IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* VIRULENCE FACTORS VIA A POPLAR TREE MODEL

A Dissertation

by

CAN ATTILA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Chemical Engineering

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Approved by:

Chair of Committee,	Thomas K. Wood
Committee Members,	Arul Jayaraman
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	Victor M. Ugaz
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ABSTRACT

Identification of *Pseudomonas aeruginosa* Virulence Factors via a Poplar Tree Model.

(December 2008)

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Chair of Advisory Committee: Dr. Thomas K. Wood

Differential gene expression of *P. aeruginosa* in a rhizosphere biofilm on poplar tree roots was examined in order to identify new virulence factors from this human pathogen. Changes in gene expression for poplar trees contacted with *P. aeruginosa* was examined as well to identify the response of poplar roots to *P. aeruginosa* infection. This is the first study of the whole-transcriptome analysis of *P. aeruginosa* on a plant tree root. The 20 most highly-induced genes of *P. aeruginosa* were examined for their role in biofilm formation, rhizosphere colonization, barley germination, and poplar tree killing assays. Seven previously uncharacterized virulence genes (PA1385, PA2146, PA2462, PA2463, PA2663, PA4150, and PA4295) were identified.

The role of PA2663, a hypothetical protein discovered in the microarrays of *P. aeruginosa* while killing poplar trees, was examined in further detail. Expression of PA2663 protein increases biofilm formation in *P. aeruginosa* PAO1 drastically. By complementing the PA2663 mutation in *trans* and by studying with DNA microarrays and RT-PCR the PA2663 mutant vs. the wild-type strain, PA2663 was confirmed to be related to biofilm formation and was found that it is the first protein to control the *psl* operon in *P. aeruginosa* PAO1. Furthermore, PA2663 protein increases pyoverdine synthesis and quorum sensing (QS)-regulated phenotypes. A biofilm formation-related hypothetical protein, PA0939, was identified

in this study.

The effects of indole and 7-hydroxyindole on *P. aeruginosa* virulence factors were also examined for the first time. Indole and 7HI repressed expression of *mexGHI-opmD* multidrug efflux pump genes and genes involved in synthesis of QS-regulated virulence factors (pyocyanin, rhamnolipid, PQS, and pyoverdine production).

In addition, the effects of an anti-cancer uracil analog, 5-fluorouracil (5-FU) on *P. aeruginosa* virulence factors and *E. coli* K-12 biofilm formation were examined. 5-FU repressed biofilm formation, abolished quorum-sensing phenotypes, and reduced virulence in *P. aeruginosa*. DNA microarray and biofilm studies with 5-FU in *E. coli* revealed that 5-FU controls biofilm formation through the AriR protein in *E. coli* K-12 strain. The effects of *lsrR* and *lsrK* mutations on *E. coli* biofilm formation were also examined by flow cell experiments.

DEDICATION

To

my parents Cevdet and Zeynep Attila

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CHAPTER I

INTRODUCTION

1.1 Background

P. aeruginosa is an opportunistic and versatile pathogenic bacterium with one of the largest sequenced genomes (Stover *et al.*, 2000). It is one of the major causes of chronic lung infections of cystic fibrosis (CF) patients and a major cause of hospital-acquired infections, thrives in many environments (Stover *et al.*, 2000). It causes disease in humans, animals (Lewenza *et al.*, 2005), insects (Jander *et al.*, 2000), nematodes (Tan *et al.*, 1999b), and plants (Rahme *et al.*, 1995). *P. aeruginosa* is notorious for its multiple virulence factors (Gallagher and Manoil, 2001) and resistance to antibiotics and disinfectants (Stover *et al.*, 2000). Although this bacterium has been examined as a model for biofilm formation (Caiazza *et al.*, 2007) and has been studied extensively, one fourth of its open reading frames (ORFs) are unknown (Lewenza *et al.*, 2005). *P. aeruginosa* infects CF patients by forming biofilms (Anderson *et al.*, 2008) and also 80% of bacterial infections are caused by bacteria living in the biofilm state (Costerton *et al.*, 1999). Thus, elucidation of *P. aeruginosa* virulence and biofilm forming genes is crucial for therapeutic approaches.

This dissertation follows the style of *Microbial Biotechnology*.

Virulence factors of *P. aeruginosa* have been examined extensively through *in vitro* studies (Handfield *et al.*, 2000). However, bacterial gene expression and virulence factors differ between *in vivo* and *in vitro* conditions. For instance, some *P. aeruginosa* strains possessing quorum sensing (QS) genes do not produce known QS signals *in vitro* (Willcox *et al.*, 2008). Virulence factors depend on environmental conditions; therefore, *in vivo* knowledge is crucial for therapeutic approaches (Scheie and Petersen, 2004). Screening bacterial mutants to characterize genetic functions with vertebrate animal models is not practical (Rahme *et al.*, 2000), since animal host models are expensive, time consuming, and tedious (Prithiviraj *et al.*, 2005b). Moreover, animal models have intrinsic correlation problems (Rumbaugh *et al.*, 1999). However, plant models are fast, readily available, and cheaper than animal models (Prithiviraj *et al.*, 2005b). Furthermore, pathogenic bacteria use virulence factors in animals and in plants (Jha *et al.*, 2005; Prithiviraj *et al.*, 2005a; Rahme *et al.*, 1997; Rahme *et al.*, 2000). Therefore, plants offer a high-throughput screening method to identify unexplored bacterial virulence and biofilm-related genes.

Poplar can be used as a model plant due to its small genome size (Tuskan *et al.*, 2004) and routine transformation system mediated by *Agrobacterium tumefaciens* (Fillatti, 1987). Although, the determinants of disease resistance are not well-studied in woody plants, poplar has diverse resistance proteins that typically have nucleotide binding sites and leucine rich repeats, in contrast to model grass plants like Arabidopsis and rice (Tuskan *et al.*, 2006).

The control of bacterial gene expression depending on population density is called quorum sensing (QS) (Bassler, 1999). Bacteria synchronize many virulence factors through QS (Miller and Bassler, 2001). The *P. aeruginosa* QS system regulates around 600 genes and many virulence-related characteristics such as biofilm formation (Davies *et al.*, 1998), host colonization (Chun *et al.*, 2004), motility (Déziel *et al.*, 2003), elastase production (Gambello

and Iglewski, 1991), rhamnolipid production (Déziel *et al.*, 2003), and pyocyanin production (Cao *et al.*, 2001). Therefore, drugs that can interfere with QS systems offer potential therapeutic approaches. Although, furanone (Givskov *et al.*, 1996) and some garlic extracts (Rasmussen *et al.*, 2005) repress QS genes of *P. aeruginosa*, these compounds are unstable or their active components are in very low concentrations (Zeng *et al.*, 2008). Therefore, these compounds can not be utilized in therapies (Zeng *et al.*, 2008). Thus, studies to find new target virulence genes and compounds are crucial for therapies of *P. aeruginosa* infections.

