cells (Fig. 5A). The addition of AHL extracts had no effect on the

Table 3. Swimming, swarming, and twitching motilities of strain 30-84 and mutants deficient in either or both quorum-sensing systems

Strain	Swimming zone (cm)		Swarming zone (cm)		Twitching zone (cm)	
	16 h		16 h	24 h	48 h	
30-84	6.36 \pm 0.16 ^a		2.28 \pm 0.12 ^a	2.89 \pm 0.23 ^a	1.85 \pm 0.06 ^a	
30-84I	6.38 \pm 0.14 ^a		1.72 \pm 0.16 ^{ab}	2.40 \pm 0.20 ^{bc}	1.73 \pm 0.12 ^a	
30-84R	6.32 \pm 0.12 ^a		1.98 \pm 0.16 ^{ab}	2.48 \pm 0.15 ^{ab}	1.75 \pm 0.11 ^a	
30-84I2	6.36 \pm 0.11 ^a		2.19 \pm 0.11 ^a	2.66 \pm 0.16 ^{ab}	1.64 \pm 0.02 ^a	
30-84R2	6.42 \pm 0.16 ^a		2.22 \pm 0.15 ^a	2.92 \pm 0.07 ^a	1.72 \pm 0.16 ^a	
30-84I/12	6.58 \pm 0.14 ^a		1.46 \pm 0.15 ^c	2.06 \pm 0.25 ^c	1.31 \pm 0.08 ^b	
30-84R/R2	6.62 \pm 0.15 ^a		1.55 \pm 0.17 ^c	2.03 \pm 0.12 ^c	1.28 \pm 0.06 ^b	

Swimming zone was determined by measuring the diameters of bacterial lawns after 16 h. Swarming motility was assayed by measuring the sizes of bacterial swarming zones after 16 and 24 h. For twitching motility assays, test strains were stab inoculated with toothpicks to the bottom of swarming plate medium, and bacterial movement, as indicated by the size of the zone between the agar and the bottom of petri plate, was measured after 48 h. Data represent the means of five replicates (\pm standard error) from one experiment.

For each treatment, values with different superscript letters are significantly different.

bacterial population densities of the culture supernatant (data not shown). However, the addition of AHL signals fully restored seed-attached population densities by 30-84I (*phzI*) and 30-84I2 (*csaI*), and slightly increased seed-attached populations 30-84I/12 (*phzI*, *csaI*).

Biofilm Formation on Wheat Roots. Strain 30-84 and several mutants were compared for biofilm formation on the roots of wheat plants grown 30 days in pasteurized soil (Fig. 5B). In this experiment, roots were thoroughly washed and then rinsed with sterile water to remove solid particles and unbound bacteria. Bacterial populations that remained on the roots after washing reflect the ability of each strain to survive within adhering biofilms on roots. The highest bacterial populations (8.46 log units/g root) were recovered from the washed roots of wheat treated with the wild-type strain. Bacterial populations of double mutants 30-84I/12 and 30-84R/2 isolated from the roots were significantly (at least 30-fold) lower than the wild type (6.98 and 6.96 log units/g root, respectively).

To determine whether the presence of wild-type strain 30-84 could improve biofilm formation by the double mutants 30-84I/12 30-84R/2 on root surfaces, strains were grown independently and then mutant cells were mixed with wild-type cells at different ratios immediately prior to seed treatment. When introduced in 1:1 or even 1:9 mixtures (wild-type strain 30-84:mutant), the double quorum-sensing mutants 30-84I/12 and 30-84R/2 established populations on roots similar to or slightly higher than that of the wild type (Fig. 5B). These results suggest that even when they account for only 1/10 of the starting population, wild-type cells contribute to biofilm formation by the 30-84I/12 and 30-84R/2 double mutants on root surfaces.

Motility Assays. To determine if loss of biofilm formation by any of the mutants was attributable to a defect in properties associated with bacterial motility, flagella-mediated swimming and solid surface translocation assays were performed. Swimming zones formed rapidly for all strains, were not different after 16 h (Table 3), and covered the entire surface after 20 h (data not shown). Swarming motility was unchanged in the single QS mutants (30-84I, 30-84R, 30-84I2, and 30-84R2) after 16 or 24 h as compared to the parental strain (Table 3), and swarming zones formed by both the wild type and single quorum-sensing mutants covered the surface by 36 h (data not shown). Twitching motility was similar in the single QS mutants as compared to the wild type after 48 h (Table 3). In contrast to the swimming assays, the double QS mutants (30-84I/12, 30-84R/2) were significantly reduced in swarming and twitching as compared to the wild type after 48 h.

Discussion

Two quorum-sensing (QS) systems, PhzR/PhzI and CsaR/CsaI, were previously identified in the biological control bacterium *P. chlororaphis* strain 30-84. The PhzR/PhzI QS system regulates phenazine antibiotic production [30, 46] and the CsaR/CsaI QS system regulates as yet unknown aspects of the bacterial cell surface [49]. Results of the present study demonstrate that both QS systems are involved in biofilm development by *P. chlororaphis* strain 30-84, but that the PhzR/PhzI system and specifically phenazine production have the greatest influence on biofilm formation. Significantly, the phenazine structural mutant *phzB* and the *phzR* and *phzI* mutants were impaired to similar levels in biofilm development, suggesting that loss of phenazines was responsible for the majority of the biofilm defect in the *phzR/phzI* mutants. Furthermore, our results show that deficiencies in biofilm production by AHL synthase mutants may be complemented by the addition of AHLs or AHL-producing strains. Genetic complementation of single QS mutants with their corresponding functional alleles also restored the wild-type phenotypes including phenazine production to the *phzR/phzI* mutants and biofilm formation to all mutants, indicating that their specific QS mutation was responsible for their deficiencies in biofilm production. The PhzR/PhzI and CsaR/CsaI QS systems also contribute to the ability of strain 30-84 to form tightly surface-adherent biofilm populations on both wheat seeds and roots. Similar to the results obtained *in vitro*, addition of total AHLs to the medium restored biofilm formation by both the *phzI*- and *csaI*-defective mutants on seeds, but not by the *phzR* and *csaR* mutants. The fact that biofilm formation by the double *phzR/csaR* transcriptional activator mutant was rescued by the presence of the wild-type strain suggests that AHL production by the wild type is not the primary mechanism involved. The observed improved establishment of biofilm populations on roots by the double mutants in mixtures with the wild type may be attributable to (1) initial surface binding by wild-type cells, or (2) production by the wild-type cells of a diffusible compound required for biofilm formation that is not produced by the QS transcriptional regulator mutants. One candidate diffusible compound is phenazine.

To specifically test the role of phenazines in biofilm formation, we compared biofilm formation by the *phzB* phenazine structural mutant and the *phzR* QS mutant to that of the wild type in both microtiter and flow cell assays. Both mutants defective in phenazine biosynthesis were significantly less able to form biofilms in microtiter plate assays than the wild type and formed virtually no biofilm in flow cells. Genetic complementation of the *phzB* mutant with the phenazine biosynthetic genes under *E. coli lac* promoter control, but without extra

copies of the *phzI/phzR* genes, restored biofilm formation by the *phzB* mutant strain 30-84Z to wild-type levels in both plate and flow cell assays. These results demonstrate that the deficiencies in biofilm production by the phenazine structural mutant were specifically attributable to loss of phenazine biosynthesis. Addition of phenazines from cell-free culture supernatants or as purified phenazine also restored biofilm formation by phenazine structural mutant to wild-type levels in microtiter plates, demonstrating that phenazines could complement the mutation. Interestingly, phenazines did not completely complement the *phzR* or *phzR/csaR* mutants, suggesting that other traits under the control of one or both of the QS systems are needed for wild-type biofilm formation.

Previous work on numerous bacteria demonstrated that QS is a global regulatory system affecting the expression of multiple genes, many of which influence aspects of biofilm development and maturation (reviewed in [29]). Several studies have demonstrated that QS is involved in bacterial motility, which plays an integral role in biofilm maturation (reviewed in [41]). In the present study, we found no differences between the mutants and the wild type in swimming, indicating that the biofilm defects in the QS mutants were not due to deficiencies in flagellar function. Similarly, single mutations in each QS system had little effect on twitching and swarming; however, the double QS mutants exhibited less twitching and swarming than the wild type. Although the exact mechanism(s) responsible for these defects in twitching and swarming motility in double QS mutants remain to be determined, these data suggest that PhzR/PhzI and CsaR/CsaI may regulate biofilm formation partly through bacterial surface migration [17].

This study is the first to report a role for phenazine production in biofilm formation. Previous work has demonstrated QS control of phenazines in bacteria [30, 43]. Phenazines are secondary metabolites that exhibit antimicrobial activity against many species of bacteria, fungi, and protozoa (reviewed in [5]). Over 6000 phenazine derivatives produced by a variety of bacteria have been identified [19, 40]. Previous work on pseudomonads has focused on phenazines because of their roles in suppressing plant pathogens and as virulence factors during host infection. *P. chlororaphis* strain 30-84 produces three phenazine derivatives, phenazine-1-carboxylic acid, 2-hydroxy-phenazine-1-carboxylic acid and 2-hydroxy-phenazine [31]. Phenazine production by *P. chlororaphis* strain 30-84 is important for its ability to inhibit the growth of fungal plant pathogens and for its ability to persist in the rhizosphere [22, 31]. We hypothesize that phenazine antibiotics produced by *P. chlororaphis* serve multiple roles in the plant rhizosphere, including inhibition of other microorganisms and biofilm development on plant surfaces, which ultimately influences bacterial persistence. Although the mechanis-

tic role for phenazines in biofilm formation remains to be determined, the inability of phenazine-deficient mutants to establish surface-adherent populations *in vitro* and on plant surfaces suggests that phenazines may play a role in surface attachment and/or survival in bacterial aggregations. Phenazines could contribute to biofilm development in various ways: (1) phenazines may serve as adhesins, analogous to the role of proteins and polysaccharides by *Staphylococcus epidermidis* [36] or the Bap protein of *S. aureus* [8]; (2) phenazines may themselves serve as signals, triggering the expression of other factors important in biofilm development; (3) phenazines may play a role in survival within the aggregate bacterial community. The consequences of life in a bacterial biofilm include the accumulation of bacterial by-products and limitations in oxygen, iron, and other nutrients. Phenazine derivatives have been implicated in several bacterial traits that may facilitate survival under these conditions, including metal mineralization and extracellular electron shuttling [15], and electron transport [1]. Previous research demonstrated that a complex web of regulatory pathways controls phenazine biosynthesis in strain 30-84 and other pseudomonads [19, 32]. Our hypothesis that phenazines play multiple roles for the producing cell in the rhizosphere may explain this complex regulation. Biofilm establishment is believed to be key step in microbial community development, structure, and survival on plants. Current work is aimed at further defining the role(s) of QS and phenazines in specific stages of biofilm establishment and growth *in vitro* and on plants, and ultimately, in the development of plant-associated microbial communities.

Acknowledgments

We thank Patricia Figuli, Shaina Schwartz, and Dr. Cheryl Whistler for technical assistance and Christopher Rensing for critical discussions. This work was supported by USDA NRICGP grant No. 2001-02684.

References

1. Abken, HJ, Tietze, M, Brodersen, J, Baumer, S, Beifuss, U, Deppenmeier, U (1998) Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Go1. *J Bacteriol* 180: 2027-2032
2. Arevalo-Ferro, C, Reil, G, Gorg, A, Eberl, L, Riedel, K (2005) Biofilm formation of *Pseudomonas putida* IsoF: the role of quorum sensing as assessed by proteomics. *Syst Appl Microbiol* 28: 87-114
3. Bauer, WD, Mathesius, U (2004) Plant responses to bacterial quorum sensing signals. *Curr Opin Plant Biol* 7: 429-433
4. Beloin, C, Ghigo, JM (2005) Finding gene-expression patterns in bacterial biofilms. *Trends Microbiol* 13: 16-19
5. Cao, H, Baldini, RL, Rahme, LG (2001) Common mechanisms for pathogens of plants and animals. *Annu Rev Phytopathol* 39: 259-284
6. Cha, C, Gao, P, Chen, YC, Shaw, PD, Farrand, SK (1998) Production

- of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol Plant Microbe Interact* 11: 1119–1129
7. Christensen, BB, Sternberg, C, Andersen, JB, Palmer Jr, RJ, Nielsen, AT, Givskov, M, Molin, S (1999) Molecular tools for study of biofilm physiology. *Methods Enzymol* 310: 20–42
 8. Cucarella, C, Solano, C, Valle, J, Amorena, B, Lasa, I, Penades, JR (2001) Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol* 183: 2888–2896
 9. Davey, ME, Caiazza, NC, O'Toole, GA (2003) Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 185: 1027–1036
 10. Davies, DG, Parsek, MR, Pearson, JP, Iglewski, BH, Costerton, JW, Greenberg, EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280: 295–298
 11. Deziel, E, Comeau, Y, Villemur, R (2001) Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpilated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *J Bacteriol* 183: 1195–1204
 12. Fuqua, C, Parsek, MR, Greenberg, EP (2001) Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* 35: 439–468
 13. Genavaux, P, Muller, S, Bauda, P (1996) A rapid screening procedure to identify mini-Tn10 insertion mutants of *Escherichia coli* K-12 with altered adhesion properties. *FEMS Microbiol Lett* 142: 27–30
 14. Heilmann, C, Gerke, C, Perdreau-Remington, F, Goetz, F (1996) Characterization of Tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infect Immun* 64: 277–282
 15. Hernandez, ME, Newman, DK (2001) Extracellular electron transfer. *Cell Mol Life Sci* 58: 1562–1571
 16. Heydorn, A, Ersboll, BK, Hentzer, M, Parsek, MR, Givskov, M, Molin, S (2000) Experimental reproducibility in flow-chamber biofilms. *Microbiology* 146: 2409–2415
 17. Klausen, M, Heydorn, A, Ragas, P, Lambertsen, L, Aaes-Jorgensen, A, Molin, S, Tolker-Nielsen, T (2003) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* 48: 1511–1524
 18. Kierek-Pearson, K, Karatan, E (2005) Biofilm development in bacteria. *Adv Appl Microbiol* 57: 79–111
 19. Laursen, JB, Nielsen, J (2004) Phenazine natural products: biosynthesis, synthetic analogues, and biological activity. *Chem Rev* 104: 1663–1686
 20. Lazazzera, BA (2005) Lessons from DNA microarray analysis: the gene expression profile of biofilms. *Curr Opin Microbiol* 2: 222–227
 21. Loo, CY, Corliss, DA, Ganeshkumar, N (2000) *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. *J Bacteriol* 182: 1374–1382
 22. Mazzola, M, Cook, RJ, Thomashow, LS, Weller, DM, Pierson, LS (1992) Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. *Appl Environ Microbiol* 58: 2616–2624
 23. McLean, RJ, Whiteley, M, Stickler, DJ, Fuqua, WC (1997) Evidence of autoinducer activity in naturally occurring biofilms. *FEMS Microbiol Lett* 154: 259–263
 24. Miller, MB, Bassler, BL (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* 55: 165–199
 25. Morris, CA, Monier, JM (2003) The ecological significance of biofilm formation by plant-associated bacteria. *Annu Rev Phytopathol* 41: 429–453
 26. O'Toole, G, Kaplan, HB, Kolter, R (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* 54: 49–79
 27. O'Toole, GA, Kolter, R (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* 30: 295–304
 28. O'Toole, GA, Kolter, R (1998) Initiation of biofilm formation in *Pseudomonas fluorescens* WC365 proceeds via multiple, convergent signaling pathways: a genetic analysis. *Mol Microbiol* 28: 449–461
 29. Parsek, MR, Greenberg, EP (2005) Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* 13: 27–33
 30. Pierson III, LS, Keppenne, VD, Wood, DW (1994) Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *J Bacteriol* 176: 3966–3974
 31. Pierson, LS III, Thomashow, LS (1992) Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. *Mol Plant Microbe Interact* 5: 330–339
 32. Pierson III, LS, Wood, DW, Pierson, EA, Chancey, ST (1998) Homoserine lactone-mediated gene regulation in plant-associated bacteria. *Annu Rev Phytopathol* 36: 207–225
 33. Pratt, LA, Kolter, R (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 30: 285–293
 34. Ramey, BE, Koutsoudis, M, von Bodman, SB, Fuqua, C (2004) Biofilm formation in plant-microbe associations. *Curr Opin Microbiol* 7: 602–609
 35. Rice, SA, Koh, KS, Queck, SY, Labbate, M, Lam, KW, Kjellberg, S (2005) Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *J Bacteriol* 187: 3477–3485
 36. Rupp, ME, Ulphani, JS, Fey, PD, Mack, D (1999) Characterization of *Staphylococcus epidermidis* polysaccharide intercellular adhesion/hemagglutinin in the pathogenesis of intravascular catheter-associated infection in a rat model. *Infect Immun* 67: 2656–2659
 37. Sauer, K, Camper, AK (2001) Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J Bacteriol* 183: 6579–6589
 38. Schuster, M, Lostroh, CP, Ogi, T, Greenberg, EP (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* 185: 2066–2079
 39. Staskawicz, B, Dahlbreck, D, Keen, N, Napoli, C (1987) Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glyciniae*. *J Bacteriol* 169: 5789–5794
 40. Turner, JM, Messenger, AJ (1986) Occurrence, biochemistry and physiology of phenazine pigment production. *Adv Microb Physiol* 27: 211–275
 41. Ulrich, RL, Hines, HB, Parthasarathy, N, Jeddeloh, JA (2003) Mutational analysis and biochemical characterization of the *Burkholderia thailandensis* DW503 quorum-sensing network. *J Bacteriol* 186: 4350–4360
 42. Von Bodman, SB, Bauer, WD, Coplin, DL (2003) Quorum sensing in plant-pathogenic bacteria. *Annu Rev Phytopathol* 41: 455–482
 43. Wagner, VE, Bushnell, D, Passador, L, Brooks, AI, Iglewski, BH (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* 185: 2080–2095
 44. Watnick, PI, Kolter, R (1999) Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol Microbiol* 34: 586–595
 45. Whitehead, NA, Barnard, AM, Slater, H, Simpson, NJ, Salmond, GP (2001) Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* 25: 365–404
 46. Wood, DW, Pierson III, LS (1996) The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* 168: 49–53
 47. Wood, DW, Gong, F, Aykin, MM, Williams, P, Pierson III, LS (1997) N-Acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *J Bacteriol* 179: 7663–7670
 48. Yoshida, A, Ansay, T, Takehara, T, Kuramitsu, HK (2005) LuxS-based signaling affects *Streptococcus mutans* biofilm formation. *Appl Environ Microbiol* 71: 2372–2380
 49. Zhang, Z, Pierson III, LS (2001) A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Appl Environ Microbiol* 67: 4305–4315

APPENDIX B

Altering the Ratio of Phenazines in *Pseudomonas chlororaphis* (*aureofaciens*) strain

30-84: Effects on Biofilm Formation and Pathogen Inhibition

Copyright © 2007, the American Society for Microbiology. All rights reserved.

ASM Journals

[HOME](#) [HELP](#) [FEEDBACK](#) [SUBSCRIPTIONS](#) [SEARCH](#)

COPYRIGHT NOTICE AND LICENSE INFORMATION

The following Appendix B was published in the April 2008, vol. 190 of the Journal of Bacteriology. It was also published online ahead of print on Feb. 8, 2008 (doi:10.1128/JB.01587-07).

The article title is “Altering the ratio of phenazines in *Pseudomonas chlororaphis* 30-84: Effects on biofilm formation and pathogen inhibition.”

Journal Name: Journal of Bacteriology

Volume Number: 190 (8)

Page Numbers: 2759-2766.

Year: 2008

Individuals

An individual's access to an ASM Journal Online is subject to the following conditions:

1. The person using ASM Journals Online may browse, search, retrieve, and view an ASM Journal Online, and may store or print single copies of individual articles, or discrete items or portions of such articles, solely for the user's own use.