1.2 Motivation

Every year 70,000 people incur CF worldwide (Willcox *et al.*, 2008) and by adolescence, 80% of these patients are infected by *P. aeruginosa* (Lyczak *et al.*, 2002). *P. aeruginosa* infections are the leading cause of mortality in CF patients (Nilsson *et al.*, 2007). The multifactorial virulence and the antibiotic resistance of *P. aeruginosa* render its treatment very arduous and sometimes impossible (Stover *et al.*, 2000). The compounds discovered to date that target the virulence genes of *P. aeruginosa* are not useful for therapeutic approaches (Zeng *et al.*, 2008). Furthermore, antibiotic resistant bacteria are emerging (Willcox *et al.*, 2008) and compounds blocking growth increase the resistance of bacteria to antibiotics (Rasmussen and Givskov, 2006). Thus, studying the genes and new compounds involved in virulence is crucial for new therapeutic approaches.

The availability of genome sequences and DNA microarrays have provided a great mechanism to explore the gene expression of the whole genome of an organism (Ye *et al.*, 2000). Bacterial gene expression varies depending on the state (biofilm vs. planktonic) (Ren *et al.*, 2004b), stage (period of the biofilm) (Domka *et al.*, 2007), and effects of different surfaces (Attila *et al.*, 2008a). As gene expression alters with the surface, to discern virulence and

infection genes, it is indispensable to examine gene expression of pathogens on a living host. The interaction of bacteria with plants is similar to the interactions of bacteria with other eukaryotes (Lugtenberg *et al.*, 2002) and the use of plant models makes it easy to screen for putative virulence factors (Prithiviraj *et al.*, 2005b). We found that *P. aeruginosa* is a pathogen to poplar trees and colonizes poplar tree roots (Attila *et al.*, 2008a). The sophisticated mechanism of infection and intrinsic colonization of *P. aeruginosa* on poplar tree roots prompted us to ask how this pathogen and poplar trees perceive and respond to each other. This study is also important to discern the response of the host to a pathogen. The first bacterial transcriptome analysis of a pathogenic bacterium, *Erwinia chrysanthemi* 3937, on African violet leaves revealed that many of the induced genes were related to virulence factors (Okinaka *et al.*, 2002) and a new study of the transcriptome analysis of *P. putida* on maize roots revealed that many efflux genes and aromatic compounds metabolism genes were induced in the rhizosphere (Matilla *et al.*, 2007).

Although the transcriptome analysis of *P. aeruginosa* with diluted sugarbeet root exudates have been previously studied (Mark *et al.*, 2005), the transcriptome analysis of this pathogen has never been explored on a woody plant tissue. Another important difference between Mark *et al.*'s study and our study is that in Mark *et al.*'s study, bacteria were not contacted with plants; therefore, colonization genes and biofilm forming genes of *P. aeruginosa* and the response of the host were not examined. Our whole-transcriptome study is the first study of *P. aeruginosa* in the rhizosphere and a previous study with another pathogenic bacterium with plants leaves had promising results (Okinaka *et al.*, 2002). Moreover, a study of *P. putida* in the rhizosphere of corn (Matilla *et al.*, 2007) was published after the on-line publication of our study "*P. aeruginosa* PAO1 virulence factors and poplar tree response in the rhizosphere" in which virulence-related genes of *P. putida* were deciphered (Matilla *et al.*, 2007).

As many of the virulence factors of *P. aeruginosa* are QS regulated (Mashburn-Warren *et al.*, 2008), targeting compounds to QS genes are important. QS molecules produced from distinct bacteria affect QS and virulence of other bacteria as in the case of AI-2 affecting the virulence genes of *P. aeruginosa* (Duan *et al.*, 2003). Indole is a signal molecule in *E. coli* (Wang *et al.*, 2001) whose derivatives as well as itself repress biofilm formation and attachment (Bansal *et al.*, 2007; Lee *et al.*, 2007a). Moreover, indole derivatives repress the expression of the type III secretion system in *P. aeruginosa* (Shen *et al.*, 2008) and can be used as anticancer agents (Higdon *et al.*, 2007). Therefore, it is promising to examine the effects of indole and indole derivatives in the virulence factors of *P. aeruginosa*. Cancer agents are approved for human therapy, which makes them readily applicable to use in human therapy. Showing that uracil controls QS in *P. aeruginosa* and examining the effects of uracil derivatives, such as 5-fluorouracil, an already approved human cancer agent (Wiebke *et al.*, 2003), can help in the discovery of new antagonists against bacterial infections.

1.3 Research objectives, importance, and novelty

The purpose of this study is to identify the genes involved in virulence of the versatile human pathogen *P. aeruginosa* PAO1 with a poplar tree model by DNA microarray analysis and characterize the twenty most up-regulated genes in DNA microarray analysis. This study is an effort to improve the current knowledge about the *P. aeruginosa* virulence genes. First, the differential gene expression of *P. aeruginosa* while killing poplar trees via a rhizosphere biofilm and the differential gene expression of poplar tree upon *P. aeruginosa* PAO1 contact was examined. This study is novel since it is the first whole-transcriptome analysis of *P. aeruginosa* in the rhizosphere and the first transcriptome analysis of a plant contacted with a pathogenic bacterium and also it is the first use of poplar trees as host model for *P. aeruginosa*. Second,

twenty of the most-induced genes of *P. aeruginosa* PAO1 were examined in detail with *in vivo* and *in vitro* virulence studies. This study is also important because *P. aeruginosa* virulence factors were examined using *in vivo* and *in vitro* studies and new *in vivo* virulence experiments were designed. Third, the role of PA2663, a hypothetical protein that we discovered in poplar tree rhizosphere, was examined in detail by DNA microarrays and quorum-sensing regulated virulence assays. This is novel since it is the first protein found to control *psl* biofilm forming operon of *P. aeruginosa*. Fourth, the effect of indole and 7-hydroxyindole on *P. aeruginosa* virulence factors was examined. This study is novel, since it is the first study of the effects of indole and 7-hydroxyindole on *P. aeruginosa* virulence factors and the pharmaceutical potential as treatments for *P. aeruginosa* infections. Fifth, the role of a *pyrF* mutation and uracil on quorum sensing regulated virulence phenotypes was examined in detail. It is novel since this is the first report of uracil as a signal. Sixth, the effects of a uracil derivative, 5-fluorouracil, on expression of virulence factors with *P. aeruginosa* and on biofilm formation of *E. coli* was examined. This is important since the bacterial effects of an already-approved human treatment anti-cancer drug are examined and this is the first examination of 5-fluorouracil for anti-virulence effects in *P. aeruginosa* and *E. coli.* Seventh, the role of LsrR and LsrK proteins was studied in *E. coli* biofilm formation using flow cells and a scanning confocal microscope.

The specific objectives were to:

- study the gene expression of *P. aeruginosa* PAO1 on poplar tree roots
- study the gene expression of poplar trees in contact with a pathogen, *P. aeruginosa*
- study the proteins of *P. aeruginosa* PAO1 via microarrays with poplar tree roots via *in vitro* experiments (biofilm formation and motility) and via *in vivo* experiments (rhizosphere colonization, poplar tree wilting assay, and barley germination)

- Determine the role of PA2663 protein by studying the gene expression of *P. aeruginosa* PAO1 PA2663 mutant and by studying the effects of the PA2663 mutation on QS phenotypes, phenotype microarrays, and pyoverdine synthesis on *P. aeruginosa* PAO1.
- Investigate the use of indole and 7-hydroxyindole as anti-virulence compounds by studying their effects on QS phenotypes, phenotype microarrays, and pyoverdine synthesis of *P. aeruginosa* PAO1.
- Investigate uracil as an internal messenger by studying the effects of *pyrF* mutation in *P. aeruginosa* PA14
- Investigate 5-fluorouracil as an anti-virulence compound by studying its effects on biofilm formation and QS phenotypes of *P. aeruginosa* PA14
- Investigate 5-fluorouracil as an anti-virulence compound by studying its effects on biofilm formation of *E. coli* and attachment genes in enterohemorrhagic *E. coli*.
- identify the *E. coli* K-12 genes affected by 5-FU with DNA microarrays
- study the effects of *lsrR* and *lsrK* mutations in *E. coli* biofilm formation by flow cell experiments

CHAPTER II

LITERATURE REVIEW

2.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium frequently found in soil, marine habitats, plants, animals, and humans (Filiatrault *et al.*, 2006; Lewenza *et al.*, 2005; Stover *et al.*, 2000). It is the third most-common pathogen associated with hospital-acquired infections (Moreau-Marquis *et al.*, 2008). *P. aeruginosa*, with 6.3 million base pairs and 5,570 open reading frames (ORFs), is one of the largest sequenced bacterial genomes (Stover *et al.*, 2000).

P. aeruginosa has more multidrug efflux pumps than *E. coli*, *B. subtilis,* and *M. tuberculosis* (Stover *et al.*, 2000), which renders it more resistant to antibiotics and difficult to treat. With more than 150 predicted outer membrane proteins and four chemotaxis systems, it is also an important bacterium for the transport and efflux of compounds (Stover *et al.*, 2000).

P. aeruginosa causes disease in humans, animals (Lewenza *et al.*, 2005), in non-mammalian eukaryotes such as insects (Jander *et al.*, 2000), nematodes (Tan *et al.*, 1999b), and in plants (Rahme *et al.*, 1995). It is even used to kill Gram-positive soil bacterium, *Bacillus subitilis* (Park *et al.*, 2005). *P. aeruginosa* is the main pathogen found in the lungs of cystic fibrosis (CF) patients (Singh *et al.*, 2000). In the US, there are around 30,000 CF patients (Lau *et al.*, 2004a), and every year 70,000 people in the world inherit CF (Willcox *et al.*, 2008). By adolescence, 80% of CF patients are infected by *P. aeruginosa* (Lyczak *et al.*, 2002). Chronic colonization of *P. aeruginosa* in CF patients causes progressive lung damage, respiratory failure, and death (Singh *et al.*, 2000). Although, it is believed that the increased salt concentration in the airway fluids decreases the activity of antimicrobial peptides and proteins in the airways of CF patients (Costerton *et al.*, 1999), the relation between the genetic defect in CF patients and

the induced vulnerability to *P. aeruginosa* has not been determined (Yahr and Parsek, 2006). Furthermore, the multifactorial virulence factors of *P. aeruginosa* (Gallagher and Manoil, 2001) and the fact that one fourth of the open reading frames are uncharacterized, (Lewenza *et al.*, 2005) make the discovery of *P. aeruginosa* virulence genes crucial.

2.2 Biofilm formation

Biofilms are highly-structured, matrix-closed bacterial communities within exopolysaccharide encasings (Costerton *et al.*, 1995; Rahme *et al.*, 1997). Ninety-nine percent of all bacteria are found in biofilms (Sauer *et al.*, 2004), and 80% of all bacterial infections are caused from bacteria living in the biofilm state (Costerton, 2004). Biofilms can be up to 1,000 times more resistant to antibiotics than planktonic cells (Klemm *et al.*, 2007). The discovery of biofilms as related to health problems occurred in the 1960s when Johannes Van Houte and Ronald Gibbons discovered that bacteria produce some gummy adhesives that grow on teeth (Torres and Kaper, 2002). Later studies have demonstrated that biofilms adhere to the lungs of CF patients and to many medical devices, such as urinary catheters, prosthetic heart valves (Potera, 1999; Stewart and Costerton, 2001). Bacteria in biofilms infect 4.3% of orthopedic and 7.4% of cardiovascular implants, which cause an annual cost of more than $3 billion in the USA (Klemm *et al.*, 2007). Biofilms not only cause problems in the medical industry, they are the cause of many industrial problems such as damage in oil pipes, water tanks, corrosion, and fouling of heat exchangers (Pasmore and Costerton, 2003).

The biofilm matrix is comprised of bacterial cells, secreted cell products, proteins, polysaccharides, DNA, and water (Allesen-Holm *et al.*, 2006; Sutherland, 2001). Biofilm matrix formation depends on both genetic and environmental factors (Sutherland, 2001). The gene expression of bacteria in the biofilm state differ from their counterparts in planktonic state (Ren

et al., 2004b); therefore, to discern biofilm gene expression, bacterial gene expression has to be determined in the biofilm state.

The biofilm life cycle is composed of five steps, (i) attachment of bacterial cells, (ii) irreversible attachment and production of exopolysaccharides, (iii) early development of biofilms, (iv) maturation of biofilms, and (v) dispersal of biofilms (Stoodley *et al.*, 2002). Therefore, the gene expression of biofilms varies temporally (Domka *et al.*, 2007). In Fig. 2.1, the five steps of biofilm cycle is shown.

2.2.1 Biofilm formation in *P. aeruginosa*

P. aeruginosa has been shown to form biofilms on different surfaces such as CF patients lungs (Govan and Deretic, 1996), contact lenses (Sankaridurg *et al.*, 2000), catheters (Donlan and Costerton, 2002), plants (Attila *et al.*, 2008a; Walker *et al.*, 2004), and teflon (Donlan and Costerton, 2002). *P. aeruginosa* is notorious for biofilm formation (Sauer *et al.*, 2004); hence, it is a model organism for biofilm formation (Caiazza *et al.*, 2007). The biofilm formation of *P. aeruginosa* depends on many factors such as quorum sensing (QS) (*las* system, *rhl* system, and 2-heptyl-3-hydroxy-4-quinolone or PQS) (Chun *et al.*, 2004; Diggle *et al.*, 2006), environmental iron concentration (Bollinger *et al.*, 2001), and nutritional conditions (Sauer *et al.*, 2004).

Exopolysaccharide is required to form a robust biofilm in *P. aeruginosa* (Kuchma *et al.*, 2007), and the biofilm matrix is important in the protection from host defense systems (Fux *et al.*, 2005). The exopolysaccharide (EPS) matrix of *P. aeruginosa* was first characterized by Wozniak *et al.* (2003). *P. aeruginosa* PAO1 EPS matrix consists of glucose, ketodeoxyoctulosonate, mannose, rhamnose, xylose, *N*-acetyl fucosamine, *N*-acetyl galactosamine, *N*-acetyl glucosamine, and *P. aeruginosa* PA14 EPS matrix consists of galactose,