2. Unless specifically authorized by ASM in its Instructions to Authors or through other written consent, the person using ASM Journals Online may not: (i) distribute a copy (electronic or otherwise) of any article, file, or material from any ASM Journal Online; (ii) post a copy of any article, file, or material from any ASM Journal Online on a website (Internet or intranet); (iii) alter any article, file, or material from any ASM Journal Online; (iv) charge for a copy (electronic or otherwise) of any article, file, or material from any ASM Journal Online; or (v) systematically print, archive, or store entire issues or a significant number of articles from any issue.

3. Any use beyond that permitted here, including reuse of text, illustrations, tables, etc., in works intended for publication or presentation in any form, requires written permission from the publisher. Affirmative written permission must be obtained from ASM. ASM does not recognize or accede to permission requests that presume permission is granted unless ASM gives notice that permission is denied. Mail or fax (do not e-mail) signed requests to: Journals Dept., American Society for Microbiology, 1752 N Street, N.W., Washington, DC 20036-2904, USA; fax, (202) 942-9355.

4. Personal accounts and/or passwords may not be shared.

5. Unauthorized copying or prohibited use may result in revocation of access to ASM Journals Online and/or may expose the user to liability for copyright infringement, including injunctive relief and damages under U.S. and/or foreign copyright laws.
6. Any copies of material from ASM Journals Online must include the copyright notice shown on the initial page of each article or, if different, on the portion reproduced.
7. This License also applies to accepted manuscripts that are published online prior to publication of the final, typeset articles in an ASM Journal ("publish ahead of print" or PAP manuscripts). PAP manuscripts are posted "as is" (i.e., as submitted by the authors at the modification stage), and do not reflect editorial changes that might be made subsequently by ASM or the authors.
8. ASM does not own the copyrights in Supplemental Material that may be linked to, or accessed through, an article. ASM does have a license to grant its licensees access to such Supplemental Material. Please review copyright ownership information for Supplemental Material and contact the copyright owner for any permissions sought. This License does not authorize the copying, storing or transmission of any Supplemental Material.

Libraries/Institutions/Companies

All library/institution/company use is governed by the above principles, by section 108 of the Copyright Act, and by any applicable Site License.

DISCLAIMER OF WARRANTIES

These publications are provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or noninfringement. Descriptions of, or references to, products or publications do not imply endorsement of that product or publication.

In no event shall the American Society for Microbiology be liable for any special, incidental, indirect or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from these journals, whether or not advised of the possibility of damage, and on any theory of liability.

ASM Journals Online is under development, and changes may be made in the publications and programs at any time. All advertising appearing in the ASM Journals Online is the sole responsibility of the advertiser. ASM Journals Online does not endorse or warrant any product advertised or any statements made in any advertisements.

Note: If you are using one of the popular "offline browsers" that allow you to download content from a site and read it later, please be aware that we impose the following restriction on their use. For us to provide reliable, continuous, and timely access to ASM Journals Online for all readers, we ask that you configure your offline browser to not request more than one page per 60 seconds. Be aware that noncompliance with this rule will result in your access to ASM Journals Online being blocked until you contact us and resolve the problem.

Copyright © 2007, the American Society for Microbiology. All rights reserved.

Altering the Ratio of Phenazines in *Pseudomonas chlororaphis* (*aureofaciens*) strain

30-84: Effects on Biofilm Formation and Pathogen Inhibition

VSRK Maddula, EA Pierson, and LS Pierson III*

Department of Plant Sciences, Division of Plant Pathology & Microbiology,

The University of Arizona, Tucson, AZ 85721.

*Corresponding author:

Department of Plant Sciences.

Division of Plant Pathology & Microbiology.

The University of Arizona.

Tucson, AZ 85721.

Tel. 520-621-9419

Fax 520-621-7186

Lsp@u.arizona.edu

Abstract

Pseudomonas chlororaphis strain 30-84 is a plant beneficial bacterium able to control take-all disease of wheat caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (*Ggt*). The production of phenazines (PZs) by strain 30-84 is the primary mechanism of pathogen inhibition, and contributes to the persistence of strain 30-84 in the rhizosphere. PZ production is regulated in part by the PhzR/PhzI quorum sensing (QS) system. Previous flow cell analyses demonstrated that QS and PZs are involved in biofilm formation in *P. chlororaphis* (26). *P. chlororaphis* produces mainly two PZs, phenazine-1-carboxylic acid (PCA) and 2-hydroxy-phenazine carboxylic acid (2-OH-PCA). In the present study, we examined the effect of altering the ratio of PZs produced by *P. chlororaphis* on biofilm formation and pathogen inhibition. As part of this study, we generated derivatives of strain 30-84 that produced only PCA or over-produced 2-OH-PCA. Using flow cell assays, we found that these PZ-altered derivatives of strain 30-84 differed from the wild type in initial attachment, mature biofilm architecture, and dispersal from biofilms. For example, increased 2-OH-PCA production promoted initial attachment and altered the 3-dimensional structure of the mature biofilm relative to the wild type. Additionally, both alterations promoted thicker biofilm development and lowered dispersal rates compared to the wild type. The PZ-altered derivatives of strain 30-84 also differed in their ability to inhibit the fungal pathogen *Ggt*. Loss of 2-OH-PCA resulted in a significant reduction in the inhibition of *Ggt*. Our findings suggest that alterations in the ratios of antibiotic secondary metabolites synthesized by an organism may have complex and wide-ranging effects on its biology.

Keywords: *Pseudomonas*, phenazine, biofilm, biocontrol,

Introduction

Bacteria in nature exist within surface-attached communities termed biofilms, and interact cooperatively and competitively with other members of the microbial community (reviewed in 13, 14, 22, 33). Biofilm communities are ‘microniches’ that differ dramatically from surrounding conditions (33), confer resistance to deleterious agents such as antibiotics and detergents (15), and enable cells to coordinately carry out functions not typically undertaken outside of the biofilm (33). Bacteria associated with plants may form biofilms on or within plant tissues (reviewed in 13, 33, 41). The associations between plants and microorganisms encompass beneficial, neutral, detrimental, and pathogenic associations. The effects of deleterious or pathogenic microorganisms on plants can be ameliorated by the presence of bacteria that reduce the impact of the pathogen on the plant. Many of these beneficial bacteria (often termed biological control bacteria) produce secondary metabolites that interfere with the pathogen’s disease potential either by direct inhibition of pathogen growth, induction of plant defenses, or enhancement of plant growth (reviewed in 53). Secondary metabolites are chemical compounds historically defined as not being involved directly in ‘normal’ growth, development or reproduction. These compounds often are produced by the bacterium during the transition from exponential to stationary growth phase. Thus, plant beneficial bacteria and the secondary metabolites they produce represent key ecological control points for manipulating plant-microbe interactions that promote plant health.

Among fluorescent pseudomonads that serve as biological control agents, several secondary metabolites, including phenazines (PZs), pyrrolnitrin, pyoluteorin, and 2,4,

diacetyl-phloroglucinol, were identified as primary mechanisms of disease control. These secondary metabolites originally were classified as antibiotics due to their ability to inhibit plant pathogens on agricultural crops (reviewed in 40, 47, 52, 54). A general hypothesis regarding these antibiotic metabolites is that they serve a competitive function by inhibiting the growth of other microorganisms. More recently, regulatory pathways that control secondary metabolite production are being elucidated (reviewed in 11, 40, 51). These studies demonstrate that many bacteria utilize combinations of conserved sensory networks to control antibiotic production in response to environmental, nutritional, population, and metabolic inputs. We hypothesize that the complexity of antibiotic regulation reflects the complexity of their roles for the producing organism. In support of this hypothesis, a recent review provides evidence that secondary metabolites serve multiple roles in a number of taxonomically diverse organisms (38).

Pseudomonas chlororaphis (aureofaciens) strain 30-84 is a biological control bacterium effective against take-all disease of wheat caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (*Ggt*). Strain 30-84 produces several secondary metabolites, including PZs. PZ production is the primary mechanism of pathogen inhibition and contributes to persistence of strain 30-84 in the rhizosphere (10, 28, 35, 46). PZ biosynthesis is tightly regulated at multiple levels, including PhzR/PhzI quorum-sensing (QS) (37, 58), GacA/GacS two-component global regulation (9, 10), RpeA negative regulation (55) and RsmA post-transcriptional regulation (unpublished). Recently, we showed that PhzR/PhzI QS mutants of strain 30-84 were defective in biofilm formation (26). Interestingly, compared to the QS mutants, a structural mutant

defective only in PZ biosynthesis was equally impaired in biofilm formation (26). Genetic and biochemical complementation of the PZ mutant restored biofilm formation to wild type levels. These results clearly demonstrated a second important role for this antibiotic secondary metabolite, e.g. involvement in the formation of biofilms.

The PZ core biosynthetic genes are conserved among pseudomonads, but often differ in their terminal modifying enzymes (27). The PZ biosynthetic genes in strain 30-84 differ from other known pseudomonads by the presence of *phzO*, which encodes a monooxygenase (16, 27). This monooxygenase aids in conversion of the primary yellow PZ derivative phenazine-1-carboxylic acid (PCA) into the orange 2-hydroxy-phenazine-1-carboxylic acid (2-OH-PCA). This enzymatic reaction results in the partial conversion of PCA to 2-OH-PCA. Thus, *P. chlororaphis* 30-84 produces primarily the yellow PCA (~80-90%), and low amounts of the orange 2-OH-PCA (~10-20%). We hypothesize that the types of PZs produced and the ratios in which they are produced play important roles in the competitive survival and persistence of the producing organism.

In the present study, we examined the effect of altering the ratio of PZs produced by *P. chlororaphis* 30-84 on biofilm formation and pathogen inhibition. As part of this study, we generated derivatives of strain 30-84 that produced only PCA (strain 30-84PCA) or over-produced 2-OH-PCA (strain 30-84O*). Using flow cell assays, we showed that these PZ-altered derivatives of strain 30-84 differed from the wild type in initial attachment, mature biofilm architecture, and dispersal from biofilms. The PZ-altered derivatives of strain 30-84 also differed in their ability to inhibit the fungal pathogen *Ggt*. Our findings suggest that alterations in the ratios of antibiotic secondary

metabolites produced may have diverse and possibly unforeseen effects on the biology of the organism.

Materials and Methods:

Bacterial strains and medium. Bacterial strains and plasmids used in this study are described in Table 1. A spontaneous rifampicin-resistant derivative of *P. chlororaphis* strain 30-84 was used in all studies (35), and all mutants were derived from this parental strain. Triparental matings into strain 30-84 or its derivatives were performed as described previously (36). All strains of *P. chlororaphis* were grown at 28°C in LB medium supplemented with 5g of NaCl /liter or in AB minimal media supplemented with 2% Casaminoacids (AB+CAA) (58). *E. coli* strains were grown at 37°C in LB with supplements. KMPE medium (35) was used for fungal inhibition assays. Antibiotics were used where appropriate at the following concentrations for *E. coli*: ampicillin (Ap) at 100 µg/ml, gentamycin (Gm) at 25 µg/ml, kanamycin sulfate (Km) at 50 µg/ml, chloramphenicol (Cm) at 30 µg/ml and tetracycline (Tc) at 25 µg/ml; for *P. chlororaphis*: Gm at 50 µg/ml, Km at 50 µg/ml, Tc at 50 µg/ml, Cm at 300 µg/ml and rifampicin (Rif) at 100 µg/ml (58).

Construction of mutants. *P. chlororaphis* strain 30-84PCA was isolated following Tn5 mutagenesis of strain 30-84 as described previously (35). A Km^r, yellow derivative that only produced PCA was identified. To determine the location of the Tn5 insertion, chromosomal DNA from strain 30-84PCA was isolated and digested with *EcoRI*. The resulting fragments were cloned into pUC18 and Km^r transformants of *E. coli* strain

DH5 α were isolated. One transformant containing plasmid pKM-38 was selected for further analysis (Table 1). Since Tn5 contains a single *Bam*HI site, pKM-38 was digested with *Bam*HI and *Eco*RI, and the resulting two fragments cloned into *Bam*HI and *Eco*RI digested pPR510. The resulting white Cm^r transformants were further screened for Km resistance and sensitivity. The Km^r transformants contained pKM-510-38R, pPR510 carrying the fragment of Tn5 encoding Km^r and flanking chromosomal DNA. The Km^s transformants contained plasmid pKM-510-38S, pPR510 carrying the other fragment of Tn5 and flanking chromosomal DNA. The DNA sequence of the adjacent chromosomal DNA regions was determined using primers to the inverted repeat region of Tn5, as described previously (42). The sequences were assembled after removal of the Tn5 sequences and compared to the nucleotide database using NCBI BLAST (1).

Derivative 30-84O* was constructed by the introduction of additional copies of *phzO*, which encodes the monooxygenase responsible for the conversion of PCA into 2-OH-PCA in strain 30-84. A 5 kb *Pst*I fragment of the PZ biosynthetic operon containing *phzO* was cloned from pLSP18-6 into pLAFR3, resulting in the isolation of pKM-*phzO**. The cloned 5 kb *Pst*I fragment also contained part of *phzC* and *phzD*. These additional regions were included because a ~2.0 kb *Xba*I-*Pst*I fragment containing only *phzO* failed to convert PCA into 2-OH-PCA in our experiments (data not shown).

To control for the effects of the Tc^r carrying pLAFR3 vector pKM-*phzO** in strain 30-84O*, we introduced pLAFR3 (no insert) into strains 30-84, 30-84Z, and 30-84PCA. The strains containing pLAFR3 were used in all assays where strains were compared to 30-84O* with appropriate antibiotic selection. For confocal scanning laser

microscopy (CSLM), strain 30-84 and its derivatives also were fluorescently labeled by introduction of the plasmid pKT2CM-GFP, which contained *gfp* under the expression of the *tac* promoter (Table 1).

Phenazine quantification. PZs were extracted from strain 30-84 and derivatives as described previously (55). Briefly, cultures were grown overnight (16-18 h) in LB and AB-CAA to late exponential phase ($OD_{620} = 1.8$) and cell-free supernatants (CFS) prepared by centrifugation ($2,600 \times g$) for 15 min. Total PZs from CFS were extracted with an equal volume of acidified benzene and the benzene phase was separated and evaporated under air. Dried PZs were dissolved in 0.1N NaOH and quantified with UV-Visible spectroscopy using 0.1 N NaOH as the blank. The absorption maxima for PCA and 2-OH-PCA were measured at 367 nm and 484 nm, respectively. The relative amounts of PCA and 2-OH-PCA were calculated by multiplying their absorption maxima by their standard extinction coefficients (21, 34). PZs also were quantified from dispersing cells in flow cell effluents of 1 to 4-d old biofilms of each 30-84 derivative. Effluent samples (50 ml) were collected in acidified benzene and the PZs extracted. The relative amounts of each PZ were calculated and standardized to the number of cells in the effluent sample. We conducted three separate experiments, each over a 4-d period.

Flow cell assay. Single-pass flow cell assays were used to visually compare biofilm formation by strain 30-84 and derivatives as described previously (26) with slight modifications. Briefly, inoculum was prepared from exponential phase cultures grown in AB-CAA and washed twice with fresh AB-CAA. These cultures were further diluted ($OD_{620} = 0.4$) with AB-CAA. For each treatment, a 300 μ l aliquot of dilute culture was

inoculated into individual flow cell chambers. After inoculation, chambers were maintained under static conditions (no flow) for 0.5 h to allow cell attachment, and then continuous flow of fresh media (160 μ l/min) was initiated. Biofilms were visualized using an Olympus BX60 light microscope or Nikon E800 confocal laser scanning microscope (CLSM) at various magnifications. Images were taken at multiple time intervals up to 6 d after inoculation. At each time interval, 5 random images were taken for each treatment and representative images are presented. Image stacks taken with the Nikon E800 confocal laser scanning microscope were rendered to get 3-dimensional images using Volocity software (Improvision Ltd., Coventry, UK. 2006). To quantify differences among mutants in biofilm architecture, biofilm parameters were calculated using ISA-2 software (6, 7). Each experiment was repeated at least twice.

Initial attachment and dispersal rates. Strain 30-84 and derivatives with altered PZ ratios were analyzed for various stages of biofilm formation, initial attachment and dispersal rate. Initial attachment was measured by imaging biofilm establishment in the flow cell assay at early time points (45 min to 6 h) after flow initiation. Five random images were taken for each treatment using Olympus BX60 light microscope and the images analyzed using ISA- 2 software to calculate percent surface coverage. Experiments were repeated twice and representative results from one experiment are presented.

Dispersal rates from biofilms were determined by modifying the flow cell apparatus to allow collection of the effluent from each of the three flow chambers independently. Effluent samples (3 ml) were collected at 24 h intervals for 3 d. Collection

of samples after 3 d was problematic because maintaining the pressure in the flow cell apparatus during sampling became more difficult due to increasing back pressure from the effluent tank. Effluent counts were determined by serial dilution plating on LB with appropriate selection. Experiments were repeated three times and representative results from one experiment are presented.

Fungal Inhibition assay. Fungal inhibition by strain 30-84 and derivatives was determined by measuring their ability to inhibit mycelial growth of *Gaeumannomyces graminis tritici* (*Ggt*) in vitro as described previously (31). Briefly, a fresh 5 mm agar plug of *Ggt* was placed in the center of KMPE plates. After 24 h, 5 μ l spots of overnights of the bacterial cultures to be tested were placed at the plate peripheries. Plates were incubated 48 h and zones of inhibition were measured in mm. Each experiment was repeated twice with 3 replicates.

Statistical analysis. Biofilm volumetric parameters, dispersal rates, and zones of inhibition were analyzed statistically. Means were compared among treatments (by time interval, where appropriate) using Analysis of Variance (ANOVA, $p < 0.05$) and protected least significant difference (LSD) multiple comparison tests (SAS Version 8.2, SAS Institute, Inc., Cary, NC, USA).

Results:

Modifying phenazine production in 30-84: The PZ biosynthetic pathway is linear (Fig. 1A) and is under QS regulation (*phzR/phzI*) (36, 57). Eight genes (*phzXYFABCD O*) constitute the PZ operon of strain 30-84 (37), of which the terminal *phzO*

monooxygenase is specific to *P. chlororaphis* 30-84 (16). This monooxygenase adds a hydroxyl group at the second carbon position of PCA leading to formation of 2-OH-PCA. *P. chlororaphis* 30-84 synthesizes three PZ derivatives PCA (~80-90%), 2-OH-PCA (~10-20%), and a small amount of the PZ derivative 2-OH-PZ (~1-2%) formed by spontaneous decarboxylation of 2-OH-PCA (35). To produce a derivative of strain 30-84 that produces only PCA, we constructed the *phzO* mutant 30-84PCA using Tn5 mutagenesis. Our sequence data indicated that the Tn5 was inserted 1,160 bp from the start of the 1,473 bp *phzO* gene, terminating the PZ biosynthetic pathway at PCA production. Generation of a mutant that produced only 2-OH-PCA was not possible because 2-OH-PCA is derived from PCA. Instead we sought to enhance the conversion of PCA to 2-OH-PCA by the introduction of extra copies of *phzO* *in trans* in strain 30-84. This was achieved by cloning a 5-kb fragment of the PZ biosynthetic cluster containing *phzC*, *phzD* and *phzO* under the control of a *lac* promoter into pLAFR3 to make pKM-*phzO**. We refer to this plasmid-containing 30-84 derivative as 30-84O*.