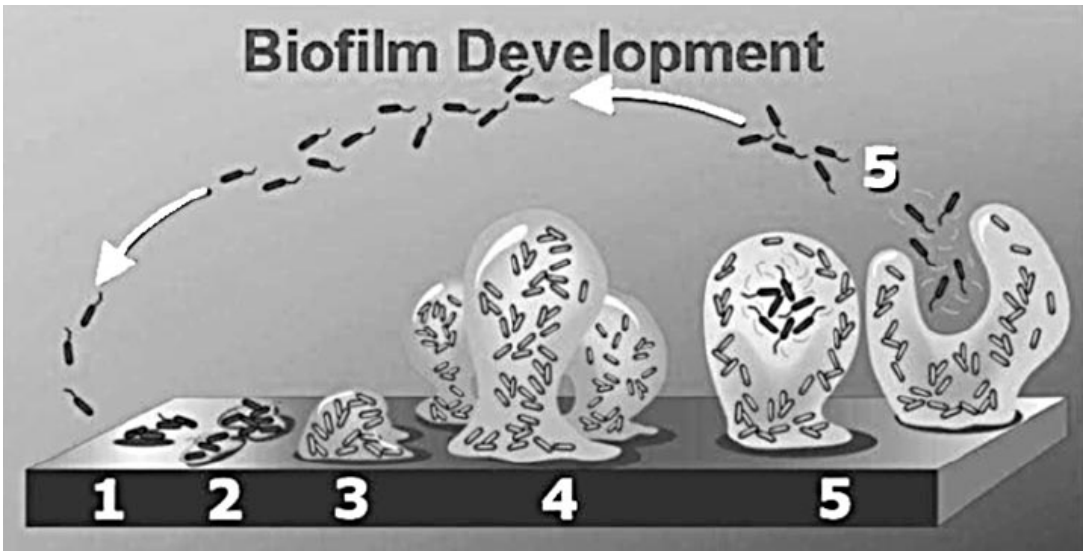

Fig. 2.1. The biofilm life cycle (Stoodley *et al.*, 2002). (1) Attachment of cells, (2) irreversible attachment of bacterial cells, (3) biofilm formation, (4) maturation of biofilm, and (5) dispersal of biofilm cells are shown.

glucose, ketodeoxyoctulosonate, mannose, rhamnose, *N*-acetyl glucosamine, *N*-acetyl quinovosamine, and other unknown sugars (Wozniak *et al.*, 2003).

The gene clusters of *P. aeruginosa* involved in biofilm formation have been studied and two biofilm forming operons, *pel* and *psl* have been identified in *P. aeruginosa* (Friedman and Kolter, 2004a; Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004). The *pel* operon affects biofilm maturation, and the *psl* operon affects biofilm initiation (Ma *et al.*, 2006). Mannose is one of the main components in the polysaccharide produced by the *psl* locus (Friedman and Kolter, 2004b; Ma *et al.*, 2006); hence, mannose is important for biofilm formation in *P. aeruginosa*.

Other biofilm affecting factors for *P. aeruginosa* include the GacA/GacS two-component response regulator that increases biofilm formation and antibiotic resistance (Parkins *et al.*, 2001). Surface attachment is also important since a *sadB* mutation impairs irreversible attachment of *P. aeruginosa* (Caiazza and O'Toole, 2004). Another important biofilm determinant is iron as it promotes both biofilm formation and pathogenecity. Iron is essential for growth and is present at low concentrations (10^{-18} M) (Kaneko *et al.*, 2007). *P. aeruginosa* has over 30 genes targeting iron (Kaneko *et al.*, 2007) and is bound to iron-carriers (siderophores) to increase availability for bacterial cells (Michel *et al.*, 2005).

The knowledge of biofilm formation and other virulence factors of *P. aeruginosa* are mostly based on *in vitro* studies (Handfield *et al.*, 2000). *In vivo* studies to discern the genetic basis of biofilms and other pathogenic factors are crucial for therapeutic approaches (Scheie and Petersen, 2004).

2.2.2 Biofilm formation in *E. coli*

E. coli is the best-studied microorganism (Lugtenberg *et al.*, 2002) and investigation of *E. coli* biofilm genes has helped to understand biofilm formation (González-Barrios *et al.*, 2006a), since the newly-characterized biofilm proteins may be related to well-studied pathways. To date, to investigate the genetic basis of biofilm formation in *E. coli*, five single time point DNA microarrays (Beloin *et al.*, 2004; Hancock and Klemm, 2007; Junker *et al.*, 2006; Ren *et al.*, 2004b; Schembri *et al.*, 2003) and one temporal study (Domka *et al.*, 2007) have been performed. Stress-response genes (*hslST, hha, soxS, ycfR, pspABCDE*, *cpxAR*, *rpoE*, and *rseA*) are common genes seen in *E. coli* biofilm cells (Beloin *et al.*, 2004; Ren *et al.*, 2004b); hence, stress genes are important in biofilm formation of *E. coli*. Carbon catabolism and sulfur metabolism genes were also differentially regulated in *E. coli* biofilms (Beloin *et al.*, 2004; Ren *et al.*, 2004b; Ren *et al.*, 2005a).

E. coli biofilm formation can be affected by different molecules in different ways. Cell signaling molecules, *N*-butyryl-*D,L*-homoserine lactone, *N*-hexanoyl-*D,L*-homoserine lactone, and *N*-octanoyl-*D,L*-homoserine lactone reduce biofilm formation (Lee *et al.*, 2007b); whereas, autoinducer 2 (AI-2) increases biofilm formation in *E. coli* (González-Barrios *et al.*, 2006b). Cyclic diguanylic acid (c-di-GMP) was also linked to increased biofilm formation in *E. coli* (Méndez-Ortiz *et al.*, 2006). Ursolic acid from *Diospyros dendo* (Ren *et al.*, 2005a) and furanone from the marine alga *Delisea pulchra* (Ren *et al.*, 2004a) decrease biofilm formation in *E. coli.* Furanone also inhibits biofilm formation in *P. aeruginosa* (Hentzer *et al.*, 2002) and in Gram-positive bacterium *B. subtilis* (Ren *et al.*, 2002). Hence, biofilm repressing or inducing chemicals are not strain-specific and can affect biofilm formation or other virulence factors in other Gram-positive and Gram-negative bacteria. Epinephrine and norepinephrine increase biofilm formation in *E. coli* O157:H7 (Bansal *et al.*, 2007). Indole, 7-hydroxyindole, and 5-

hydroxyindole decrease biofilm formation in both pathogenic and non-pathogenic *E. coli* (Bansal *et al.*, 2007; Lee *et al.*, 2007a; Lee *et al.*, 2007b). Isatin increases biofilm formation in pathogenic *E. coli* and does not affect the biofilm formation in non-pathogenic *E. coli* K-12 strains (Lee *et al.*, 2007a). Therefore, the effects of biofilm-related chemicals can diverge in pathogenic and in non-pathogenic *E. coli* strains.

2.3 Plant-bacteria interactions

Depending on the bacteria and the plant host, plant-bacteria interactions can be beneficial, saprophytic, or pathogenic (Lugtenberg *et al.*, 2002). Bacteria can promote plant growth by producing nitrogen, micronutrients, and phytohormones (Lugtenberg and Dekkers, 1999) and protect plants from fungal pathogens (Troxler *et al.*, 1997). Likewise, the rhizosphere provides sugars and amino acids for bacterial growth (Lugtenberg and Dekkers, 1999). Furthermore, bacteria can help plants for phytoremediation, a process to degrade hazardous chemicals with plants (Lugtenberg and Dekkers, 1999). In plant-bacteria interactions, the host and the bacteria must be chosen deliberately. For instance, *P. aeruginosa* is a pathogen of lettuce (Filiatrault *et al.*, 2006) and *Arabidopsis* (Walker *et al.*, 2004); however, it is not a pathogen to sugar beets (Mark *et al.*, 2005).