PZ production in derivatives 30-84PCA and 30-84O* were characterized visually and quantitatively. Compared to strain 30-84, 30-84PCA is yellow whereas 30-84O* is red when grown to late exponential phase in liquid or solid LB or AB-CAA media (Fig 1B). Similar total amounts of PZ were produced by 30-84, 30-84PCA and 30-84O* (Table 2). However, strain 30-84PCA produces only PCA whereas strain 30-84O* produces 2.5 fold more 2-OH-PCA than wild type. As expected, since 2-OH-PCA is derived from PCA, this strain is reduced in the amount of PCA produced. 30-84O* makes only 70% and 50% of the amount of PCA produced by wild type and 30-84PCA,

respectively. The PZ ratios produced by wild type and the PZ-altered derivatives were consistent across media tested (LB, AB-CAA) (Table 2, and data not shown) and in the flow cell effluent of 1 to 4-d old biofilms. For example, the mean ratio of PZs produced by wild type in dispersing and LB grown cells (n = 14) was 86% PCA and 14% 2-OH-PCA ($\pm 5\%$). Dispersing and LB grown cells of 30-84PCA produced 98% PCA ($\pm 1\%$), whereas dispersing and LB grown cells of 30-84O* produced 60% PCA and 40% 2-OH-PCA ($\pm 6\%$).

Effect of altered PZ ratios on initial attachment. The early attachment phase of biofilm formation by strains 30-84, 30-84PCA and 30-84O* was analyzed in flow cell assays by light microscopic imaging (1000X) at early time points (Fig. 2). Five random images from each flow cell chamber were taken at 45 min. and 2, 4, and 6 h after flow initiation and the images were analyzed using ISA Software (6, 7) to calculate percent surface coverage (Table 3). Images from as early as 45 minutes after inoculation demonstrate that 30-84O* covers more surface area than either 30-84 or 30-84PCA, and this difference in initial attachment is still apparent after 6 h (Fig. 2.). Mean surface area coverage from two independent experiments for 30-84 and 30-84PCA was approximately 1% after 45 minutes, whereas it was already 44% for 30-84O* (Table 3). Interestingly, over the 6 h period, surface coverage of 30-84 and 30-84PCA increased from 1% to 41% and 49% respectively, as compared to a 44% to 59% increase for 30-84O*. The surface coverage by 30-84O* appeared to be more uniform than that of 30-84 and 30-84PCA, whereas the attached cells of 30-84 and 30-84PCA appeared to have a more aggregated, clustered distribution (Fig. 2).

PZ effects on biofilm architecture. Differences in mature biofilm architecture formed by strains 30-84, 30-84PCA and 30-84O* after 5 d were observed in flow cell assays using CLSM and light microscopy (Fig.3). These differences in 3-dimensional structure were apparent in all views (cross section, bird's eye, and basal; Fig. 3AB) and were quantified from the volumetric parameters (homogeneity, thickness, porosity, and bio-volume; Table 4). Even after 5 d, the initial colonization patterns (seen after 45 min to 6 h) persisted in the mature biofilms. For example, strains 30-84 and 30-84PCA did not uniformly cover the attachment surface (similar to the 6 h images, Fig. 2) and produced biofilms with mushroom-like clusters separated by open channels. Strain 30-84PCA produced a thicker, denser biofilm with significantly more cells than 30-84 or 30-84O* as determined from the thickness and bio-volume parameters, respectively (Table 4). The bio-volume and thickness of 30-84PCA biofilms were 4 and 3.5 times greater, respectively, than those of the wild type. In contrast, 30-84O* produced biofilms that more uniformly covered the attachment surface (similar to the 6 h images, Fig. 2) with comparatively less structure. Biofilms of 30-84O* were significantly more homogeneous than either the wild type or 30-84PCA (Table 4). 30-84O* also produced a thicker biofilm than 30-84 (2.7X), however it was similar in cell volume. The net result is that 30-84O* produced thicker, but more porous biofilms with less 3-dimensional structure than the wild type.

PZ effects on bacterial dispersal from biofilms. To determine whether the PZ-altered derivatives differed from wild type in dispersal, effluent samples were collected downstream of each flow cell and bacterial populations determined by serial dilution plating as

an indicator of dispersal rates. Dispersal rates for 30-84 were ~2.5X and ~12X higher than the dispersal rates for 30-84PCA and 30-84O*, respectively (Table 5). Dispersal from the biofilm increased very little from day 1 to day 2, but after day 2 dispersal rates for 30-84, 30-84PCA and 30-84O* increased 27X, 16X, and 13X, respectively.

Fungal inhibition assay. To determine whether the 30-84 derivatives differed in their ability to inhibit *Ggt*, we measured the zone of inhibition between the leading edge of the fungal mycelia and bacterial spots. 30-84 and 30-84O* produced similar zones of inhibition (Table 6). In contrast, 30-84PCA was significantly reduced in its ability to inhibit the fungus (4-fold smaller zones) compared to 30-84O* and the wild type. As expected, 30-84Z, which produces no PZs, did not inhibit fungal growth.

Discussion:

Altering the ratio of PZs produced by strain 30-84 alters biofilm attachment. Our data suggest that more efficient conversion of PCA to 2-OH-PCA results in better initial attachment. Biofilms of 30-84O* covered a greater percentage of the flow cell surface area than 30-84 or 30-84PCA; this difference was evident from 45 minutes after initiation of flow and persisted to 6 h when the majority of the surface was covered. Previous studies demonstrated that biofilm development occurs as a series of defined steps including: reversible attachment, irreversible attachment, microcolony formation, maturation into 3-dimensional mushroom-like structures with open channels, and dispersal (22, 44). It is believed that reversible attachment takes place within a short time (e.g. seconds to minutes). In this study, the earliest time point we were able to reliably

measure for all three treatments was 45 minutes after flow initiation, the period during which attachment becomes irreversible. Our data suggest that increasing 2-OH-PCA aids in irreversible attachment.

Several factors have been shown to play roles in the initial stages of biofilm formation in different bacterial systems. Factors shown to be important include: flagella, type IV pili, cup fimbriae and *sad* genes that affect cell surface structures and properties in *P. aeruginosa* (23, 29); production of adhesins such as LapA, Lap D and regulatory domains such as GGDEF and EAL in plant associated *P. fluorescens* and *P. putida* (reviewed in 13, 20, 23); and production of the adhesins rhicadhesin and Raps in members of *Rhizobiaceae* (13). Alternatively, Dietrich et al. (17) demonstrated that addition of high levels of the PZ pyocyanin to a pyocyanin-defective mutant of *P. aeruginosa* strain PAO1 induced global changes in gene expression patterns, suggesting that pyocyanin may be serving as a regulatory signal. Although the exact mechanism by which 2-OH-PCA aids in attachment is unknown, it is possible that 2-OH-PCA production acts directly as an adhesion, or that it serves as a signal inducing the expression of additional genes that are involved in initial attachment, including changes in bacterial cell surface structures or properties. It would be interesting to see if the increase in 2-OH-PCA induces the production of adhesins like LapA, LapD and Raps in *P. chlororaphis* 30-84.

Altering the ratio of PZs produced by strain 30-84 alters mature biofilm architecture and dispersal. Changing the ratios of PZs also produced significant changes in mature biofilm architecture. In our flow cell analysis of 5-d old mature biofilms, we found that

strain 30-84 forms a biofilm approximately 19 μm in thickness with mushroom-shaped clusters of cells. Production of only PCA resulted in substantially thicker mushroom-shaped clusters (66 μm) with a 4-fold greater cell density than the wild type. More efficient conversion of 2-OH-PCA resulted in biofilms that were thicker than wild type (52 μm) with a similar cell density. These biofilms did not appear to produce the distinctive mushroom-like clusters, but were more homogenous and porous than wild type biofilms. It is interesting that changing the ratio of PZs normally produced by *P. chlororaphis* results in thicker biofilms. However production of only PCA by strain 30-84PCA resulted in biofilms with a greater cell density, despite its having less efficient initial attachment than 30-84O*.

Differences in these architectural parameters may reflect differences in reproduction, mortality, or dispersal from the biofilm. In the present study, we looked at cell dispersal as the biofilms mature. Dispersal rates of viable cells from the maturing biofilm were generally highest for 30-84, intermediate for 30-84PCA, and lowest for 30-84O*. The higher rate of dispersal by 30-84 may explain its production of thinner biofilms as compared to 30-84PCA or 30-84O*. However, it is unclear why 30-84O*, which has the lowest dispersal rate, does not produce the thickest biofilm. Clearly dispersal rate alone does not explain biofilm thickness.

The molecular mechanism for dispersal of sessile biofilm bacterial cells to free living planktonic cells is not well understood. Various factors or processes causing dispersal have been reported. These include: sudden changes in nutrient availability (23); chelators like EDTA (4); biosurfactant production such as rhamnolipids in *P. aeruginosa*

(8); the chemotaxis regulator 'BdlA' in *P. aeruginosa* (32); oxygen availability and the presence of nitric oxide (NO) in *P. aeruginosa* (5). In our current study, altered PZ ratios resulted in decreased dispersal rates in both 30-84PCA and 30-84O* derivatives. Because PZs are known redox compounds, one possibility is that altering the PZ ratio in 30-84PCA and 30-84O* reduces nitrosative stress. If this is the case, less production of reactive nitrogen intermediates such as NO might reduce bacterial dispersal (5). Further studies are needed to determine whether these two PZ derivatives affect nitrosative stress or other known mechanisms.

Altering the ratio of PZs produced by strain 30-84 affects fungal inhibition. In the present study, we showed that loss of 2-OH-PCA production results in a significant reduction in the inhibition of the fungal pathogen *Ggt*. Similar results were reported by Toohey et al. (49) who demonstrated that 2-OH-PCA has greater antibiotic activity than PCA against an array of bacterial and fungal organisms. Interestingly, in our assay increased conversion of PCA to 2-OH-PCA by 30-84O* did not improve in *Ggt* inhibition significantly, compared to the wild type. This may indicate that the wild type already produces an optimum amount of 2-OH-PCA for fungal inhibition, or alternatively, the 2.5 fold increase is not sufficient to produce a measurable increase in fungal inhibition.

Ecological perspectives. PZs belong to a large class of well-known heterocyclic secondary metabolites produced by a variety of bacteria (11, 19, 25, 27, 50). Over 100 natural derivatives of PZs have been described with effects on a large variety of micro- and macro-organisms (25, 50). PZs effectively control a wide range of plant pathogenic

fungi and are a well-characterized mechanism of bacterial plant disease control (2, 11, 40, 51). The PZ pyocyanin also is known to be an important virulence factor in infections caused by the opportunistic human pathogen, *P. aeruginosa* (reviewed in 38).

Although, as suggested recently by Laursen and Nielsen (25), “little is yet known about the physiological function of PZs in their natural environment”, the complexity of the roles of secondary metabolites including PZs in the ecology and life-style of the organism is beginning to be recognized (17, 25, and reviewed in 38). Similar to strain 30-84, the production of PZs in other PZ-producing bacteria appears to be controlled by complex sensory networks that may include one or more QS systems, two-component regulation, post transcriptional regulation, and other mechanisms (11, 24, 40, 51). These observations provide support for the hypothesis that the complexity of regulation of secondary metabolites reflects the complexity of their roles. This hypothesis is consistent with the evolutionary theory that metabolically costly antibiotics are subject to natural selection and are more likely to be maintained if they serve multiple functions (56).

In agriculture, antibiotic production by plant-associated microorganisms represents an environmentally compatible method of disease control. Commonly proposed approaches for improving microbial disease control include increasing secondary metabolite production, altering the structure of antibiotics produced, or introducing antibiotic pathways into other bacteria (3, 12, 48). However, our findings suggest that alterations in the ratios of antibiotic secondary metabolites produced may have wide-ranging effects on the biology of the organism. Therefore, central to intelligent manipulation of these complex interactions to improve plant health is to move beyond the

conception that antibiotics function only in limiting target pathogens, and to focus on the many roles antibiotics play for the producing organism and their relative importance in microbe-microbe and microbe-host interactions.

Acknowledgments:

We thank Dr. Carl Boswell for invaluable assistance with CSLM. We also thank Anne Estes and Gerardo Puopolo for helpful comments and suggestions. This work was supported by funding from the Office of the Vice President, The University of Arizona, to LS Pierson III.

References:

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389-3402.
2. **Anjaiah, V., N. Koedam, B. Nowak-Thompson, J. E. Loper, M. Höfte, J. T. Tambong, and P. Cornelis.** 1998. Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 derivatives toward *Fusarium* spp. and *Pythium* spp. *Mol. Plant-Microbe Interact.* **11**:847-854.
3. **Bakker, P. A., D. C. Glandorf, M. Viebahn, T. W. Ouwens, E. Smit, P. Leeftang, K. Wernars, L. S. Thomashow, J. E. Thomas-Oates, and L. C. van Loon.** 2002. Effects of *Pseudomonas putida* modified to produce phenazine-1-carboxylic acid and 2, 4-diacetylphloroglucinol on the microflora of field grown wheat. *Antonie Van Leeuwenhoek.* **81**:617-624.
4. **Banin, E., K. M. Brady, and E. P. Greenberg.** 2005. Chelator-Induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. *Appl. Environ. Microbiol.* **72**: 2064–2069.
5. **Barraud, N., D. J. Hassett, S. H. Hwang, S. A. Rice, S. Kjelleberg, and J. S. Webb.** 2006. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J. Bacteriol.* **188**:7344-7353.
6. **Beyenal, H., C. Donovan, Z. Lewandowski, and G. Harkin.** 2004. Three-dimensional biofilm structure quantification. *J. Microbiol. Methods* **59**:395-413.

7. **Beyenal, H., Z. Lewandowski, and G. Harkin.** 2004. Quantifying biofilm structure: facts and fiction. *Biofouling* **20**:1-23.
8. **Boles, B. R., M. Thoendel, and P. K. Singh.** 2005. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Mol. Microbiol.* **57**:1210-1223.
9. **Chancey, S. T., D. W. Wood, and L. S. Pierson III.** 1999. Two-component transcriptional regulation of *N*-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Appl. Environ. Microbiol.* **65**:2294-2299.
10. **Chancey, S. T., D. W. Wood, E. A. Pierson, and L. S. Pierson III.** 2002. Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *Appl. Environ. Microbiol.* **68**:3308-3314.
11. **Chin-A-Woeng, T. F. C., G. V. Bloemberg, and B. J. J. Lugtenberg.** 2003. Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytologist* **157**:503-523.
12. **Chin-A-Woeng, T. F. C., J. E. Thomas-Oates, B. J. J. Lugtenberg, and G. V. Bloemberg.** 2001. Introduction of the *phzH* gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. *Mol. Plant-Microbe Interact.* **14**:1006-1015.
13. **Danhorn, T., and C. Fuqua.** 2007. Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* **61**:401-422.

14. **Davey, M. E., and G. A. O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* **64**:847-867.
15. **Davies, D.** 2003. Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Disc.* **2**:114-122.
16. **Delaney, S. M., D. V. Mavrodi, R. F. Bonsall, and L. S. Thomashow.** 2001. *phzO*, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30-84. *J. Bacteriol.* **183**:318-327.
17. **Dietrich, L. E., A. Price-Whelan, A. Petersen, M. Whiteley, and D. K. Newman.** 2006. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **61**:1308-1321.
18. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. *Gene* **52**:147-154.
19. **Giddens, S. R., Y. Feng, and H. K. Mahanty.** 2002. Characterization of a novel phenazine antibiotic gene cluster in *Erwinia herbicola* Eh1087. *Molec. Microbiol.* **45**:769-783.
20. **Hinsa, S. M., and G. A. O'Toole.** 2006. Biofilm formation by *Pseudomonas fluorescens* WCS365: a role for LapD. *Microbiol.* **152**: 1375-1383.
21. **Jayatilake, G. S., M. P. Thornton, A. C. Leonard, J. E. Grimwade, and B. J. Baker.** 1996. Metabolites from an antarctic sponge-associated bacterium, *Pseudomonas aeruginosa*. *J. Nat. Prod.* **59**:293-296.

22. **Kierrek-Pearson, K., and E. Karatan.** 2005. Biofilm development in bacteria. *Adv. Appl. Microbiol.* **57**: 79–111.
23. **Klausen, M., M. Gjermansen, J. U. Kreft, and T. Tolker-Nielsen.** 2006. Dynamics of development and dispersal in sessile microbial communities: examples from *Pseudomonas aeruginosa* and *Pseudomonas putida* model biofilms. *FEMS Microbiol. Lett.* **261**:1-11.
24. **Lapouge, K., M. Schubert, F. H. Allain, and D. Haas.** 2007. Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol. Microbiol.* **67**:241-253.
25. **Laursen, J. B., and J. Nielsen.** 2004. Phenazine natural products: biosynthesis, synthetic analogues, and biological activity. *Chem. Rev.* **104**:1663-1686.
26. **Maddula, V. S. R. K., Z. Zhang, E. A. Pierson, and L. S. Pierson III.** 2006. Quorum sensing and phenazines are involved in biofilm formation by *Pseudomonas chlororaphis (aureofaciens)* strain 30-84. *Microb. Ecol.* **52**:289-301.
27. **Mavrodi, D. V., W. Blankenfeldt, and L. S. Thomashow.** 2006. Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. *Annu. Rev. Phytopathol.* **44**:417-445.
28. **Mazzola, M., R. J. Cook, L. S. Thomashow, D. M. Weller, and L. S. Pierson III.** 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. *Appl. Environ. Microbiol.* **58**:2616-2624.

29. **Merritt, J.H., K. M. Brothers, S. L. Kuchma, and G. A. O'Toole.** 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *J. Bacteriol.* **189**:8154-8164.
30. **Miller, W.G., J. H. J. Leveau, and S. E. Lindow.** 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Mol. Plant-Microbe Interact.* **13**:1243-1250.
31. **Morello, J. E., E. A. Pierson, and L. S. Pierson III.** 2004. Negative cross-communication among wheat rhizosphere bacteria: effect on antibiotic production by the biological control bacterium *Pseudomonas aureofaciens* 30-84. *Appl. Environ. Microbiol.* **70**:3103-3109.
32. **Morgan, R., S. Kohn, S. Hwang, D. J. Hassett, and K. Sauer.** 2006. BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. *J. Bacteriol.* **188**:7335-7343.
33. **Morris, C. E., and J. M. Monier.** 2003. The ecological significance of biofilm formation by plant-associated bacteria. *Annu. Rev. Phytopathol.* **41**:429-453.
34. **Olson, E., and J. Richards.** 1967. Structure of the orange pigment from *Pseudomonas aureofaciens*. *J. Org. Chem.* **32**:2887-2890.
35. **Pierson, L. S., III, and L. S. Thomashow.** 1992. Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. *Mol. Plant Microbe Interact.* **5**:330-339.

36. **Pierson, L. S., III, V. D. Keppenne, and D. W. Wood.** 1994. Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *J. Bacteriol.* **176**:3966-3974.
37. **Pierson, L. S., III, T. Gaffney, S. Lam, and F. Gong.** 1995. Molecular analysis of genes encoding phenazine biosynthesis in the biological control bacterium *Pseudomonas aureofaciens* 30-84. *FEMS Microbiol. Lett.* **134**:299-307.
38. **Price-Whelan, A., L. E. Dietrich, and D. K. Newman.** 2006. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat. Chem. Biol.* **2**:71-78.
39. **Quigley, N. B., and P. R. Reves.** 1987. Chloramphenicol resistance cloning vector based on pUC9. *Plasmid* **17**:54-57.
40. **Raaijmakers, J. M., M. Vlami, and J. T. de Souza.** 2002. Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* **81**:537-547.
41. **Ramey, B. E., M. Koutsoudis, S. B. von Bodman, and C. Fuqua.** 2004. Biofilm formation in plant-microbe associations. *Curr. Opin. Microbiol.* **7**: 602-609.
42. **Rich, J. J., and D. K. Willis.** 1990. A single oligonucleotide can be used to rapidly isolate DNA sequences flanking a transposon Tn5 insertion by the polymerase chain reaction. *Nucleic Acids Res.* **18**:6673-6676.
43. **Sambrook, J., E. F. Fritsch, and T. Maaniatis.** 1989. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
44. **Sauer, K., A. H. Rickard, and D. G. Davies.** 2007. Biofilms and Biocomplexity. *Microbe* **2**:347-353.