Plant-bacteria interactions provide a great opportunity to examine bacterial virulence factors *in vivo*. Tan *et al.* (1999a) revealed that in *P. aeruginosa*, the same genes are necessary to kill both *Arabidopsis thaliana* and *C. elegans*. This conservation of virulence factors in animals and in plants is also studied in other human pathogenic bacteria such as *Enterococcus faecalis* (Jha *et al.*, 2005) and *Staphylococcus aureus* (Prithiviraj *et al.*, 2005a). Correlating the data from animal infection models with the data from clinical isolates possesses intrinsic problems (Rumbaugh *et al.*, 1999) and also animals models are expensive, tedious, and time-

consuming (Prithiviraj *et al.*, 2005b). Screening bacterial mutant libraries with any vertebrate animal is not practical (Rahme *et al.*, 2000). However, plant models are fast, readily-available, and relatively cheaper than animals, which makes plant models indispensable for high throughput methods to discover virulence genes (Filiatrault *et al.*, 2006; Prithiviraj *et al.*, 2005b). Furthermore, the first bacterial transcriptome analysis of a pathogenic bacterium, *Erwinia chrysanthemi* 3937, on African violet leaves revealed that many of the induced genes are in virulence-related factors, iron uptake, xenobiotic resistance, and chemotaxis (Okinaka *et al.*, 2002).

Plants also produce a variety of molecules having potential pharmaceutical value in the case of ursolic acid inhibiting biofilm formation in *E. coli* (Ren *et al.*, 2005a) and garlic extracts repressing the QS and virulence genes of *P. aeruginosa* (Rasmussen *et al.*, 2005). Plants producing acyl-homoserine lactonase repress the QS signaling of *E. carotovora* and become more resistant to the infections caused by this pathogenic bacterium (Dong *et al.*, 2001). Genetically-modified plants that produce bacterial QS molecules are also becoming important in disease control and manipulation of plant-bacteria interactions (Fray *et al.*, 1999).

2.3.1 *P. aeruginosa*-plant interactions

Rahme *et al.* (1995) revealed that *P. aeruginosa* uses many of the same virulence factors in both plants and animals. Additional publications also show that many *P. aeruginosa* virulence genes are important for virulence with plants and animals (Rahme *et al.*, 1997; Rahme *et al.*, 2000; Silo-Suh *et al.*, 2002; Tan *et al.*, 1999a; Tan *et al.*, 1999b). Although, *P. aeruginosa* colonizes alfalfa seedlings (Silo-Suh *et al.*, 2002), cucumber roots (Lugtenberg and Dekkers, 1999), lettuce leaves (Filiatrault *et al.*, 2006), sweet basil roots (Walker *et al.*, 2004), sugar beet roots (Mark *et al.*, 2005), tobacco leaves (Weir *et al.*, 2008), wheat roots (Lugtenberg and

Dekkers, 1999), and *Arabidopsis* roots (Walker *et al.*, 2004), *Pseudomonas*-plant interactions are not well understood (Lugtenberg *et al.*, 2002).

P. aeruginosa infections on *Arabidopsis* (Plotnikova *et al.*, 2000) and lettuce leaves (Filiatrault *et al.*, 2006), and wounded stems (Silo-Suh *et al.*, 2002) have been studied. However, these models can not be used as a characteristic feature for colonization since plant roots and surrounding areas are favorable for bacterial colonization (Molina *et al.*, 2003). Therefore, elucidation of gene expression of *P. aeruginosa* on plant roots is important, until our study (Chapter III), the gene expression of a bacterium on a plant root had not been examined. Only Mark *et al.* (2005) studied the influence of sugar beet root exudates on the *P. aeruginosa* PAO1 transcriptome. Although this study was important, the root exudates were diluted in a different medium, which decreases the effects of the root exudates, and the examined transcriptome was not obtained from colonized cells, making it possible that colonization-related genes may no longer be elicited.

Similar to our study, the sugar root exudates induced expression of PA0984, which encodes an immunity protein, and the expression of PA2146, a hypothetical protein, found in our microarray analysis and important for barley germination (Chapter III) (Mark *et al.*, 2005). Moreover, PA1458 (*cheA*, response regulator) and PA1092 (*fliC*, flagellin type B) were repressed in our study (Chapter III) as seen with sugar beet root exudates (Mark *et al.*, 2005).

After our study, the whole-transcriptome of *P. putida* on maize roots was performed (Matilla *et al.*, 2007). As in our study (Chapter III), the chemotaxis and transporter genes were differentially regulated in *P. putida* upon contacting the maize roots (Matilla *et al.*, 2007). Also, a new study of *P. aeruginosa* on *Nicotiana tabacum* leaves (Weir *et al.*, 2008) shows that transporter genes of *P. aeruginosa* are differentially regulated upon the contact with plants.

Table 2.1. Partial list of the induced or repressed genes of *P. aeruginosa* upon poplar tree root contact (Chapter III), upon exposure to sugar beet root exudates (Mark *et al.*, 2005), and upon contact with *N. tabacum* leaves (Weir *et al.*, 2008), as well as for *P. putida* upon contact with maize roots (Matilla *et al.*, 2007).

PA#	PP#	Fold change upon poplar root contact	Fold change upon contact with sugar beet root exudates	Fold change upon contact with *N. tabacum* leaves	Fold change of *P. putida* upon contact with maize roots	Function
Carbon compound catabolism						
PA0231	-	-1.1	1.7		-	Beta-ketoadipate enol-lactone hydrolase
PA4148	-	2.8			-	Putative enzyme (probable short-chain dehydrogenase)
PA4149	-	3.5			-	Conserved hypothetical protein (acetoin catabolism protein homolog, acoX)
PA4150	-	4.6			-	Probable dehydrogenase E1 component
PA4151	-	5.7	10.3		-	Acetoin catabolism protein AcoB
PA4152	-	3.5			-	Probable hydrolase
PA4153	-	6.5			-	2,3-butanediol dehydrogenase
Cell wall/LPS/capsule						
PA1385	-	3.2			-	Probable glycosyl transferase
PA3540	-	1.4	3.7		-	GDP-mannose 6-dehydrogenase AlgD
PA3545	-	1.5	5.1		-	Alginate-c5-mannuronan-epimerase AlgG
PA3548	-	1.2	3.2		-	Alginate o-acetyltransferase AlgI
PA3551	-	1.2	2.2			Phosphomannose isomerase/guanosine 5'-diphospho-*D*-mannose pyrophosphorylase
Central intermediary metabolism						
PA3296	-	1.3		-3.9	-	Alkaline phosphatase
Chaperons & heat shock proteins						
PA0538	-	2.6			-	Disulfide bond formation protein
Chemotaxis, flagella, motility and attachment						
PA0412	-	1.1	2.2			Methyltransferase PilK
PA1092	-	-11.3	1.4		-	Flaggellin type BH

Table 2.1. (continued)

PA#	PP#	Fold change upon poplar root contact	Fold change upon contact with sugar beet root exudates	Fold change upon contact with *N. tabacum* leaves	Fold change of *P. putida* upon contact with maize roots	Function
PA1093	-	-9.8			-	Middle portion 58% similar to flagellin protein
PA1094	-	-7.0			-	Flagellar capping protein FliD
PA1458	-	-3.5	1.2		-	Probable chemotaxis signal transduction histidine kinase
PA2788	-	-6.5			-	Probable chemotaxis transducer
PA3349	-	-5.7			-	Probable chemotaxis protein
PA3351	-	-8.6			-	Hypothetical protein (61% similarity to FlgM)
PA3361	-	-21.1			-	Fucose-binding lectin PA-IIL
PA3385	-	-6.1			-	Alginate and motility regulator Z
-	PP4331	-	-	-	5.0	Conserved hypothetical protein
-	PP4359	-	-	-	3.7	Flagellar protein FliL
-	PP4391	-	-	-	4.3	Flagellar basal-body rod protein FlgB
-	PP4987	-	-	-	5.9	Chemotaxis protein
Colicin immunity protein						
PA0981	-	5.7			-	Hypothetical protein
PA0984	-	10.6	1.8		-	Membrane proteins; secreted factors (toxins, enzymes, alginate)
DNA replication, recombination, and repair						
-	PP1476	-	-	-	33.7	Conserved hypothetical protein
-	PP3966	-	-	-	11.7	ISPpu14 transposase OrfI
Efflux pumps						
-	PP1271	-	-	-	17.4	Multidrug efflux MFS transporter putative
Energy metabolism						
PA0519	-	2.6			-	Nitrite reductase precursor
PA0523	-	2.6			-	Nitric-oxide reductase subunit C
PA0524	-	2.6			-	Nitric-oxide reductase subunit B

Table 2.1. (continued)

PA#	PP#	Fold change upon poplar root contact	Fold change upon contact with sugar beet root exudates	Fold change upon contact with *N. tabacum* leaves	Fold change of *P. putida* upon contact with maize roots	Function
Fatty acid and phospholipid metabolism						
PA3300	-	2.6			-	Long-chain-fatty-acid-CoA ligase
Hypothetical, unclassified, unknown function						
PA0529	-	-7.5			-	Conserved hypothetical protein (60% similar to hypothetical protein YiiM *E. coli* (function unknown))
PA1096	-	-6.1	1.5		-	Hypothetical protein
PA2146	-	3.5	5.8		-	Hypothetical protein (92% similar to hypothetical *yciG* gene product of *E. coli*)
PA2222	-	2.6	2.8		-	Hypothetical protein, cell surface antigen, glycine cleavage, aminomethyl transferase
PA2459	-	7.0			-	Hypothetical protein
PA2461	-	19.7			-	Hypothetical protein
PA5138		2.8			-	Hypothetical protein (type I export signal, HisJ, ABC-type amino acid transport/signal transduction systems, periplasmic component)
Membrane proteins						
PA1960	-	2.8			-	Hypothetical protein (3 predicted transmembrane helices, integral membrane protein in *P. syringae*, cold-shock domain family protein in *Methylococcus capsulatus* str.)
PA2663	-	2.8			-	Hypothetical membrane protein
PA3278	-	2.8			-	Hypothetical protein (3 predicted transmembrane helices, ABC transporter related, predicted zinc finger)
PA3279	-	2.1		-6.2	-	Phosphate-specific outer membrane porin OprP precursor
PA4295	-	3.0		-6.2	-	Hypothetical protein (peptidase in *P. fluorescens*)

Table 2.1. (continued)

PA#	PP#	Fold change upon poplar root contact	Fold change upon contact with sugar beet root exudates	Fold change upon contact with *N. tabacum* leaves	Fold change of *P. putida* upon contact with maize roots	Function
Metabolism						
-	PP2694	-	-	-	10.2	Aldehyde dehydrogenase family protein
-	PP2847	-	-	-	24.6	Urease accessory protein
-	PP3352	-	-	-	31.5	Phenylacetic acid degradation protein PaaI putative
Motility and attachment						
PA1080	-	-2.0	1.3		-	Flagellar hook protein FlgE
PA1460	-	-2.8	1.5		-	MotC
PA4549	-	4.3			-	Type 4 fimbrial biogenesis protein FimT
Protein secretion and export apparatus						
PA1691	-	2.1			-	Translocation protein in type III secretion
PA1694	-	1.5	1.5		-	Translocation protein in type III secretion
PA1712	-	2.3			-	Exoenzyme S synthesis protein B, type III secretion
PA1718	-	2.3			-	Type III secretion protein PscE
Putative peptidase						
PA5181	-	-3.2		3.6	-	Probable oxidoreductase
Regulators and sensor proteins						
-	PP0700	-	-	-	30.7	Transmembrane senesor putative
-	PP1066	-	-	-	17.6	Sigma 54 dependent response regulator
-	PP2127	-	-	-	19.8	Sensor histidine kinase
Secreted factors						
PA3319	-	1.4		-5.4	-	Non-hemolytic phospholipases C precursor
Transcription, RNA processing, and degradation						
PA2840	-	-6.5			-	Probable ATP-dependent RNA helicase
PA5239	-	-6.1			-	Transcription termination factor Rho

Table 2.1. (continued)

PA#	PP#	Fold change upon poplar root contact	Fold change upon contact with sugar beet root exudates	Fold change upon contact with *N. tabacum* leaves	Fold change of *P. putida* upon contact with maize roots	Function
Transcriptional regulator						
PA0160	-	-7.5			-	Hypothetical protein (type I export signal, next to transcriptional regulator gene)
PA0513	-	3.2			-	Energy metabolism; transcriptional regulators; biosynthesis of cofactors, prosthetic groups and carriers
PA0874	-	-11.3	1.4		-	Hypothetical protein (type I export signal, next to transcriptional regulator, transcriptional regulator in *P. syringae* and *P. fluorescens*)
Translation, post-translational modification, degradation						
PA2742	-	-5.7			-	50S ribosomal protein L35
PA3162	-	-5.7			-	30S ribosomal protein S1
PA3656	-	-5.3			-	30S ribosomal protein S2
PA4241	-	-6.5			-	30S ribosomal protein S13
PA4242	-	-5.3			-	50S ribosomal protein L36
PA4246	-	-5.7			-	30S ribosomal protein S5
PA4258	-	-5.3			-	50S ribosomal protein L22
PA4259	-	-5.9			-	30S ribosomal protein S19
PA4260	-	-5.3			-	50S ribosomal protein L2
PA4261	-	-5.3			-	50S ribosomal protein L23
PA4263	-	-5.3			-	50S ribosomal protein L3
PA4268	-	-5.3			-	30S ribosomal protein S12
PA4272	-	-5.7			-	50S ribosomal protein L10
PA4273	-	-5.7			-	50S ribosomal protein L1
PA4568	-	-5.7			-	50S ribosomal protein L21

Table 2.1. (continued)

PA#	PP#	Fold change upon poplar root contact	Fold change upon contact with sugar beet root exudates	Fold change upon contact with *N. tabacum* leaves	Fold change of *P. putida* upon contact with maize roots	Function
Transport of small molecules						
PA0280	-	1.4		4.2	-	Sulfate transport protein CysA
PA0281	-	1.9		3.9	-	Sulfate transport protein CysW
PA0282	-	1.4		2.7	-	Sulfate transport protein CysT
PA0323	-	2.6			-	Probable binding protein component of ABC transporter
PA0450	-	1.7		-6.0	-	Probable phosphate transporter
PA1019	-	2.6			-	Cis,cis-muconate transporter MucK
PA3280	-	1.7		-7.1	-	Pyrophosphate specific outer membrane protein
PA3376	-	2.1		-6.0	-	Probable ATP-binding component of ABC transporter
PA3384	-	1.7		5.2	-	ATP-binding component of ABC phosphonate transporter
PA4861	-	1.5		-5.9	-	Probable ATP-binding component of ABC transporter
Virulence associated protein						
PA4674	-	2.8			-	Conserved hypothetical protein (VapI, 69% similar to virulence-associated protein in *Dichelobacter nodosus*)

A partial list of repressed and induced genes of *P. aeruginosa* upon poplar tree root contact, upon sugar beet root exudates, and upon contact with *N. tabacum* leaves, and the partial list of induced or repressed genes of *P. putida* upon maize root contact are shown in Table 2.1.