45. **Staskawicz, B. J., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J. Bacteriol.* **169**:5789-5794.
46. **Thomashow, L. S., and D. M. Weller.** 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. *J. Bacteriol.* **170**:3499-3508.
47. **Thomashow, L. S.** 1996. Biological control of plant root pathogens. *Curr. Opin. Biotechnol.* **3**:343-347.
48. **Timms-Wilson, T. M., R. J. Ellis, A. Renwick, D. J. Rhodes, D. V. Mavrodi, D. M. Weller, L. S. Thomashow, and M. J. Bailey.** 2000. Chromosomal insertion of phenazine-1-carboxylic acid biosynthetic pathway enhances efficacy of damping-off disease control by *Pseudomonas fluorescens*. *Mol. Plant-Microbe Interact.* **13**:1293-1300.
49. **Toohey, J. I., C. D. Nelson, and G. Krotkov.** 1965. Toxicity of phenazine carboxylic acid to some bacteria, algae, higher plants, and animals. *Can. J. Bot.* **43**:1151-1155.
50. **Turner, J. M., and A. J. Messenger.** 1986. Occurrence, biochemistry and physiology of phenazine pigment production. *Adv. Microb. Physiol.* **27**:211-275.
51. **Walsh, U. F., J. P. Morrissey, and F. O'Gara.** 2001. *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Curr. Opin. Biotechnol.* **12**:289-295.

52. **Weller, D. M.** 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* **26**:379-407.
53. **Weller, D. M.** 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathol.* **97**:250-256.
54. **Whipps, J. M.** 1997. Developments in the biological control of soilborne plant pathogens. *Adv. Bot. Res.* **26**:1-133.
55. **Whistler, C. A., and L. S. Pierson III.** 2003. Repression of phenazine antibiotic production in *Pseudomonas aureofaciens* strain 30-84 by RpeA. *J. Bacteriol.* **185**:3718-3725.
56. **Wink, M.** 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* **64**:3-19.
57. **Wood, D. W., and L. S. Pierson III.** 1996. The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**:49-53.
58. **Zhang, Z., and L. S. Pierson III.** 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Appl. Environ. Microbiol.* **67**:4305-4315.

Figure Legends:

Figure 1. (A) Simplified PZ biosynthetic pathway. PZs are derived from chorismate precursor of which PCA is the primary phenazine compound synthesized. Subsequently PCA is hydroxylated by *phzO*, a monooxygenase. **(B) Liquid cultures of the PZ derivative mutants compared to 30-84WT.** Cultures were grown in AB-CAA with selection in 24-well plates. Images of strain 30-84, 30-84O*; and 30-84PCA after 24 h are given.

Figure 2. Effect of altered PZ ratio on the initial attachment stage of biofilm formation. The early attachment phase of biofilm formation by strains 30-84, 30-84PCA and 30-84O* was observed and analyzed in a flow cell assay by taking light microscope images at early time points. Five random images for each treatment and time point combination were taken. Each experiment was repeated twice and representative data are presented. **(A) Representative images taken 45 min and (B) 6 h after the start of media flow.** All the images were obtained at 1000X magnification using Olympus BX60 light microscope.

Figure 3. Effect of altered phenazine ratio on biofilm architecture. Mature biofilm architecture was observed and analyzed in a flow cell assay using fluorescently labeled derivatives of strains 30-84, 30-84PCA and 30-84O*. **(A) Bird's-eye and (B) Cross-sectional 3D images obtained from CLSM of 5-d old mature biofilms of 30-84WT, 30-84PCA and 30-84O*.** Random Z-series image stacks were obtained using Nikon

E800 confocal laser scanning microscope at 400X magnification and rendered to get 3-dimensional images using Volocity software. **(C) Basal view of the 5-d old mature biofilms of 30-84WT, 30-84PCA and 30-84O***. Images are taken using an Olympus BX60 light microscope at 100X magnification.

Figures:

Figure 1. Maddula et al.

Figure 1A.

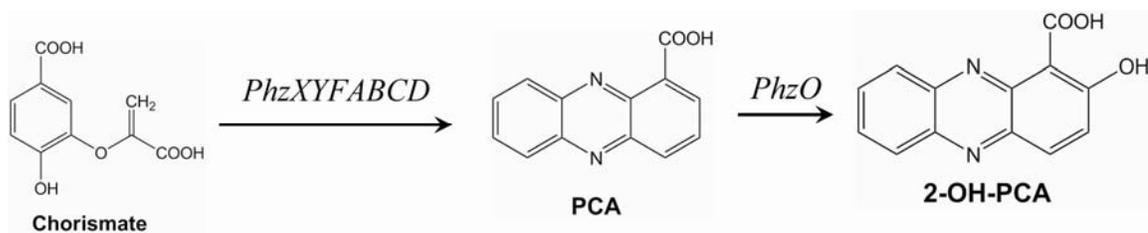


Figure 1B.

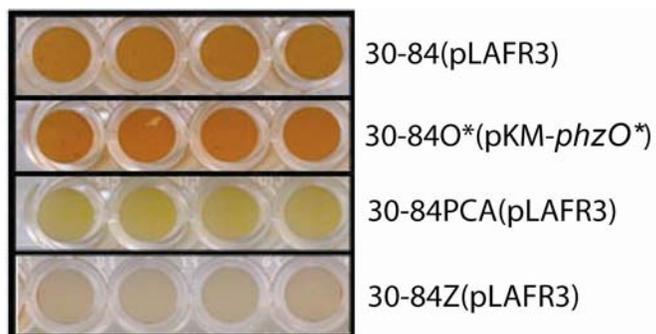


Figure 2: Maddula et al.

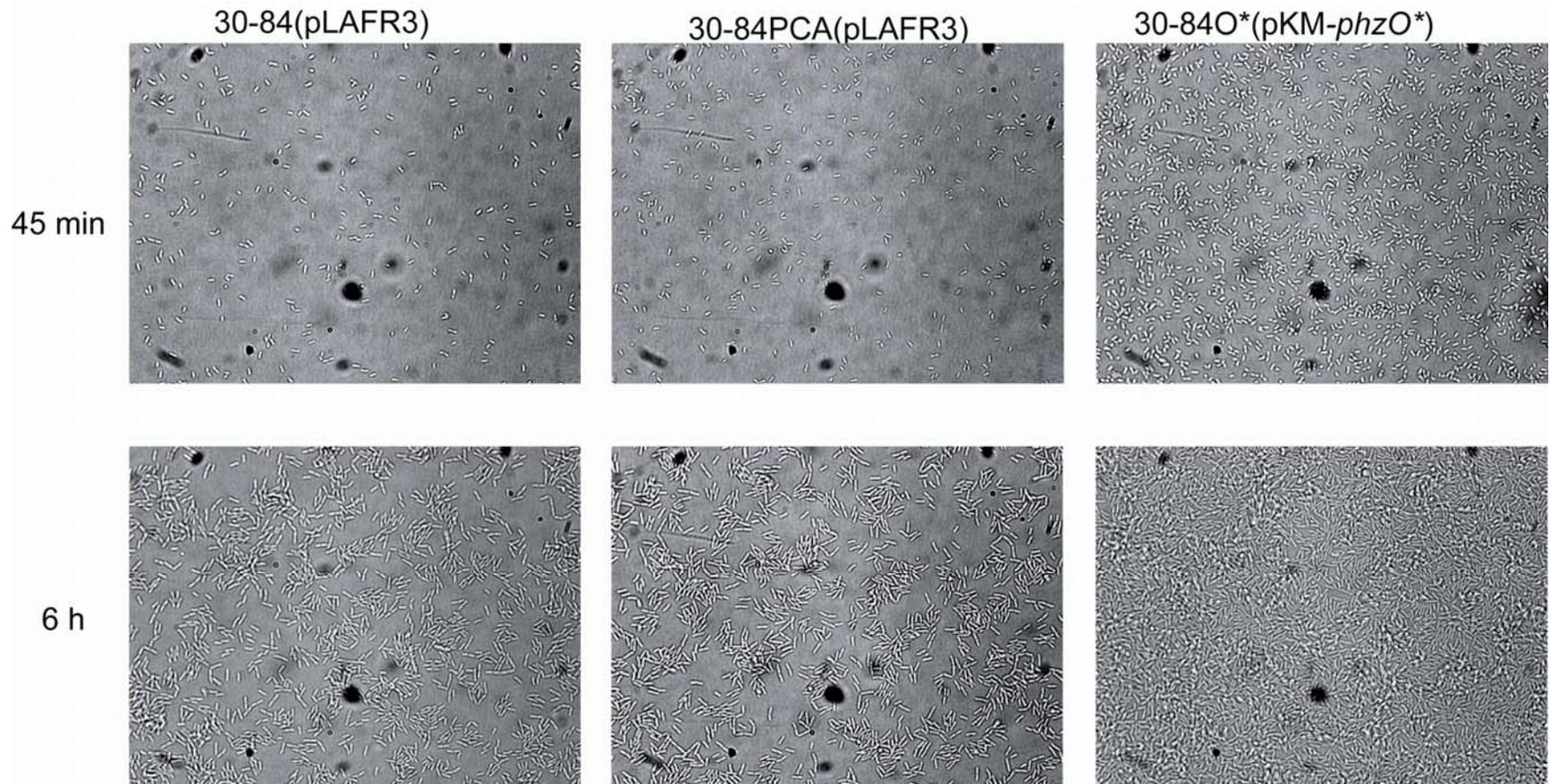


Figure 3. Maddula et al.

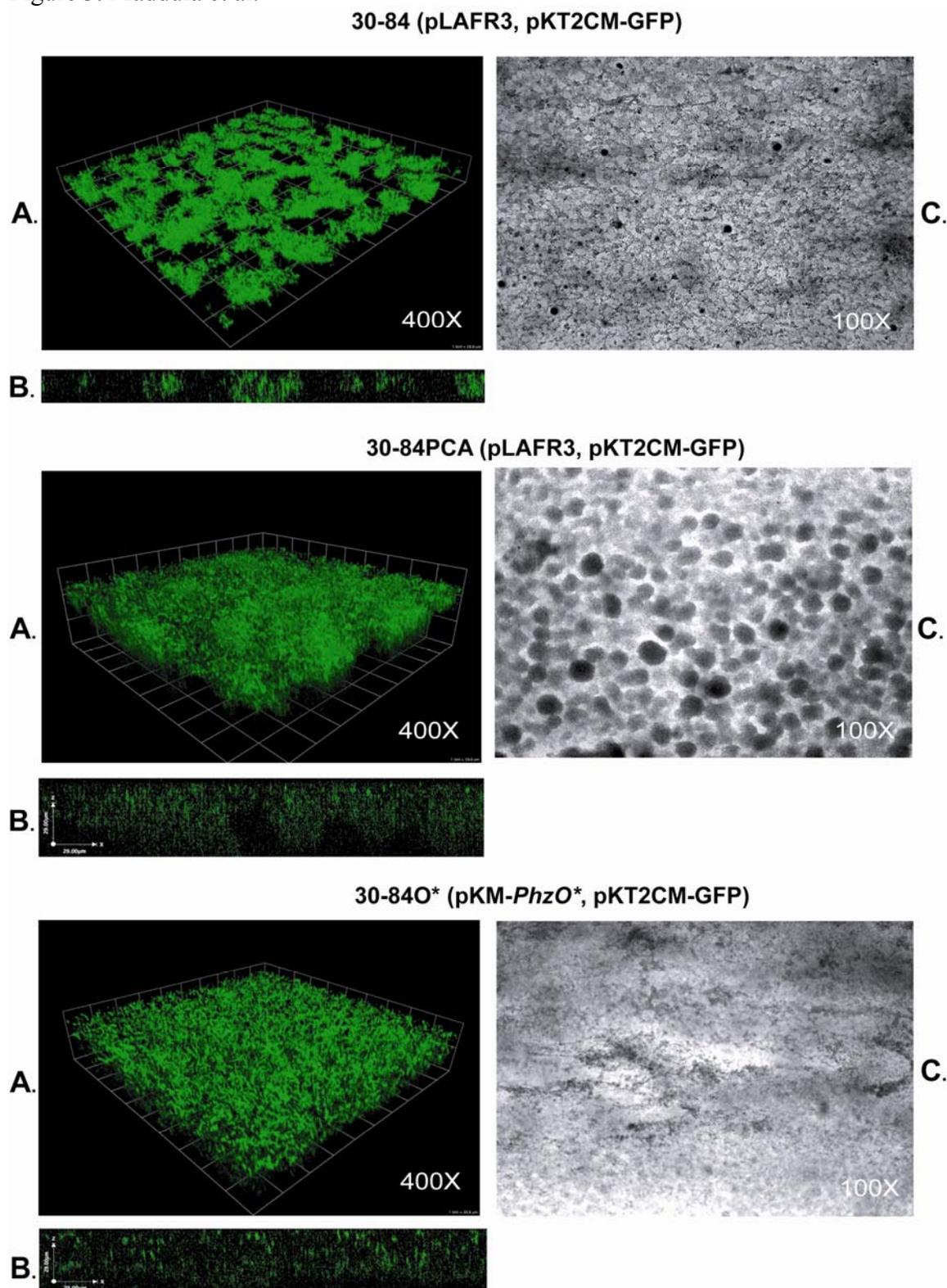


Table 1. **Bacterial strains and plasmids used in this study**

Strain or plasmid	Description ^a	Reference or source
<i>Pseudomonas chlororaphis</i> strains.		
30-84	Wild type, Rif ^r	35
30-84PCA	PCA ⁺ , 2-OH-PCA ⁻ , Rif ^r Km ^r , <i>phzO</i> ::Tn5	This study
30-84O*	30-84 containing plasmid pKM- <i>phzO</i> *	This study
30-84Z	Phz ⁻ Rif ^r <i>phzB</i> :: <i>lacZ</i> genomic fusion	36
<i>E. coli</i> strains		
DH5 α	F ⁻ <i>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1</i> Δ (<i>argF-lacZYA</i>)1169 Φ 80 <i>lacZ</i> Δ M15 λ	GIBCO-BRL
HB101	F ⁻ <i>hsdS20</i> (r _B ⁻ m _B ⁻) <i>supE44 recA1 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-5</i> λ	GIBCO-BRL
Plasmids		
pLAFR3	IncP1 <i>cos</i> ⁺ <i>rlx</i> ⁺ Tc ^r	45
pLSP 18-6	pLAFR3 carrying a 9.2 kb <i>EcoR</i> I fragment of 30-84 chromosome with <i>phzFABCD</i> and <i>phzO</i>	35
pKM- <i>phzO</i> *	pLAFR3 carrying a 5.0 kb <i>Pst</i> I fragment of pLSP 18-6 with <i>phzCD</i> and <i>phzO</i>	This study
pUC18	ColE1 Ap ^r	43

pPR510	ColE1 Cm ^r	39
pKM-38	pUC18 containing the 9.2 kb chromosomal <i>EcoRI</i> fragment carrying Tn5 within <i>phzO</i> . Km ^r	This study
pKM-510-38R	pPR510 containing <i>EcoRI-BamHI</i> fragment of pKM-38, Km ^r	This study
pKM-510-38S	pPR510 containing <i>BamHI-EcoRI</i> fragment of pKM-38, Km ^s	This study
pPROBE-KT'	pVS1 replicon, p15a origin of replication, <i>gfp</i> transcriptional fusion, Km ^r	30
pHP24Ω-Cm	Contains <i>cat</i> , Cm ^r	18
pMAL-p2X	Commercial vector containing the <i>tac</i> promoter <i>Ptac</i>	New England Biolabs
pLAF 13-9	pLAFR3 containing a 3.5 kb <i>BamHI-HindIII</i> fragment with a promoterless <i>lacZ</i> gene.	LS Pierson, unpublished
pKT2CM-GFP	pPROBE-KT' containing the 4.3 <i>EcoRI</i> fragment carrying <i>cat</i> , a 150 bp <i>EcoRI-BamHI</i> PCR amplicon carrying <i>Ptac</i> , and the 3.5 kb <i>BamHI-HindIII</i> fragment carrying a promoterless <i>lacZ</i> gene. Km ^r Cm ^r <i>lac</i> ⁺ <i>gfp</i> ⁺	EA Pierson, unpublished

^a Ap^r, Cm^r, Km^r, Rif^r, and Tc^r denote resistance to ampicillin, chloramphenicol, kanamycin, rifampicin, and tetracycline, respectively, and Km^s denotes sensitivity to kanamycin.

Table. 2: Production of PCA and 2-OH-PCA by strain 30-84 and altered PZ derivatives:

PZs were quantified using the UV-Vis spectral peaks for PCA and 2-OH-PCA (367nm and 484nm, respectively) multiplied by their respective extinction coefficients. Extractions were repeated 3 times and representative data ($\mu\text{g/ml}$) from one extraction are shown below. Since total amounts of PZs differed between extractions, data for each extraction were standardized by calculating the ratio of PCA, 2-OH-PCA or total PZs produced by each derivative relative to the wild type. Mean ratio data are given parenthetically.

Strain	PCA $\mu\text{g/ml}$	2-OH-PCA $\mu\text{g/ml}$	Total PZs $\mu\text{g/ml}$
30-84 (pLAFR3)	28.5 (1.0)	6.7 (1.0)	35.0 (1.0)
30-84PCA (pLAFR3)	34.1 (1.4)	0.0 (0.1)	34.1 (1.1)
30-84O* (pKM- <i>phzO</i> *)	18.4 (0.7)	14.6 (2.5)	33.0 (1.0)
30-84Z (pLAFR3)	0 (0)	0 (0)	0 (0)

Table. 3. **Percentage of the slide surface area covered by the 30-84 derivatives at early time points.** Surface coverage area is calculated from images taken of the flow cell chambers at early time points after inoculation. Images were taken randomly with a bright field microscope. Images were analyzed for areal porosity (P) using ISA-2D software and percent surface coverage (1-P) is calculated from the porosity measurement. Data are the mean surface area coverage and standard deviation based on five images. Experiments were repeated twice and representative values from one experiment are shown.

Percent surface area coverage at different time points	30-84 (pLAFR3)	30-84PCA (pLAFR3)	30-84O* (pKM- <i>phzO</i> *)
At 45min	1 % ± 0.01	1 % ± 0.01	44 % ± 0.01
At 2h	2 % ± 0.02	1 % ± 0.01	54 % ± 0.02
At 4h	34 % ± 0.02	39 % ± 0.04	56 % ± 0.07
At 6h	41 % ± 0.02	49 % ± 0.04	59 % ± 0.07

Table 4: **Biofilm volumetric parameters.** Mature biofilm (5 d) image stacks were taken using confocal scanning laser microscope (CSLM). Biofilm volumetric parameters were calculated from the image stacks using ISA-3D software. Confocal images were taken from two experiments with minimum 3 image stacks for each treatment. Average values of three image stacks from one representative experiment are shown below. Differences in thickness and bio-volume between treatments are given parenthetically as fold difference compared to wild type.

Parameter	30-84 (pLAFR3)	30-84PCA (pLAFR3)	30-84O* (pKM- <i>phzO</i> *)
Homogeneity (%)	79 ^b	76 ^b	83 ^a
Thickness (μm)	19 ^c	66 ^a	52 ^b
Porosity (%)	91 ^b	86 ^b	96 ^a
Bio-volume (μm ³)	2.0 x 10 ^{6b} (1X)	8.8 x 10 ^{6a} (4X)	2.0 x 10 ^{6b} (1X)

For each parameter, treatment values with different superscript letters are significantly different.

Table 5: **Dispersal rates by 30-84, 30-84PCA and 30-84O* from biofilms.** Samples of effluent from individual flow cell chambers were collected 1, 2, and 3 d after the initiation of flow, serially diluted, and plated on solid media. Data are pooled from three replicate flow chamber experiments and expressed as mean cfu/ml $\times 10^6$. In each experiment, three replicate plates were counted for each sample.

Time interval	30-84 (pLAFR3)	30-84PCA (pLAFR3)	30-84O* (pKM- <i>phzO</i> *)
1d	1.74 ^a	0.62 ^b	0.14 ^c
2d	1.40 ^a	1.15 ^a	0.27 ^c
3d	46.53 ^a	18.63 ^b	3.60 ^c

For each time interval, treatment values with different superscript letters are significantly different.

Table 6: **Fungal inhibition assay.** Fungal inhibition by 30-84, 30-84PCA and 30-84O* was expressed as distance in mm from the leading edge of *Ggt* mycelia to the bacterial spot. Experiments were repeated twice with 3 replicates and data presented are pooled from both experiments.

Strain	Zone of inhibition (mm)
30-84 (pLAFR3)	8.08 ^a
30-84PCA (pLAFR3)	1.82 ^b
30-84O* (pKM- <i>phzO</i> *)	8.08 ^a
30-84Z (pLAFR3)	0.00 ^c

Treatment values with different superscript letters are significantly different.

APPENDIX C

Towards Understanding the Mechanisms by which Phenazines Contribute to Biofilm Formation

Introduction.

PZs belong to a large class of well-known heterocyclic nitrogen containing secondary metabolites produced by a variety of bacteria (Laursen and Nielson, 2004). PZs have been studied as antibiotics and virulence factors for many years. It has been noted in recent reviews that the complexity of the roles of secondary metabolites, including PZs, in the ecology and life-style of the organism are just beginning to be recognized (Dietrich et al., 2006; Laursen and Nielsen, 2004; and Price-Whelan et al., 2006).

In our previous experiments, we showed that PhzR/PhzI QS mutants of strain 30-84 were defective in biofilm formation (Maddula et al., 2006). Interestingly, compared to the QS mutants, a structural mutant defective only in PZ biosynthesis was equally impaired in biofilm formation (Maddula et al., 2006). Genetic and biochemical complementation of the PZ mutant restored biofilm formation to wild type levels. These results clearly demonstrated a second important role for this antibiotic secondary metabolite, e.g. involvement in the formation of biofilms. However, the mechanism(s) by which PZs affect biofilm development is unknown.

Several lines of evidence suggest that PZs may alter cell surface properties or may serve as signals that regulate other genes involved in biofilm formation. For example, the

outer membrane of Gram-negative bacteria has lipopolysaccharide (LPS) as the major component. In *P. aeruginosa*, the outer membrane LPS may contain 10 or more phosphate residues for each molecule (Rivera et al., 1988). The presence of these phosphates gives a net negative charge to the bacterial cell surfaces (Hancock, 1984). As a result, they may need to overcome repulsive forces on negatively charged surfaces in order to attach and establish as biofilms. We hypothesize that PZs may alter cell surface properties (eg. hydrophobicity) and may help bacteria to attach to surfaces when present.

Recently, Dietrich et al., 2006 provided evidence that pyocyanin (PYO) can act as a secondary signal that alters gene expression patterns in *P. aeruginosa*. A second line of evidence from our previous work shows that PZs in strain 30-84 are regulated by complex regulatory systems. These observations suggest that PZ expression may affect bacterial physiology in complex ways. These ideas led us to hypothesize that PZs influence genes involved in cell adhesion and biofilm development. We also recognize that PZs may influence biofilm development via other, as yet unknown mechanisms.

In this chapter, we tested our two hypotheses regarding the mechanisms by which PZs affect biofilm development. To determine whether PZs altered properties of the cell surface, I added various concentrations of PZs to metabolically-inactivated bacteria and measured cell adhesion plate assay and in flow cell assays. To determine whether PZs could act as signal molecules that altered the expression of genes involved in biofilm development, I utilized *P. aeruginosa* Affymetrix GeneChips (Schuster et al., 2003). We compared whole genome transcription profiles of 30-84WT (PZ⁺) and 30-84Z (PZ⁻) to

identify genes regulated by PZs. The detailed methodology used in this study is presented at the end of this Appendix.

Results and Discussion

PZs alter the surface properties of the cell reducing the repulsive forces. In this experiment we metabolically inactivated cells of 30-84WT (PZ⁺) and 30-84Z (PZ⁻) by adding kanamycin at 50mg /L concentration. Kanamycin, an aminoglycoside antibiotic, inhibits protein synthesis in bacteria by binding to the 30s ribosomal subunit and preventing translation (Synder and Champness, 2003). Metabolically inactivated cells were untreated, treated with solvent only, or treated with purified PZs at different concentrations (12.5 µg/ml; 25 µg/ml; 50 µg/ml; 75 µg/ml and 100 µg/ml). The ability of the treated cells to adhere to surfaces in a microtiter plate assay was measured. In our assays, metabolically inactivated cells of both 30-84 and 30-84Z showed significant improvement in surface attachment in response to the addition of PZs (Figs. 1A and 2A). The increase in the number of attached cells in response to addition of PZs in both 30-84 and 30-84Z increased with the amount of PZ from 0 to 50µg/ml (Fig 1A and 2A). Subsequent increases in amount of PZ added (75 and 100 µg /ml) produced no significant improvement in attachment, indicating a threshold had been reached. Dosage response differences were visually dramatic (Fig 1A and 2A). Statistical analysis of quantitative results demonstrated a significant improvement in attachment with addition of PZs compared to the two controls (no addition or solvent only). However, differences among treatments that received PZ amendments were not significant (e.g. no significant dosage effect). Data were 12 replicates derived from 3 different plates with 4 wells in each plate. Assays using 30-84 and 30-84Z showed similar improvements in attachment. The lack of difference may be due to the fact that the bacteria were treated with PZs at very low cell

densities ($OD_{620} \sim 0.5$) where little or no QS induced PZ gene expression would have occurred.

In order to confirm the results obtained with the static microtiter plate assay, we used a flow cell assay with inoculum prepared from metabolically inactive cells treated with 0, 50 and 100 $\mu\text{g/ml}$ of PZs. The numbers of attached cells were compared between treatments from 10 random images taken for each chamber at every time point. Results from the flow cell assays are in agreement with results from the plate assays. Addition of PZs significantly improved attachment of metabolically inactive 30-84Z to the glass surface. Manual counts of attached cells indicated addition of 50 $\mu\text{g/ml}$ of PZ significantly improved the number of cells adhered to the glass surface (~ 100 cells per view) compared to no PZ addition control treatment. Interestingly, the highest concentration (100 $\mu\text{g/ml}$) of added PZ seemed to significantly lower the number of adhered cells (~ 50 cells per view) compared to the 'no PZ addition' treatment. A similar trend persisted over different time periods (up to 2d), though at the later time points more cells were washed off. This decrease in the number of cells adhering over time is to be expected because the cells are metabolically inactive and the continuous flow of media through the chambers washes away cells over time.

Data from both assays demonstrate that addition of PZs to metabolically inactivated cells improved attachment of cells to the surface. PZs are non-polar compounds and we hypothesize PZs may reduce surface repulsion between the cell surface and other surfaces to allow attachment. More importantly, metabolic inactivation enabled us to determine the direct contribution of PZs to adhesion by strain 30-84. These

experiments avoided any possible secondary effects on gene expression (e.g. genes that may be induced due to PZ production). These results were in agreement with our hypothesis that PZs change bacterial cell wall properties aiding attachment. To our knowledge these are first experiments to show that PZs improve adhesion to metabolically inactive bacterial cells.

Previous work demonstrated that pyocyanin (PYO) produced by *P. aeruginosa* strains PAO1 and PA14 served as a secondary signal that altered gene expression patterns (Dietrich et al, 2006). In this experiment, a single (200 µg) dose of PYO was exogenously added to a PYO null mutant and gene expression patterns were followed using microarrays (Dietrich et al., 2006). However, use of a high concentration of PYO (200 µg) without allowing the cells to adapt *may not reflect* the true influence of PZs on gene expression patterns (Goh et al., 2002). We compared gene expression patterns between 30-84 (PZ⁺) and 30-84Z (PZ⁻) using Affymetric GeneChip microarrays. We believe that since our experiments allow the normal regulation of PZ production to occur that our experimental comparison is a better reflection of the natural effects and concentrations of PZs with less secondary effects due to sudden exogenous PZ addition. We believe the use of *P. aeruginosa* Affymetrix GeneChips is a valid method because both pseudomonads produce PZs and utilize similar multilevel regulatory systems for PZ expression. The lists of genes which changed in expression at least two fold in all 3 replicate comparisons are presented in the Table 1 and 2. We observed 59 genes were up-regulated in presence of PZs (Table 1) and 43 genes were down-regulated in presence of PZs (Table 2). More interestingly, among the PZ - regulated genes, we observed genes

that were previously shown to have potential roles in biofilm formation (e.g. cell wall, exopolysaccharide, fimbriae, and pili). Experiments for qRT-PCR validation of microarray data are currently under way.

Materials and Methods

Measurement of cell surface properties of the metabolically –inactivated cells in presence of PZs:

a) *Microtiter plate assay*. A modified microtiter plate assay (Maddula et al., 2006 and Maddula et al., 2008) was used. Initially, cells were grown to early log phase ($OD_{620} \sim 0.5$, e.g. little or no QS induced PZ gene expression). Kanamycin, a protein synthesis inhibitor was then added at a concentration (50mg/L) to metabolically inactivate bacterial cells. Pure PZ (P-9500, Sigma chemical company, St. Louis, MO) was added to the metabolically inactive cells at different concentrations 12.5 μ g/ml; 25 μ g/ml; 50 μ g/ml; 75 μ g/ml and 100 μ g/ml. These cells were then incubated for 24 h in a polystyrene plate with or without various concentrations of PZs. The effect of added PZ on cell attachment by metabolically inactive cells was measured by staining the adhered cells with crystal violet after removal of non-adhered cells. This methodology is similar to adhesion plate assay explained in our previous chapters.

b) *Flow cell assay*. A modified flow cell assay (Maddula et al., 2006 and Maddula et al., 2008) was used to quantify bacterial cell attachment as explained below. Cells were grown to early log phase ($OD_{620} \sim 0.5$, e.g. little or no QS induced PZ gene expression) and kanamycin, was added at 50mg/L. Three separate aliquots of the metabolically inactivated cultures then were amended with pure sigma PZ at different concentrations (0 μ g/ml; 50 μ g/ml and 100 μ g/ml). An aliquot (600 μ L) of these 3 treated cultures were inoculated into individual flow cell chambers, which were pre-rinsed with AB-CAA + Km^{50} . After inoculations, chambers were kept under static conditions for 0.5 h in an

inverted position to aid bacterial attachment to the microscopic cover slip on the flow cell chambers. After the initial static phase, the flow of fresh AB-CAA +Km⁵⁰ was started and maintained at 160 μ L/min/chamber. This flow of media allowed removal of non-adherent bacteria from the flow cell chambers. To quantify the number of adherent bacteria in each flow cell chamber, 10 random images were taken using Olympus bright field microscope at 1000X magnification. Individual attached bacteria were counted manually. These results then were compared to determine whether exogenously added PZs improved the adhesion of the metabolically inactivated cells.

Microarrays: Taking advantage of the strong similarity between *P. chlororaphis* strain 30-84 and *P. aeruginosa* PAO1, *P. aeruginosa* Affymetrix GeneChips were used to determine whether PZs produced by strain 30-84 affected the expression of PZ ‘regulons’ that may be involved in biofilm formation in strain 30-84. Expression profiles of wild type strain 30-84 (PZ⁺) and 30-84Z (PZ⁻) were compared. The pair-wise comparison was replicated on 3 separate microarrays (since one GeneChip is used for each hybridization, dye swaps were not required). Standard methods for culture growth, RNA extraction, cDNA probe generation, and microarray processing were used as explained in detail below (74).

Affymetrix *P. aeruginosa* GeneChip array: This array represents the annotated genome of *P. aeruginosa* strain PAO1 and includes 5,549 protein-coding sequences, 18 tRNA genes, a representative of the ribosomal RNA cluster and 117 genes present in strains other than PAO1. In addition, 199 probe sets corresponding to all intergenic regions exceeding 600 base pairs were also included (Affymetrix).

RNA extraction protocol

Thirteen ml cultures (LB, pH 7.2 medium) were inoculated and grown with aeration at 28°C to a specific cell density ($OD_{620} \sim 1.4$). Prior to the RNA extraction, RNA was stabilized by adding 1ml Qiagen's RNAProtect reagent to culture samples (0.5 ml). Total RNA was purified using the Qiagen RNeasy Mini kit with on-column DNase treatment according to the manufacturer's instructions.

Preliminary preparations:

Before carrying out the RNA extraction the following solutions were made fresh.

1. For preparation of RLT buffer with β -mercaptoethanol, 10 μ l β -mercaptoethanol was added per 1ml Buffer RLT and mixed. (RLT buffer is stable for 1 month after addition of β -mercaptoethanol).
2. For the preparation of TE buffer containing 8 mg/ml lysozyme, initially TE buffer was made with DEPC treated sterile double distilled water (ddH₂O) with the recipe indicated below and lysozyme was added just prior to the RNA preparation.

TE buffer: (DEPC treated) Trizma-Base 121.1 FW, Na₂-EDTA 372.24 FW

For 50 ml: 0.06 g Trizma-Base

1. 0.019 g Na₂-EDTA
 2. 50 ml DEPC-treated H₂O
 3. Adjust pH to 8.0
3. For each sample, 25-50 mg acid-washed glass beads (150-600 μ m diameter) were weighed into a 2 ml Safe-Lock eppendorf tube.

4. DNase I stock was prepared by dissolving solid DNase I in 550 μ l of the provided RNase-free water. This is done by injecting the RNase-free water into the solid DNase I using an RNase-free syringe and needle. The solution was mixed by inversion. NOTE: Do not vortex. Later the DNase I stock was eluted into single use tubes and stored at -20°C up to 9 months. Thawed aliquots can be stored at $2-8^{\circ}\text{C}$ up to 6 weeks.
5. Before the first use of Qiagen RNeasy Mini kit, 4 volumes 100% ETOH were added to RPE buffer to make the working solution.
6. For each individual treatment, the required concentration of bacteria was determined (1 volume) from OD_{620} values. These values were based on a standard curve comparison of dilution counts versus OD_{620} .

Protocol for enzymatic lysis & Mechanical disruption:

1. Two volumes RNAProtect was pipetted into RNase-free PP tube (tube must be 4X larger than culture).
2. One volume bacterial culture was added to the tube. This tube was vortexed for 5 s and incubated for 5 min at RT.
3. Tubes were centrifuged for 10 min at $5000 \times g$ (a pellet may not be visible).
4. Resulting supernatant was decanted and remaining supernatant removed by dabbing the inverted tube on a clean Kimwipe. Little of the supernatant might remain [$<80 \mu\text{l}$ per $100 \mu\text{l}$ TE(lyso) buffer to be used].

NOTES: 1. Pellets could be stored for 2 weeks at -20°C or 4 weeks at -80°C .

2. All the following extraction steps were carried out continuously during one period.

5. TE (lyso) buffer (200 μ l) was added and vortexed for 10 sec and later incubated by shaking tubes at RT for 10 min. NOTE: This incubation can be longer since RNA was stabilized.
6. RLT buffer (700 μ l) with β -mercaptoethanol was added and vortexed for 5-10 s (make sure pellet is completely re-suspended).
7. Suspension was transferred into 2 ml Safe-Lock tube with glass beads.
8. Bacterial cell suspension was disrupted using TissueLyzer adapter attached to the vortex for 5 min at maximum speed.
9. Tubes were centrifuged for 10 s at max speed. Supernatant was transferred (400 μ l from $<5 \times 10^8$ or 850 μ l from $>5 \times 10^8$) into a new tube.
10. Absolute 100% ETOH from Sigma (470 μ l) was added to the supernatant. Tubes were mixed well by shaking vigorously. NOTE: Do not centrifuge.

RNA extraction with Qiagen RNeasy Minikit with in-column DNA digestion:

1. Lysate (700 μ l) including any precipitate that might have formed was transferred to an RNeasy spin column placed in a supplied 2 ml collection tube. Lid was closed gently and centrifuged 15 s at 10,000 rpm ($>8,000 \times g$). Flow through was discarded and the collection tube was re-used. This was repeated to load remaining lysate.
2. RW1 (350 μ l) buffer was added to the column and centrifuged for 15 s at 10,000 rpm. Flow through was discarded and the collection tube was re-used.

3. Previously prepared DNase I stock (10 μ l) was added to 70 μ l RDD buffer and mixed by gentle inversion. This was centrifuged for 2 s to collect residual liquid from sides.
4. DNase I (80 μ l) was added to mix directly to the column and incubated at RT for 15 min.
5. RW1 buffer (350 μ l) was added and columns were incubated for 5 min. Later columns were centrifuged for 5 s at 10,000 rpm. Flow-through and collection tubes were discarded.
6. Column was placed in a new, supplied 2 ml collection tube. RPE buffer (500 μ l) was added and centrifuged for 15 s at 10,000 rpm. Flow through was discarded and collection tube was re-used.
7. RPE buffer (500 μ l) was added, and centrifuged for 2 min at 10,000 rpm. NOTE: Flow through should not touch spin column
8. Column was placed in new, supplied 1.5 ml microfuge tube. RNase-free water (30-50 μ l) was added and incubated 3-5 min before centrifuging. Centrifugation was done 1 min at 10,000 rpm to elute the RNA.

NOTE: Yields significantly increased with a 3-5 min incubation step before centrifuging.
9. If expected yield > 30 μ g, repeat step 5 using another 30-50 μ l RNase-free water.
10. High-quality RNA thus obtained was analyzed for purity using bioanalyzer at Genomics Shared Service Laboratory (GSSL) at the Arizona Cancer Center.

Labeling, Hybridization, and analysis:

The purified RNA was labeled and hybridized according to the GeneChip® Expression Analysis protocol provided by the manufacturer at GSSL. The GSSL has implemented Silicon Genetic's GeneSpring software to display, normalize, and mine microarray data. Statistical analysis tools used include: BRB ArrayTools from NCBI, Clustering tools.

References

1. **Danhorn, T., and C. Fuqua.** 2007. Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* **61**:401-22.
2. **Dietrich LE, Price-Whelan A, Petersen A, Whiteley M, Newman DK.** 2006. The phenazine pyocyanin is a terminal signaling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **61**:1308-1321.
3. **Goh, E. B., G. Yim, W. Tsui, J. McClure, M. G. Surette, and J. Davies.** 2002. Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc Natl Acad Sci U S A.* **99**:17025-17030.
4. **Hancock, R. E. W.** 1984. Alterations in outer membrane permeability. *Ann. Rev. Microbiol.* **38**:237-264.
5. **Jefferson KK.** 2004. What drives bacteria to produce a biofilm? *FEMS Lett* **236**: 163-173.
6. **Kierek-Pearson, K., and E. Karatan.** 2005. Biofilm development in bacteria. *Adv. Appl. Microbiol.* **57**: 79–111.
7. **Klausen, M., M. Gjermansen, J. U. Kreft, and T. Tolker-Nielsen.** 2006. Dynamics of development and dispersal in sessile microbial communities: examples from *Pseudomonas aeruginosa* and *Pseudomonas putida* model biofilms. *FEMS Microbiol. Lett.* **261**:1-11.