2.3.2 Bacterial colonization in the rhizosphere

Numerous bacterial genes are involved in plant root colonization (Somers *et al.*, 2004). Chemotaxis, motility, stress response, biofilm formation, biosynthesis of phytohormones, and nutrient acquisition genes are induced in *A. tumefaciens*, *Rhizobium*, and *P. fluorescens* (Somers *et al.*, 2004). The genes involved in root colonization are also involved in animal tissue colonization (Lugtenberg *et al.*, 2001). Many microbial species colonize the rhizosphere and the production of EPS may help bacteria colonize roots and increase survival (Morris and Monier, 2003). Plant root colonization is the first step in biofilm formation, and bacterial cells attached to the plant roots are postulated to be similar to biofilm cells (Somers *et al.*, 2004). Biofilms on roots have similar properties; however, biofilms vary on aerial parts of plants due to the variations in water availability (Morris and Monier, 2003). Biofilm formation can be pathogenic or beneficial to the plants as in the case of *Cyanobacterium* biofilms which serve as a glue for plant attachment to rocks in waterfalls (Jäger-Zürn and Grubert, 2000).

Root exudates serve as chemoattractants and induce the expression of plant-infection genes (Zhu *et al.*, 1997). The rhizosphere is a dynamic environment that allows one to study the bacterial intra- and inter-species communication networks (Dunn and Handelsman, 2002). Elucidation of genes involved in rhizosphere competition is extremely significant for bioremediation, control of plant diseases (Lugtenberg and Dekkers, 1999), and possibly for the control of human diseases. Transcriptome analysis of *P. putida* on maize roots revealed that many efflux genes and metabolism of aromatic compounds genes are induced in the rhizosphere

(Matilla *et al.*, 2007). A correlation between the efflux pumps and virulence has been established (Aendekerk *et al.*, 2005). Moreover, further examination of plant exudates will help discover novel compounds to inhibit and interfere with QS and bacterial infections as in the case of pea exudates mimicking the bacterial homoserine lactone signals (Teplitski *et al.*, 2000) and sweet basil exudate, rosmarinic acid, exhibiting antibacterial activity (Walker *et al.*, 2004).

2.4 Quorum sensing

QS is involved in host colonization (Chun *et al.*, 2004), adhesion (Miller and Bassler, 2001), acid tolerance (Suntharalingam and Cvitkovitch, 2005), biofilm formation (Davies *et al.*, 1998), motility (Déziel *et al.*, 2003), and sporulation (Miller and Bassler, 2001). QS is bacterial communication by which certain target genes are induced or repressed depending on population density. Bacterial signaling occurs in two ways; (i) the signals are produced from another organism, (ii) the signals are produced from the organism itself (Lyon and Novick, 2004). Cell density dependent signals are called QS signals (Jayaraman and Wood, 2008). QS synchronizes bacterial behaviors. The discovery of QS goes back to the 1970s with the study of light production in deep-sea marine organisms (Nealson, 1977). QS is used by both Gram-positive and Gram-negative bacteria and is controlled by a complex regulatory network at transcriptional and post transcriptional levels (Liang *et al.*, 2008; Miller and Bassler, 2001). QS bacteria utilize signal molecules depending on the cell density, usually Gram-negative bacteria utilize acylated homoserine lactones (AHLs) and Gram-positive bacteria utilize oligopeptides (Lyon and Novick, 2004; Miller and Bassler, 2001).

2.4.1 Quorum sensing in *P. aeruginosa*

QS in *P. aeruginosa* regulates many of complex virulence factors (Mashburn-Warren *et al.*, 2008), including expression of approximately 600 genes in *P. aeruginosa* (Schuster *et al.*, 2003; Wagner *et al.*, 2003). In *P. aeruginosa*, three quorum sensing systems are described, the *las* system (Passador *et al.*, 1993) with signal molecule *N*-(3-oxo-dodecanoyl)-*L*-homoserine lactone (3-oxo-C_{12}-HSL), the *rhl* system (Latifi *et al.*, 1995) with signal molecule *N*-butyryl-*L*-homoserine lactone (C_4-HSL), and the *Pseudomonas* quinolone system (PQS) (Pesci *et al.*, 1999) with signal molecule 2-heptyl-3-hyrodxy-4-quinolone.

These three QS systems are intertwined (Diggle *et al.*, 2006; Miller and Bassler, 2001). LasR regulates the expression of *rhlR* and rhamnolipid biosynthesis (Ochsner and Reiser, 1995) and is required for PQS production (Pesci *et al.*, 1999). The *las* and *rhl* systems are believed to work sequentially (Miller and Bassler, 2001). PQS, the third QS system in *P. aeruginosa*, regulates the production of elastase, rhamnolipids, and pyocyanin (Diggle *et al.*, 2006). Elastase production is regulated by *lasR* (Passador *et al.*, 1993), and rhamnolipid and pyocyanin production is regulated by the *rhl* operon (Pearson *et al.*, 1997; Wagner *et al.*, 2006a). PQS is a hydrophobic compound transported in *P. aeruginosa* through membrane vesicles (Mashburn and Whiteley, 2005).

All the three QS systems in *P. aeruginosa* regulate many virulence factors. Davies *et al.* (1998) first revealed that QS is important for biofilm formation in *P. aeruginosa*. The *lasI* mutant formed less biofilm, and the biofilm architecture was flat compared to the wild-type strain (Davies *et al.*, 1998). The *lasR* mutant can not proliferate in the gut of *C. elegans* and is less virulent to *Arabidopsis* (Tan *et al.*, 1999b) and mice (Rumbaugh *et al.*, 1999). The *rhlI* mutant is less virulent in mice (Pearson *et al.*, 2000; Rumbaugh *et al.*, 1999), and we showed that

the *pqsA* mutant is less virulent to poplar trees (Attila *et al.*, 2008a). Both 3-oxo-C_{12}-HSL and C_4-HSL were detected and measured in the sputum of CF patients (Erickson *et al.*, 2002).