8. **Laursen, J. B., and J. Nielsen.** 2004. Phenazine natural products: biosynthesis, synthetic analogues, and biological activity. *Chem. Rev.* **104**:1663-1686
9. **Lazazzera BA.** 2005. Lessons from DNA microarray analysis: the gene expression profile of biofilms. *Curr Opin. Microbiol.* 8:222-227.
10. **Maddula VSRK, Zhang Z, Pierson EA, and Pierson LS III.** 2006. Quorum sensing and phenazines are involved in biofilm formation by *Pseudomonas chlororaphis (aureofaciens)* strain 30-84. 2006. *Microbial Ecology.* 52:289-301.
11. **Maddula VSRK, Pierson EA, and Pierson LS III.** 2008. Altering the ratios of phenazines in *Pseudomonas chlororaphis (aureofaciens)* 30-84: Effect on biofilm formation and pathogen inhibition. 2008. *J. bacterial.* doi:10.1128/JB.01587-07.
12. **Price-Whelan, A., L. E. Dietrich, and D. K. Newman.** 2006. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat. Chem. Biol.* **2**:71-78.
13. **Rivera, M., R. E. Hancock, J. G. Sawyer, A. Haug, and E. J. McGroarty.** 1988. Enhanced binding of polycationic antibiotics to lipopolysaccharide from an aminoglycoside-supersusceptible, *tolA* mutant strain of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* **32**: 649–655.
14. **Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg.** 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa*

quorum-controlled genes: a transcriptome analysis. *J. Bacteriol.* **185**:2066-2079.

15. **Synder, L., W. Champness.** 2003. *Molecular genetics of bacteria*. Second edition. ASM press, Washington, D. C.
16. **Vasil, ML.** 2003. DNA microarrays in analysis of quorum sensing: strengths and limitations. *J. Bacteriol.* 185:2061-2065.

Figure legends:

Figure 1: Effect of exogenous PZ addition on attachment of metabolically inactivated 30-84 wild type bacterial cells. **A.** Photograph of polystyrene plates showing differences in attached bacterial cells. Attached bacterial cells were stained with crystal violet (0.1%); degree of blueness is proportional to number of attached bacteria. Individual wells have 30-84 (PZ⁺) cells that were untreated or treated with solvent ethanol (ETOH) or treated with PZ at different concentrations 12.5µg/ml; 25µg/ml; 50µg/ml; 75µg/ml and 100µg/ml. **B.** The graph presented shows the OD₅₄₀ values as a measure of cell adhesion to plastic surfaces for treatments using metabolically inactivated 30-84 (PZ⁺) cells. Treatments included metabolically inactivated 30-84 cells that were untreated or treated with solvent ethanol (ETOH) or treated with PZ at different concentrations (12.5µg/ml; 25µg/ml; 50µg/ml; 75µg/ml and 100µg/ml). Treated cells were incubated in 24-well polystyrene plates for 24 h. After incubation, unattached cells were removed by vigorous tapping on absorbent paper. The remaining adherent bacteria were fixed to the plates and stained with crystal violet (0.1%). Excess stain was removed by rinsing twice with distilled water. Adherent cells were decolorized with a 20% acetone + 80% ethanol mix and the amount of crystal violet (proportional to the density of adherent cells) was quantified as OD₅₄₀. Experiments were replicated at least 3 times and values from one representative experiment were shown here. Data presented are averages of 12 replicate wells with error bars.

Figure 2: Effect of exogenous PZ addition on attachment of metabolically inactivated 30-84Z cells. **A.** Photograph of polystyrene plates showing differences in

attached bacterial cells. Attached bacterial cells were stained with crystal violet (0.1%); degree of blueness is proportional to number of attached bacteria. Individual wells have 30-84Z (PZ⁻) cells that were untreated or treated with solvent ethanol (ETOH) or treated with PZ at different concentrations similar to the treatments of 30-84 as explained above.

B. The graph presented shows the OD₅₄₀ values as a measure of cell adhesion to plastic surfaces for treatments using metabolically inactivated 30-84Z (PZ⁻) cells. Treatments included metabolically inactivated 30-84Z cells that were untreated or treated with solvent ethanol (ETOH) or treated with PZ at different concentrations similar to the treatments of 30-84 as explained above.

Figure 3: Effect of exogenous PZ addition on attachment of metabolically inactive 30-84Z measured in flow cell assay. The graph presented shows the number of attached cells per view in each flow cell chamber at different time intervals. Metabolically inactivated 30-84Z cells were treated with ETOH solvent only or with different concentrations of PZs (50µg/ml and 100µg/ml). Treated cells were inoculated into individual flow cell chambers. Flow cells were incubated for 30 min under no flow conditions to allow cell attachment. After incubation, flow was initiated and ten random images (1000X) were taken at different time intervals (45 min, 3 h, 5 h, 8 h, 1 d and 2 d). Attached cells were quantified by manually counting cells per view. Experiments were replicated at least 3 times and values from one representative experiment were shown here. Data presented are the means from 10 replicate images at each time point with error bars.

Figure 1 A:

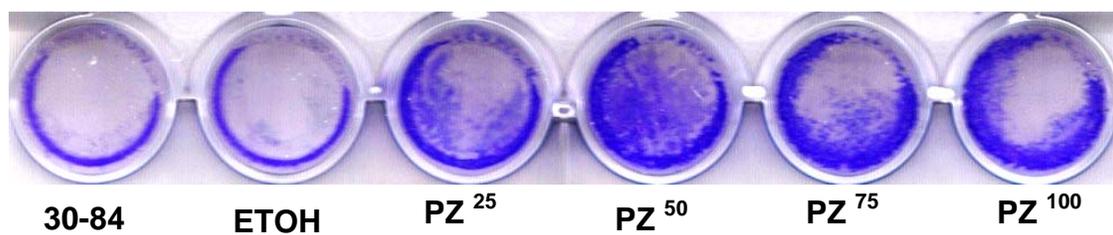


Figure 1B:

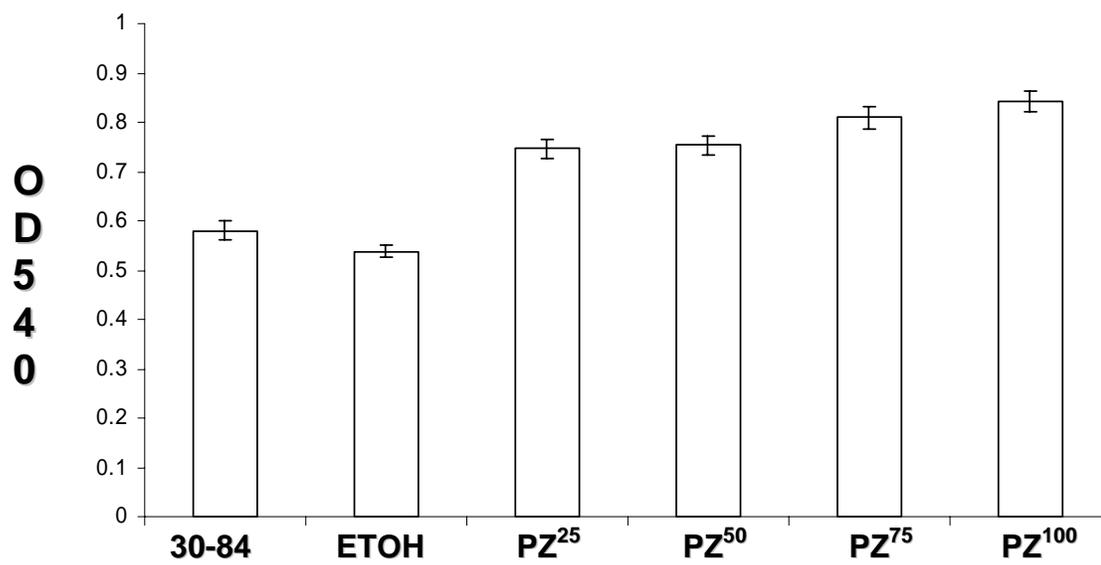


Figure 2 A:

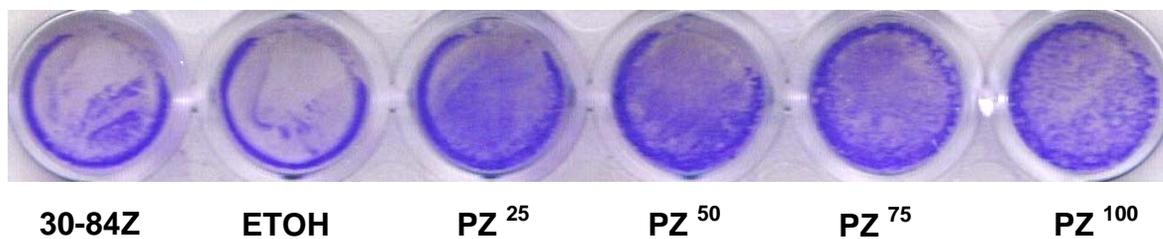


Figure 2B:

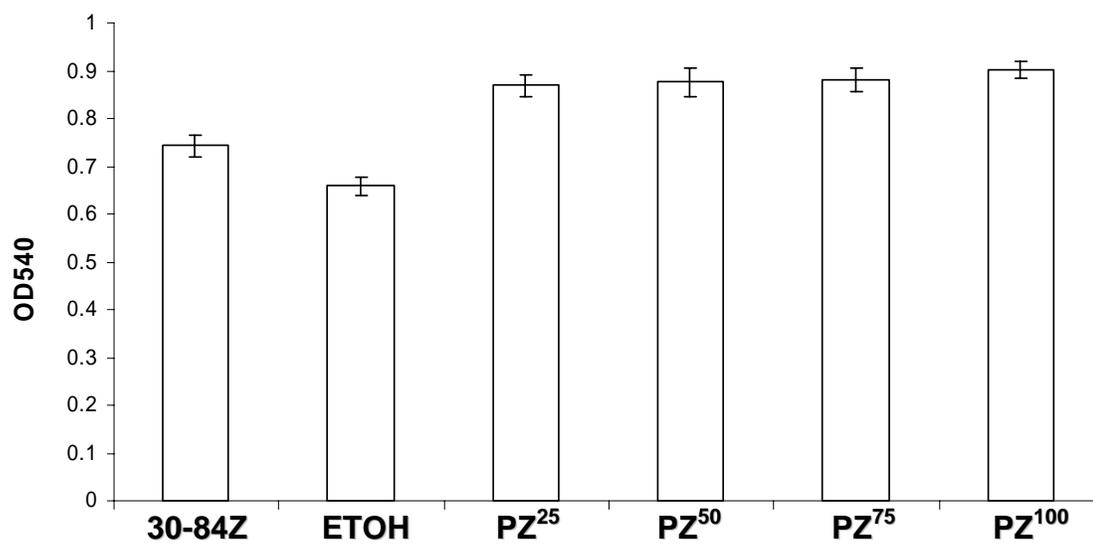


Figure 3:

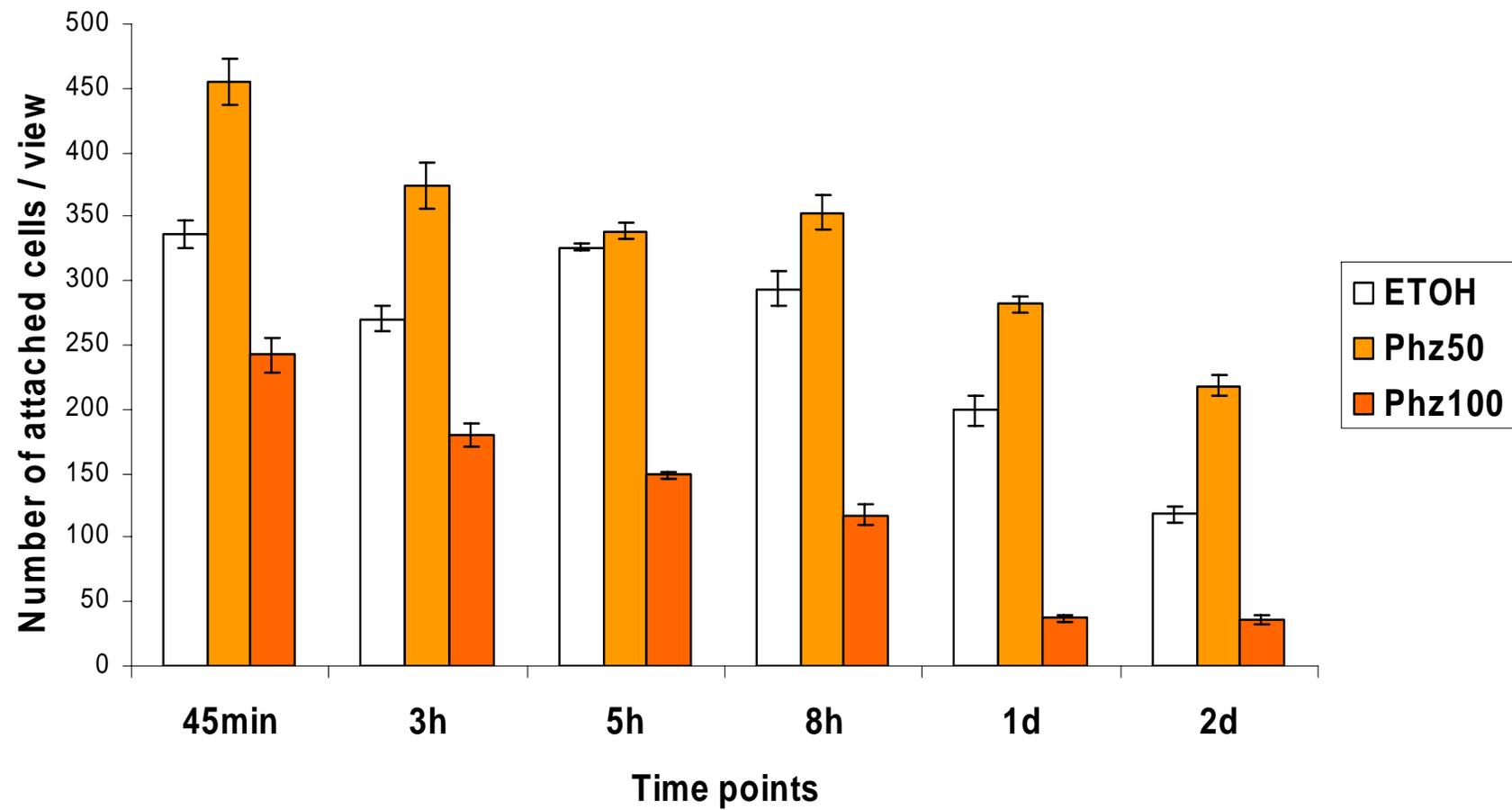


Table 1: **List of genes UP-regulated in the presence of PZs.** Gene expression differences were measured in a whole genome microarray comparisons of 30-84WT (PZ⁺) and 30-84Z (PZ⁻) using *P. aeruginosa* PAO1 Affymetrix GeneChip genome arrays. Total RNA was extracted from LB cultures grown to exponential phase (OD₆₂₀ = ~ 1.4) with Qiagen RNeasy minikit. Manufactures recommendations were followed for cDNA synthesis, labeling and hybridization and were carried out in collaboration with AZCC Microarray Core Facility. Comparisons were replicated 3 times and data were presented for genes having at least 2 fold mean change in all 3 experiments.

*Locus ID	Descriptions	Mean fold change
Secondary metabolities		
PA1905	<i>phzG2</i> , probable pyridoxamine 5 -phosphate oxidase	10.18
Motility and Attachment		
PA2130	Probable fimbrial biogenesis usher protein; Motility & Attachment	6.64
PA2128	Probable fimbrial protein (precursor); Motility & Attachment	3.30
Cell wall property		
PA5238	probable O-antigen acetylase; Cell wall / LPS / capsule; Membrane proteins; Putative enzymes	6.87
PA3984	Int apolipoprotein N-acyltransferase; Cell wall / LPS / capsule; Translation, post-translational modification, degradation	3.94
PA2873	hypothetical protein; Membrane proteins	3.84
PA3150	<i>wbpG</i> LPS biosynthesis protein WbpG; Cell wall / LPS / capsule	2.69
Energy / Metabolism		
PA3738	XerD, integrase/recombinase; DNA replication, recombination, modification and repair	7.25
PA0106	<i>coxA</i> cytochrome c oxidase, subunit I; Energy metabolism	5.15
PA1671	Stk1 serine-threonine kinase; Translation, post-translational modification, degradation	4.26
PA0668	<i>tyrZ</i> tyrosyl-tRNA synthetase 2; Amino acid biosynthesis and metabolism; Translation, post-translational modification, degradation	3.12

PA1393	<i>cysC</i> adenosine 5 -phosphosulfate (APS) kinase Central intermediary metabolism; Nucleotide biosynthesis and metabolism; Amino acid biosynthesis and metabolism	2.97
--------	--	------

Transcriptional Regulators

PA2316	Probable transcriptional regulator	4.25
PA4307	<i>pctC</i> chemotactic transducer; Adaptation, protection; Chemotaxis	4.22
PA4036	Probable two-component sensor	3.92
PA3599	Probable transcriptional regulator	3.22
PA0475	Probable transcriptional regulator	3.09
PA1911	probable transmembrane sensor; Membrane proteins; Transcriptional regulators	2.58
PA2267	Probable transcriptional regulator	2.51

Transport

PA4304	Probable type II secretion system protein	8.19
PA1496	probable potassium channel; Membrane proteins; Transport of small molecules	5.50
PA2677	Probable type II secretion protein	4.55
PA0304	<i>potI</i> polyamine transport protein; Membrane proteins; Transport of small molecules	4.15
PA5097	probable amino acid permease; Membrane proteins; Transport of small molecules	3.43
PA1419	probable transporter; Membrane proteins; Transport of small molecules	2.57

Putative Enzymes

PA0421	hypothetical protein (putative enzyme)	9.04
PA4576	Probable ATP-dependent protease (putative enzyme)	5.64
PA4678	<i>rimI</i> peptide n-acetyltransferase; Translation, post-translational modification, degradation	4.34
PA5150	Probable short-chain dehydrogenase (putative enzyme)	4.20
PA5188	Probable 3-hydroxyacyl-CoA dehydrogenase (putative enzyme)	4.00
PA1062	hypothetical protein; (putative enzyme)	3.05
PA1027	Probable aldehyde dehydrogenase (putative enzyme)	2.60
PA0249	probable acetyltransferase (putative enzyme)	2.50

Intergenic regions

5087407-5086695	Intergenic region between PA4581 and PA4582, 5086695-5087407, (-) strand	5.88
1063544-1064555	Intergenic region between PA1003 and PA1004, 1063544-1064555, (+) strand	4.34
1324109-	Intergenic region between PA1244 and PA1245, 1324109-	4.14

1324793 1324793, (+) strand
3129070- Intergenic region between PA2852 and PA2853, 3129070-
3129728 3129728, (+) strand 2.97

Hypothetical proteins

PA0729, PA1955, PA5109, PA2030, PA0014, PA3783, PA0076, PA0941, PA1579,
PA0062, PA0827, PA3274, PA3445, PA2021, PA1050, PA1234, PA1906, PA4346,
PA3840, PA3613, PA2872, PA3600, PA0729, PA1955, PA5109, PA2030, PA0014,
PA3783, PA0076, PA0941, PA1579, PA0062, PA0827, PA3274, PA3445, PA2021,
PA1050, PA1234, PA1906, PA4346, PA3840, PA3613, PA2872, PA3600

* Locus ID indicates *P. aeruginosa* PAO1 annotations.

Table 2: **List of genes DOWN-regulated in the presence of PZs.** Gene expressions differences were measured in a whole genome microarray comparison of 30-84WT (PZ⁺) and 30-84Z (PZ⁻) using *P. aeruginosa* PAO1 Affymetrix GeneChip genome arrays. Total RNA extracted from LB grown exponential phase (OD₆₂₀ = ~ 1.4) cultures with Qiagen RNeasy minikit. Manufactures recommendations were followed for cDNA synthesis, labeling and hybridization and were carried out in collaboration with AZCC Microarray Core Facility. Comparisons were replicated 3 times and data were presented for genes having at least 2 fold mean change in all 3 experiments.

Locus ID*	Descriptions	Mean fold change
Secondary metabolites		
PA5375	<i>betT1</i> choline transporter; Membrane proteins; Transport of small molecules	6.12
PA2195	<i>hcnC</i> hydrogen cyanide synthase; Central intermediary metabolism	5.01
Efflux / Transport		
PA1718	<i>pseE</i> type III export protein; Protein secretion/export apparatus	11.31
PA1877	probable secretion protein; Protein secretion/export apparatus	4.30
PA4365	probable transporter; Transport of small molecules; Membrane proteins	2.90
PA1238	probable outer membrane component of multidrug efflux pump; Transport of small molecules	2.63
Transcriptional Regulators		
PA0477	probable transcriptional regulator	3.02
Energy / Metabolism		
PA3152	<i>hisH2</i> glutamine amidotransferase; Amino acid biosynthesis and metabolism	5.41
PA3584	<i>glpD</i> , glycerol-3-phosphate dehydrogenase; central intermediary metabolism; Energy metabolism	3.68
PA4977	probable acetolactate synthase large subunit; Amino acid biosynthesis and metabolism	3.54

Stress

PA4083	probable pili assembly chaperone; Motility & Attachment; Chaperones & heat shock proteins	4.64
PA3842	probable chaperone; heat shock proteins; Secreted Factors (toxins, enzymes, alginate)	3.56

Cell division

PA4513	probable oxidoreductase; (putative enzyme)	4.37
PA1800	<i>tig</i> , trigger factor; cell division; chaperones & heat shock proteins	3.47

Putative Enzyme

PA3295	probable HIT family protein,(putative enzyme)	3.90
PA4209	probable O-methyltransferase; (putative enzyme)	2.95
PA1990	probable peptidase (putative enzyme)	2.80
PA3568	probable acetyl-CoA synthetase (putative enzyme)	2.33
PA3994	probable epoxide hydrolase (putative enzyme)	2.21

Intergenic region

1997509- 1996806	Intergenic region between PA1897 and PA1898, 1996806-1997509, (-) strand	5.00
---------------------	--	------

Hypothetical proteins

PA0258, PA5446, PA2453, PA2224, PA2236, PA3786, PA0251, PA4736, PA4584, PA2793, PA5108, PA2819, PA2318, PA2372, PA3110, PA1042, PA2117, PA3843, PA2870, PA4757, PA0121, PA5540

* Locus ID indicates *P. aeruginosa* PAO1 annotations.

APPENDIX D

Studies on Effects of *rpeA/rpeB* Two-component Regulators in *Pseudomonas* spp.

Introduction

A mutation in *rpeA* (repressor of phenazine expression), a homologue of a two-component sensor-kinase in many other bacteria, was identified previously in *P. chlororaphis* strain 30-84 (Whistler and Pierson, 2003). Subsequent work showed that RpeA encodes a conserved two-component histidine sensory kinase that acts as a repressor for PZ biosynthesis. Mutation in *rpeA* allowed excess production of PZs in rich media. The mutation also allowed PZ production even under low nutrient conditions where PZs are not normally made by the wild type. The *rpeA* mutation also bypassed the QS requirement for PZ production, enabling a *phzR/rpeA* double mutant to produce PZs (Whistler and Pierson, 2003). More detailed experiments characterizing *rpeA/B* homologs of *Pseudomonas aeruginosa* PAO1 and PA14 and determining the roles of these two-component regulators on global gene expression in microarray experiments are currently under way.

In my previous work, we demonstrated that a structural mutant defective only in PZ biosynthesis was impaired in biofilm formation (Maddula et al., 2006). Genetic and biochemical complementation of the PZ mutant restored biofilm formation to wild type levels. This observation led us to wonder about the biofilm phenotype of a strain that synthesizes more PZs than the 30-84 wild type levels. In order to determine affect of excess PZ production, we compared the previously characterized strain 30-84*rpeA* (PZ over producer) and 30-84 wild type in microtiter plate and chamber slide biofilm assays.

Results and discussion

Effects of increased phenazine production on biofilm formation: To study the effect of increased PZ production, strain 30-84*rpeA* was used in adhesion plate assay in comparison with 30-84 wild-type. Mutant strain 30-84*rpeA* synthesizes larger amounts of PZs than 30-84WT in a QS independent manner (Whistler and Pierson, 2003). PZ synthesis also was observed in minimal media in strain 30-84*rpeA* under conditions where 30-84 lacks PZ bio-synthesis (Whistler and Pierson, 2003). A time course of cell adhesion rate (Fig.1A) showed that 30-84 WT and 30-84*rpeA* adhesion levels at early points were not significantly different. At time points later than ~ 20 h after inoculation and before 42 h, strain 30-84*rpeA* had significantly greater adhesion than 30-84WT. We also compared adhesion in a simple chamber slide assay, which allowed direct visualization of cells attached (Figs. 1B and 1C). Similarly, these data show that 30-84*rpeA* biofilms have more surface coverage than 30-84WT biofilms. 30-84*rpeA* biofilms covered the entire slide area, whereas 30-84WT biofilms had a patchy coverage of the slide area. Taken together, these results indicate that increased PZ levels improved adhesion ability and biofilm formation of the strain 30-84. These results are also in agreement with our earlier published results that show that biofilm formation by a PZ structural mutant is significantly impaired compared to the PZ-producing wild type.

In the microtiter plate assays, the later time points provide results that showed an opposite trend to early time points. This is typical for organisms that produce a large amount of exopolysaccharide (EPS) over time. We speculate that a build up of EPS causes an increase in cell-to-cell adhesion, which may be greater than the cell to surface

adhesion forces. This hypothesis is supported by observations of the 30-84*rpeA* biofilms at 42 and 48 h, which appeared to have a sticky, filmy consistency. The experimental protocol of the adhesion plate assay involves the forcible removal of un-adhered cells, which removes almost the entire biofilm. Thus, you can not use the adhesion assay at those time points as a measure of biofilm formation. However, it was apparent from my observations that only the 30-84*rpeA* treatment produced sufficient EPS by 42 h to produce this affect.

Materials and Methods:

Bacterial strains and media: Bacterial strains and plasmids used in this study are described in Table 1. All the mutants used in this study were derived from a spontaneous rifampicin-resistant derivative of a parental strain *P. chlororaphis* strain 30-84 (Pierson and Thomashow, 1992). All the strains used in this study were grown in regular media described elsewhere (Zhang and Pierson, 2001). LB media with 5g of NaCl /liter, AB minimal media supplemented with 2% Casaminoacids (AB+CAA) were used as previously described (Zhang and Pierson, 2001). Antibiotics were used at the following concentrations ($\mu\text{g}/\mu\text{l}$) for *Escherichia coli*: ampicillin (100), gentamycin (25), kanamycin (50), and tetracycline (25); for *P. chlororaphis*: gentamycin (50), kanamycin (50), rifampicin (100), and tetracycline (50).

Quantification of Pyocyanin (PYO). PYO quantification data is not presented in the Results section. I included this methodology because I standardized the protocol for PYO quantifications as part of the RpeA/RpeB two-component regulators characterization for

P. aeruginosa strain PAO1 and strain PA14 and wanted the protocol to be available in my dissertation.

PYO strains and mutant derivatives were grown in LB broth with suitable selection at 28°C for 16-18 h. Cell-free supernatants (CFS) of these cultures were obtained by centrifuging the cultures 2600 x g for 15 min. CFSs were extracted twice with half the volume of chloroform. The blue-colored chloroform phase was separated from the aqueous phase after spinning tubes at 2600 x g for 1 min. PYOs were re-extracted from the chloroform phase using acidified water (0.2 N HCL). The extracted PYOs are pink colored. Absorption maxima were quantified at OD₅₂₀ using a UV-Vis spectrometer. The approximate amount of PYO in a sample was calculated by multiplying absorption maxima with their standard extinction coefficients (Essar et al., 1990).

Microtiter plate assay. Plate assay was carried out in 24-well polystyrene plates as described previously (Maddula et al. 2006). In the present experiments, we included triplicate wells with no inoculum or wells with strain 30-84 as negative and positive controls, respectively. Plates were incubated at 28°C for 32-38 h under static conditions and final cell densities determined (OD₆₂₀). Unattached cells were removed by inversion of the plate, followed by vigorous tapping on absorbent paper. The remaining adherent bacteria were fixed to the plates for 20 min at 50°C and then stained for 1 min with 1.25 ml /well of crystal violet (0.1%). Excess stain was removed by inversion of the plate followed by two washings (each 1.25 ml /well) with distilled water. To quantify the amount stain, which is directly proportional to number of adherent bacteria, the contents

were decolorized with a 20% acetone + 80% ethanol solution (1 ml/well) for 5 min to release the dye into solution. A sample of 200 μ l of the 1 ml decolorizing solution was taken into a new 96-well plate and readings were taken with spectrophotometer at OD₅₄₀. All the experiments were repeated at least twice.

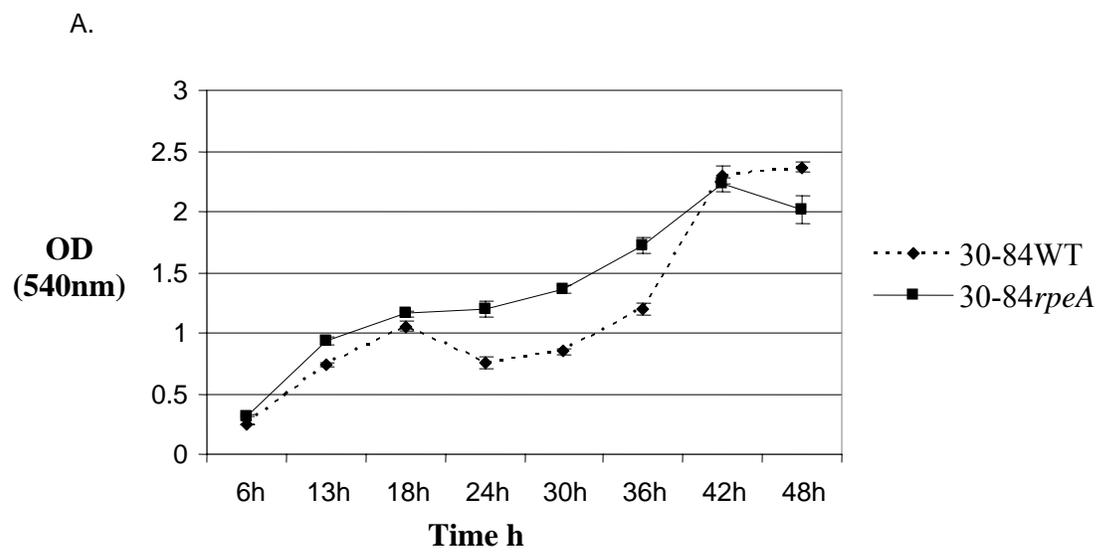
Chamber slide assay. Chamber slide assays were carried out in 8-well Lab-Tek chamber slide with cover (#177402) from Nalge Nunc International. Treatments were prepared from overnight cultures grown in 2% AB-CAA, which were adjusted to OD₆₂₀ of 0.8 by dilution with AB-CAA. The chamber slide containing 350 μ l of 2% AB-CAA then was inoculated with 3.5 μ l of OD adjusted inoculum (at a 1:100 ratio). We used at least 2 replicates per treatment. Inoculated chamber slides were incubated under static conditions at 28°C for 24 h. After the incubation period, the cultures were removed from the chambers using a pipette and the remaining unattached bacteria were removed by rinsing twice with 350 μ l of sterile water. Then the media chamber was detached from the slide and attached bacterial cells were heat fixed and visualized using an Olympus BX60 light microscope. Five random images were taken and representative image was presented.

References

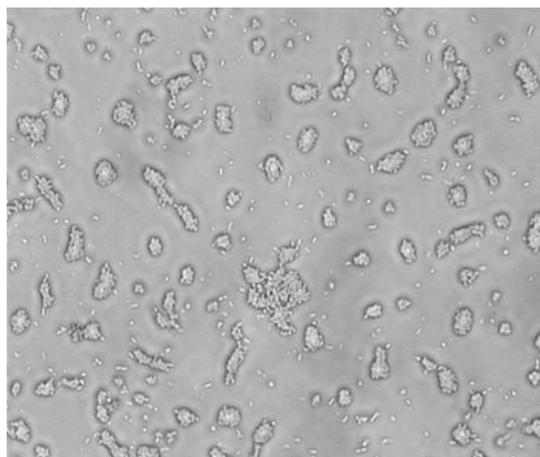
1. **Essar, D. W., L. Eberly, A. Hadero, and I. P. Crawford.** 1990. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J Bacteriol.* **172**:884–900.
2. **Maddula, V. S. R. K., Z. Zhang, E. A. Pierson, and L. S. Pierson III.** 2006. Quorum sensing and phenazines are involved in biofilm formation by *Pseudomonas chlororaphis (aureofaciens)* strain 30-84. *Microb. Ecol.* **52**:289-301.
3. **Pierson, L. S., III, and L. S. Thomashow.** 1992. Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. *Mol. Plant Microbe Interact.* **5**:330-339.
4. **Whistler, C. A., and L. S. Pierson III.** 2003. Repression of phenazine antibiotic production in *Pseudomonas aureofaciens* strain 30-84 by RpeA. *J. Bacteriol.* **185**:3718-3725.
5. **Zhang, Z., and L. S. Pierson III.** 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Appl. Environ. Microbiol.* **67**:4305-4315.

Figure 1. Cell adhesion by wild type *P. chlororaphis* strain 30-84 and the PZ-overproducing derivative, 30-84*rpeA*. (A) The graph represents cell adhesion over a time 2 d time periods. A time course of cell adhesion was determined using the optical density (540 nm) of crystal violet-stained cells adhering to the polystyrene as a measure of cell adhesion. Replicate cultures of each treatment were inoculated at the same time. At time intervals up to 48 h, replicates of each treatment were assayed for cell adhesion. Data presented are the mean of 9 replicates and standard errors were less than 0.06 at all time points. Data from one representative experiment is shown below. (B & C) Light microscopic images of 30-84WT and 30-84*rpeA* (B and C, respectively). Images show cells adhered to the chamber slides after 24 h of incubation. Five random images were taken and a representative image from one experiment is shown for each treatment.

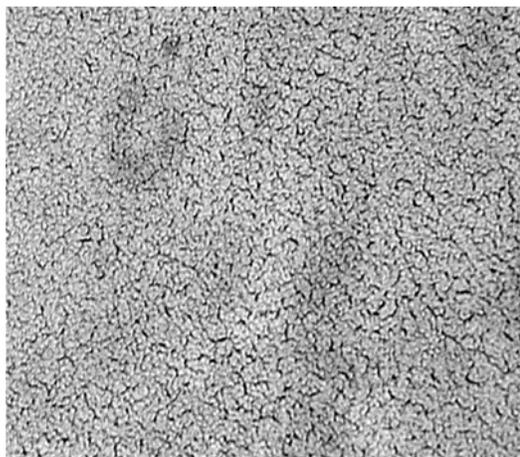
Fig. 1.



B.



C.



APPENDIX E

Standardizing Protocol for pRL-27 Tn5 Mutagenesis for *Pseudomonas chlororaphis* strain 30-84 and *P. cedrella* strain PU43.

Introduction:

Determining specific effects of genes on the phenotype of the producing bacteria is done by creating mutations of specific genes and observing for the loss of a particular phenotype. Tn5 mutagenesis is a well used method of creating non-specific mutations as it offers stable, non-leaky mutations in a comparatively short time. Wild-type Tn5 is widely used in Gram-negative bacteria due to its broad-host range (Berg and Berg 1983). Numerous Tn5 versions carrying variety of selectable markers and reporter genes have been developed (de Lorenzo and Timis 1994) for ease in selection for different bacteria. Of the many, the Tn5 vectors, a series of 'plasposons' for mutagenesis and one-step cloning of the Tn mutation is very useful (Dennis and Zylstra 1998). Certain mutations within Tn5 aided in increased efficiency of transposition. Goryshin and Reznikoff 1998 reported on a combination of mutations both in *tnp*, transposase gene and the Tn5 inverted repeats improved transposition 1000-fold compared to the wild-type Tn5. Larsen et al ., 2002, developed a Tn5 vector pRL27 that combines the useful features of the plasposon vectors with the new, hyperactive Tn5 element for use in *Xanthobacter autotrophicus* Py2.

Features of pRL27 plasposon vector: It has an RP4 origin of transfer (*oriT*), which allows plasmid transfer after conjugation with *E. coli* donors. More importantly it has a mutant version of the *tetAp::Tn5 tnp* gene construct, which encodes a hyperactive transposase.

An added advantage is the location of this *tetAp::Tn5 tnp* gene construct outside the transposon allowing stable transposon insertion. The Tn5 inverted repeats were optimized and placed flanking to *aph* gene, which encodes kanamycin resistance as a selection marker. The DNA replication origin *oriR6K* is adjacent to the *aph* gene facilitating subsequent one-step cloning of the transposon insertion mutants. Further this *oriR6K* requires the π protein encoded by *pir* gene, allowing transposon insertion in the recipient non-*pir* recipient hosts.

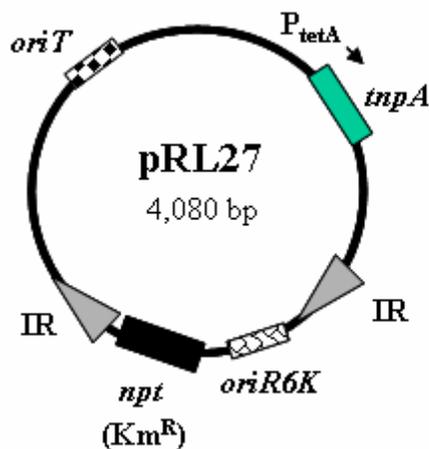


Illustration 1. Map of Tn5-pRL27 transposon vector (by Dr. Pierson).

Materials and Methods:

Transposon Mutagenesis: 3 ml of overnight cultures of the donor pRL27 and recipient strains (30-84 and PU43) were grown in LB media up to mid-logarithm phase (OD_{620} ~0.6 -0.8). For growing the donor pRL27 vector, LB medium was amended with Km at 50 mg/L concentration. One ml of the overnight culture of both donor and recipient were centrifuged for 1 min. Pellets were re-suspended in 200 μ L of LB broth. For getting biparental mating mix, 30 μ L of the re-suspended cells of each culture were spotted onto

nitrocellulose filters placed on LB+PABA¹⁰ plates (donor followed by recipient). Para-amino benzoic acid (PABA) is a supplement that reduces PZ production which interferes with conjugation due to effects on the E. coli donor (LS Pierson, personal comm.). Controls of donor alone and recipient alone also were spotted on separate filters in the same plate. These plates were incubated at 28°C for at least 24 h. After incubation, the filters containing the mating mix were re-suspended in 2 ml of sterile ddH₂O. A 75-100 µL aliquot of the re-suspended mating mix was spread on

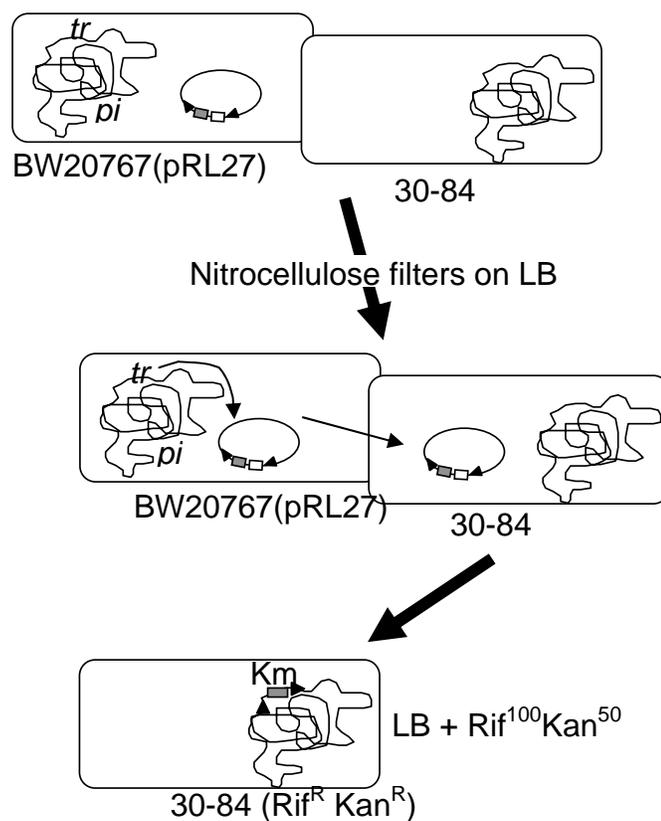


Illustration 2. Diagram of Biparental mating (by Dr. Pierson):

suitable selection plates (LB+Rif^{r100}+Km⁵⁰) and incubated at 28°C for 24-48 h for selecting Tn5-pRL27 mutants. The Tn5-pRL27 mutants then were patched onto plates with LB+Km⁵⁰ plates and later screened for interesting phenotypes on different media.

NOTES:

1. Plates of pRL27 should be started fresh out glycerol stocks. Plates older than 7-10 days yield less or no Tn5 mutants.
2. Overnight cultures of the donors and recipients should be grown fresh only up to mid-logarithmic stage of growth.

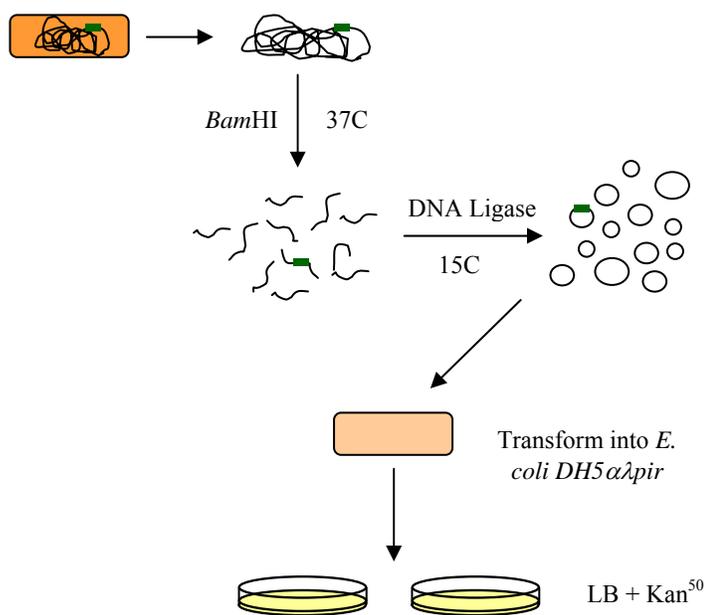


Illustration 3. Pictorial depiction of pRL-mutants plasposon generation and one-step cloning

One-step cloning of Tn5-pRL27 mutants:

Initially, total genomic DNA from each of mutant was extracted. For obtaining fast and high quality genomic DNA, a PUREGENE (Gentra Systems, Minneapolis, MN) chromosomal

extraction kit was used. Genomic DNA extraction was carried out according to the manufacture's directions for DNA extractions of Gram-negative bacteria. Extracted genomic DNA was digested with specific restriction enzymes such as *Bam*HI, which does not cut within the transposon. Subsequently, chromosomal fragments were self-ligated to circularize the fragments. These circularized chromosomal fragments were transformed into a special strain of *E. coli*, DH5 α / λ *pir*, which contains the *pir* gene encoding the π replication protein. This allows replication of the single chromosomal fragment that contains the Tn5-pRL27. Replication of this fragment as a plasmid is possible due to the presence of *oriR6K* origin of replication within the transposed region. For sequencing of Tn5 mutated regions plasmids were isolated and the adjacent chromosomal DNA regions flanking Tn5-pRL27 were sequenced using primers the following primers: (**tpnRL17-1** 5'-AACAAGCCAGGGATGTAACG-3' and **tpnRL13-2** 5'-CAGCAACACCTTCTTCACGA-3'). The sequences obtained were compared to the GenBank database using NCBI blast for identification the gene(s) disrupted by the Tn5-pRL27.

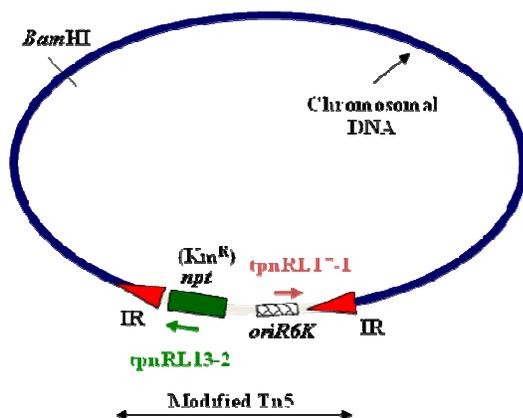


Illustration 4. Circularized chromosomal fragments with pRL27::Tn5 insertion.

Results and Discussion:

Pseudomonas cedrella PU43: Strain PU43 was isolated from the wheat rhizosphere and later determined to be a negative signaling strain (Morello et al 2004). Strain PU43 produces an unknown signal molecule that can inhibit PZ biosynthesis in *P. chlororaphis* 30-84 (Morello et al 2004). In order to genetically identify the genes responsible for the unknown negative signal production, a transposon mutagenesis approach was taken. Using the Tn5-pRL27 plasmid vector, a series of PU43-Tn5 mutants were isolated as described above. These PU43-Tn5 mutants were distributed among microbial genetics students (MIC428L) for initial screening for the loss of negative signal production. This initial screen included patching PU43-Tn5 mutants on a lawn of either strain 30-84WT or 30-84ZN and looking for colonies that lost the ability to inhibit PZ biosynthesis (identified by loss of white halo). Negative signaling strains produce a white halo surrounding the colony when spotted on a lawn of 30-84 WT or 30-84ZN. The halo appears as a white zone on the orange lawn of 30-84WT and is indicative of a zone where negative signal production inhibits orange PZ production. Similarly, the white halo on a 30-84ZN lawn is indicative of a zone where negative signal production inhibits β -galactosidase production by 30-84ZN (*phzB::lacZ*) (e.g. interferes with the transcription of the PZ operon). Mutants of negative signaling strains no longer produce a halo on 30-84WT or 30-84ZN. Further, probable PU43-Tn5 mutants that lost the ability to inhibit PZ biosynthesis were re-screened by Dr. Betsy Pierson using 96-well plate assay she developed. The identity of the interrupted genes within the PU43-Tn5 negative signaling mutants was determined via one-step cloning as described above. Some of the genes

identified by this process are presented below (Table 1). As the insertion is random, these negative signal PU43-Tn5 mutants may have mutations in genes that:

- 1) synthesize the negative signal,
- 2) are involved in the synthesis of the precursor / inducer molecules of the negative signal, or
- 3) are involved in the negative signal transport machinery.

In our initial screen and one-step cloning of mutants unable to produce the negative signal, PU43-Tn5 mutants with insertions in the shikimic aromatic acid synthesis pathway, the global *gacA* regulatory system, global transcriptional factors, transport permeases, and *TonB* domain proteins (Table 1).

The next step in determining whether these genes play a role in negative signaling is to complement mutants by introduction of a wild-type copy of the gene of interest. Introduction of the wild-type gene *in trans* should restore negative signal production if the gene of interest is responsible for the mutant phenotype. From complementation experiments, we may conclude that negative signal production is under *gacA* global regulation. Attempts to biochemically characterize the negative signal have been unsuccessful. In our future experiments, we plan to compare biochemical profiles of the supernatants wild type and complemented strains (containing the negative signal) to the mutant strain (no negative signal) to try to identify the diffusible product that is the negative signal in strain PU43.

Pseudomonas chlororaphis 30-84: Strain 30-84::Tn5 mutants were generated as described above. This standardized mutagenesis protocol also was used in the microbial

genetics class (MIC428L) for generating interesting 30-84::Tn5 mutants. We were particularly interested in mutants with altered PZ biosynthesis. Identification of altered PZ biosynthesis mutants is easy since visual differences in PZ production can be detected by colony phenotype on the plates. Selected mutant phenotypes were white, dark orange, red, and with PZ diffusion differences (probably mutations in PZ transport). In the initial assays to standardize the protocols one-step cloning, we focused on three mutants: two having the dark orange phenotype and one having the yellow phenotype. The results of our analysis showed that the mutants with the dark orange phenotypes had insertions in a probable transcriptional regulator similar to *pruU*. The mutant with the yellow phenotype had an insertion in *glcD*, a sub unit of glycolate oxidase (Table 2). We will need to complement these mutants in order to confirm that the mutant phenotype was due to these specific genes.

To conclude, these protocol standardizing experiments aided in adapting pRL-27 mutagenesis to our model system (strain 30-84) and related *Pseudomonas cedrella* strain PU43. We were able successfully characterize pRL27-Tn5 mutants by one-step cloning and we identified a number of interesting genes.

Table 1: Results summary for PU43::Tn5 mutants

Serial No.	Mutant ID	Best hit with tpn13-2 primer sequence	Best hit with tpn17-1 primer sequence	Probable mutated gene	Probable functions of the gene
1. 2. 3. 4.	DR4 DR6 20D4 20H4	D-erythrose-4-phosphate dehydrogenase (Pf5, Pf 0-1) Score = 198 bits (100), Expect = 4e-47, Identities = 181/208 (87%)	D-erythrose-4-phosphate dehydrogenase (Pf5, Pf 0-1) Score = 365 bits (184), Expect = 3e-97, Identities = 339/392 (86%)	D-erythrose-4-phosphate dehydrogenase	Precursor for Shikimic acid pathway leading to secondary metabolite production including PZs.
5.	20D3	Glycine dehydrogenase (Pf5, Pf 0-1, DC3000) Score = 1033 bits (521), Expect = 0.0, Identities = 801/888 (90%)	Glycine dehydrogenase (Pf5, Pf 0-1) Score = 289 bits (146), Expect = 1e-74, Identities = 245/278 (88%)	Glycine dehydrogenase	Converts glycine, water, and NAD ⁺ into glyoxylate, NH ₃ , NADH, H ⁺ . Participates in glyoxylic acid cycle (similar to Citric Acid Cycle in eukaryotes) → alternate pathway for glyoxylate biosynthesis. Excess glycine → could inhibit other pathways
6.	S36	<i>gacA</i> (P fluorescens)	<i>gacA</i> (P fluorescens, 30-84)	<i>gacA</i>	Global sensory kinase
7.	9E2	<i>tonB</i> domain protein (Pf5, Pf 0-1) Score = 396 bits (200), Expect = 8e-107, Identities = 356/408 (87%),	<i>tonB</i> domain protein Score = 349 bits (176), Expect = 2e-92, Identities = 347/404 (85%)	<i>tonB</i> domain protein (Pf5, Pf 0-1)	TonB proteins are essential components in iron-siderophore uptake in bacteria, apparently functioning as energy transducers in coupling the energized state of the cytoplasmic membrane to outer-membrane receptor

					function.
8.	18G5	Putative transcriptional regulator and ExodeoxyrobonucleaseI (Pf5, Pf 0-1) Score = 194 bits (98), Expect = 6e-46, Identities = 119/126 (94%)	Putative transcriptional regulator and ExodeoxyrobonucleaseI (Pf5, Pf 0-1) Score = 194 bits (98), Expect = 6e-46 Identities = 119/126 (94%)	Putative transcriptional regulator and ExodeoxyrobonucleaseI	Can be regulating transcription, but cant say much as no experimental evidence present
9.	S32	Ribonuclease H II DNA polymerase III (<i>dnaE</i>) (Pf 5) Score = 394 bits (199), Expect = 3e-106, Identities = 342/385 (88%),	Lipid-A-disaccharide synthase and Ribonuclease H (Pf 0-1) Score = 83.8 bits (42), Expect = 2e-12, Identities = 99/118 (83%)	Ribonuclease H	
10.	S27	Flagellar –M-ring protein, <i>fliF</i> (Pf 5) Score = 389 bits (196), Expect = 2e-104, Identities = 244/260 (93%)	Flagellar –M-ring protein, <i>fliF</i> (Pf 5) Score = 607 bits (306), Expect = 4e-170, Identities = 647/762 (84%)	Flagellar –M-ring protein, <i>fliF</i>	Involved in assembly of the basal body of flagellar complex. Transport similar to TypeIII sec pathway
11.	284	Arginine/ornithine antiporter (Pf 5) Score = 432 bits (218), Expect = 1e-117, Identities = 392/450 (87%)	Arginine/ornithine antiporter (Pf 5) Score = 258 bits (130), Expect = 5e-65, Identities = 270/318 (84%)	Arginine/ornithine antiporter	Involved in the transport of aminoacid in and out of the cell. May be involved in secondary metabolite synthesis
12.	290	<i>MoxR2</i> (Pf 5) ATPase associated with several cellular activities, <i>AAA3</i> (Pf 0-1) Score = 569 bits (287), Expect = 9e-159, Identities = 659/784 (84%)	No Hit was found Hit only with vector regions, May be <i>BamHI</i> site is flanking immediately to Tn5 insertion region. Score = 99.6 bits (50), Expect = 3e-17 Identities = 50/50 (100%)	<i>MoxR2</i>	ATPase , Not enough info available

13.	295	Acyl-coA dehydrogenase like (Pf 0-1, Pf 5) Score = 375 bits (189), Expect = 3e-100, Identities = 306/345 (88%)	Acyl-coA dehydrogenase like (Pf 0-1, Pf 5, PAO1) Score = 250 bits (126), Expect = 1e-62, Identities = 269/317 (84%)	Acyl-coA dehydrogenase like	Involved in beta-alanine metabolism, fatty acid metabolism, Propanoate metabolism, valine – leucine-iosleucine degradation.
14.	345	rRNA- 5S ribosomal RNA and Biotin-acetyl –coA carboxylase ligase (Pf0-1, Pf 5) Score = 339 bits (171), Expect = 2e-89, Identities = 256/284 (90%)	Tyrosyl – tRNA synthase and Peptidase (M23/M37 family) (Pf 5) Score = 523 bits (264), Expect = 5e-145, Identities = 423/476 (88%)	Seems Tn5 was inserted in between rRNA-5S ribosomal RNA and Tyrosyl –tRNA synthase	
15.	346	Sodium/alanine transporter/symporter and L- asparaginase I (DC3000, PAO1) Score = 174 bits (88), Expect = 6e-40, Identities = 163/188 (86%)	Sodium/alanine transporter/symporter and L- asparaginase I (DC3000, Pf 5, Pf 0-1) Score = 456 bits (230), Expect = 1e-124, Identities = 563/675 (83%)	Sodium/alanine transporter/symporter and L- asparaginase I	Transport
16.	370	Rod shape – determining protein, <i>mreD</i> (Pf 5) Score = 367 bits (185), Expect = 7e-98, Identities = 368/429 (85%)	Rod shape – determining protein, <i>mreC</i> (P fluorescens, Pf 5) Score = 579 bits (292), Expect = 1e-161, Identities = 450/503 (89%)	Rod shape – determining protein, <i>mreD</i>	The mre genes are responsible for rod shape and mecillinam sensitivity in E. coli. <i>mreD</i> is aprt of the operon <i>mreBCD</i> for peptidoglycan synthesis.
17.	371	Phosphate transport system permease protein 2 (or) Phosphate ABC-transporter	Phosphate transport system permease protein 2 (or) Phosphate ABC-transporter	Phosphate transport system permease protein 2 (or) Phosphate ABC-transporter	Transport of phosphate.

		permease protein (Pf 0-1, Pf 5) Score = 1136 bits (573), Expect = 0.0, Identities = 823/904 (91%),	permease protein (Pf 0-1, Pf 5) Score = 418 bits (211), Expect = 2e-113, Identities = 436/511 (85%)	permease protein	
18.	376	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECDP synthase) (KT2440, Pf 0-1) Score = 454 bits (229), Expect = 4e-124, Identities = 574/686 (83%),	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECDP synthase) (Pf0-1)	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECDP synthase) Score = 432 bits (218), Expect = 1e-117, Identities = 475/553 (85%)	Enzyme in non-mevalonate isoprenoid (terpenoid) biosynthesis, terpenoids secondary metabolites like quinones and also are phytoalexins in plants which can be synthesized in mevalonate dependent or independent pathways.
19.	351	Heat shock protein 15 (Pf 5, DC3000) RNA binding S4 (Pf0-1) Score = 228 bits (115), Expect = 4e-5, Identities = 352/431 (81%)	EAL domain protein (Putative diguanylate diphosphodiesterase) – Pf 5, Pf0-1 Score = 454 bits (229), Expect = 4e-124, Identities = 569/678 (83%)	Between HSP 15 and EAL domain protein	???
20.	289	Protein-export protein <i>SecB</i> Score = 254 bits (128), Expect = 7e-64, Identities = 224/256 (87%) and Glutaredoxin, <i>GrxC</i> or	Hits with vector region only	Probably SecB	Type II secretory pathway

		rhodanese- like glutaredoxin, <i>GrxC</i> (Pf 5, Pf 0-1)			
--	--	---	--	--	--

Table 2: Results summary for 30-84::Tn5 mutants

Serial No.	Mutant ID (Phenotype)	Best hit with tpn13-2 primer sequence	Best hit with tpn17-1 primer sequence	Probable mutated gene	Probable functions of the gene	Possible effects if gene is interrupted
30-84 #2 30-84 #3	Dark orange Dark orange	<i>purU</i> , PFL_4786 Formyltetrahydrofolate deformylase (Pf5, PfO1) Score = 1135 bits (1258), Expect = 0.0 Identities = 793/897 (88%)	PFL_4787 H-NS family protein, transcriptional regulator. (Pf-5, PfO-1) Score = 351 bits (388), Expect = 5e-94 Identities = 283/338 (83%)	Intergenic region between the oppositely oriented genes PFL_4786 and PFL_4787.	probably inhibiting function of either <i>purU</i> or PFL_4787 <i>purU</i> , function in nucleotide transport and metabolism. PFL_4787 being a putative transcriptional regulator may regulate other genes	Inhibition of nucleotide transport and metabolism, mis-regulation of genes under control of PFL_4787.
30-84 #4	Yellowish	<i>glcD</i> , PFL_2269, sub unit of glycolate oxidase (Pf5, PfO1)	<i>glcC</i> , PFL_2270 glc operon transcriptional activator (Pf5, PfO1) Score = 682 bits (756), Expect = 0.0 Identities = 508/600 (84%)	Intergenic region between the oppositely oriented PFL_2269 and PFL_2270.	<i>glcC</i> , transcriptional regulator for the glc operon. <i>glcD</i> , energy production and conversion	Probably interruption of the proper functioning of glc operon involved in energy production and conversion.

References:

1. **Berg, C. M., C. A. Grullon, A. Wang, W. A. Whalen, and D. E. Berg.** 1983. Transductional instability of Tn5-induced mutations: generalized and specialized transduction of Tn5 by bacteriophage P1. *Genetics* **105**:259-263.
2. **de Lorenzo, V. and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol.* **235**:386-405.
3. **Dennis, J. J. and G. J. Zylstra.** 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. *Appl. Environ. Microbiol.* **64**:2710-2715.
4. **Goryshin, I. Y. and W. S. Reznikoff.** 1998. Tn5 in vitro transposition. *J. Biol. Chem.* **273**:7367-7374.
5. **Larsen, R. A., M. M. Wilson, A. M. Guss, and W. W. Metcalf.** 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. *Arch. Microbiol.* **178**:193-201.
6. **Zhou, M. and W. S. Reznikoff.** 1997. Tn5 transposase mutants that alter DNA binding specificity. *J. Mol. Biol.* **271**:362-373.