2.4.2 Interactions of quorum sensing molecules with different bacteria

AI-2 is a QS molecule in *E. coli* (Li *et al.*, 2007). AI-2 increases biofilm formation in *E. coli* (González-Barrios *et al.*, 2006b) and attachment of EHEC to HeLa cells (Bansal *et al.*, 2008). *P. aeruginosa* does not have a *luxS* gene homolog to produce AI-2; however, AI-2 affects the expression of virulence genes in this bacterium (Duan *et al.*, 2003). Numerous AHLs from different bacteria were shown to act as signal molecules such as *N*-(3-hydroxy-7-cis-tetradecenoyl) homoserine lactone from *Rhizobium leguminosarum* which acts as a signal molecule and has bacteriocin activity for *R. leguminosarum* (Lithgow *et al.*, 2000). Also, enzyme AiiA from Gram-positive bacterium *Bacillus* sp. 24081 represses the acylhomoserine lactone QS signaling in *E. carotovora* (Dong *et al.*, 2000). These results show that QS-related molecules that are produced from different bacteria can affect the QS and virulence factors in other bacteria deficient in producing these chemicals. Determining the chemical signals, their receptors their target genes, and the affected pathways are the essence of deciphering bacterial communication (Miller and Bassler, 2001).

2.4.3 Quorum sensing inhibitors

To date, there are few known anti-virulence compounds (Cegelski *et al.*, 2008); anti-virulence compounds are an important way to fight infectious diseases because unlike antimicrobials, anti-virulence compounds do not affect growth and so there is less chance of developing resistance (Hentzer *et al.*, 2002). When bacteria encounter growth inhibitors, they develop resistance, which makes treatment of bacterial infections more difficult (Rasmussen and

Givskov, 2006). Compounds controlling the expression of virulence genes and quorum sensing are important for the design and development of anti-virulence compounds (Lesic *et al.*, 2007).

2.4.3.1 Furanone

Analogs of autoinducers (e.g. 3-oxohexanoyl homoserine lactone) inhibit bioluminescence in *V. fischeri* (Eberhard *et al.*, 1986) and furanone has a similar structure to AHLs (Givskov *et al.*, 1996). Furanone is produced from the alga *Delisea pulchra* (de Nys *et al.*, 1993). Furanone and furanone derivatives possess antitumor, antiviral, anti-inflammatory, and antimicrobial activities (Kim *et al.*, 2008). Furanone inhibits the bioluminescence of *V. harveyi* (Manefield *et al.*, 2000). In *V. harveyi*, furanone interferes with the binding of the QS master regulator protein $LuxR_{vh}$ to its target promoters (Defoirdt *et al.*, 2007). In *P. aeruginosa*, furanone represses biofilm formation, mice infection (Hentzer *et al.*, 2003), and pyoverdine production (Ren *et al.*, 2005b). A transcriptome analysis of furanone showed it repressed 93 genes of *P. aeruginosa* and 30% of these genes were QS genes (Hentzer *et al.*, 2003). Furanone inhibits QS in *E. coli* by inhibiting AI-2 signaling (Ren *et al.*, 2004a). Furanone also inhibits QS in *Serratia liquefaciens* MG1 (Rasmussen *et al.*, 2000) and the expression of virulence genes in *B. anthracis* (Jones *et al.*, 2005).

2.4.3.2 Indole

In 1866, Baeyer and Knop discovered indole, a building block of indigo (Higasio and Takayuki, 2001). Indigo is a dye widely used in the textile industry with an annual production of 22,000 tons per year (Ensley *et al.*, 1983; Maugard *et al.*, 2002). A variety of bacteria produce indole from *L*-tryptophan such as *E. coli* (Crawford and Yanofsky, 1958), *Vibrio vulnificus* (Dalsgaard *et al.*, 1999), *Haemophilus influenzae* (Stull *et al.*, 1995), *Pasteurella multocida*

(Clemons and Gadberry, 1982), *Klebsiella oxytoca* (Liu *et al.*, 1997), and *Proteus vulgaris* (DeMoss and Moser, 1969), NCBI BLAST search shows more than 27 genera utilize tryptophanase (*tnaA*) to convert tryptophan into indole, pyruvate, and ammonia (Stewart and Yanofsky, 1985). Indole is an extracellular *E. coli* signal (Wang *et al.*, 2001) that represses biofilm formation, motility, acid resistance (Bansal *et al.*, 2007; Domka *et al.*, 2006; Lee *et al.*, 2007b; Lee *et al.*, 2008), and attachment of EHEC to epithelial cells (Bansal *et al.*, 2007). Indole works in a QS fashion (Lee *et al.*, 2007a) primarily at temperatures less than 37°C in *E. coli* (Lee *et al.*, 2008). Indole increases the expression of multidrug exporter genes in *E. coli* (Hirakawa *et al.*, 2005) and *S. enterica* (Nikaido *et al.*, 2008). Indole induces biofilm formation of *P. fluorescens* and *P. aeruginosa* (Lee *et al.*, 2007b). In this study, we show that indole induces the multidrug efflux pump and antibiotic resistance and represses QS-regulated virulence factors of *P. aeruginosa*.

2.4.3.3 7-Hydroxyindole

Many bacterial oxygenases such as dioxygenases from *P. putida* PpG7 (Ensley *et al.*, 1983), *Ralstonia picketti* PKO1 (Fishman *et al.*, 2005), and *Burkholderia cepacia* G4 (Rui *et al.*, 2005) readily convert indole to indirubin, indigo, and hydroxyindoles (Rui *et al.*, 2005). Indole derivative (CBR-4830) has been shown to inhibit *P. aeruginosa* growth through a multi-drug efflux pump, *mexAB-oprM* (Robertson *et al.*, 2007) and indole-3-acetic acid increases the virulence of *Agrobacterium tumefaciens* (Gafni *et al.*, 1995) and inhibits the expression of type III secretion system (Shen *et al.*, 2008). Indole derivatives are even used as anticancer agents (Higdon *et al.*, 2007); therefore, indole derivatives may play an important role in bacterial virulence and therapies. 7-hydroxyindole represses biofilm formation of *E. coli* K-12 and EHEC and induces the biofilm formation of *P. aeruginosa* (Lee *et al.*, 2007a). In this study, we show

that 7-hydroxyindole represses QS-related virulence phenotypes and colonization of *P. aeruginosa* in guinea pigs and increases the antibiotic resistance.

2.4.3.4 5-fluorouracil

5-fluorouracil (5-FU) is an anticancer drug used for more than 50 years against malignancies occurring in the gastrointestinal tract, breast, head, and neck (Malet-Martino *et al.*, 2002). Below inhibitory concentrations, 5-FU decreases biofilm formation in *S. epidermis* (Hussain *et al.*, 1992). In this study, we discovered that uracil influences biofilm formation and other QS-regulated virulence factors in *P. aeruginosa* and that the uracil derivative, 5-FU, inhibits biofilm formation and other QS-regulated virulence factors. 5-FU is used as colon cancer drug and *E. coli* colonizes in the intestine; therefore, the effects of 5-FU on biofilm formation and attachment phenotypes of *E. coli* K-12 and EHEC strains are examined here.

2.5 Virulence factors of *P. aeruginosa*

P. aeruginosa is notorious for its multiple virulence factors such as adhesins, biofilm formation, cyanide production, elastase production, hemagglutinin, motility, phenazines, pyocyanin, rhamnolipid, type III secretion, siderophores, and colonization (Gallagher and Manoil, 2001; Holder *et al.*, 2001; Lamont *et al.*, 2002; Lugtenberg *et al.*, 2001; Smith and Iglewski, 2003; Stover *et al.*, 2000). Many of these virulence factors are QS regulated, which suggests that successful infection starts after a certain bacterial density is reached (Pukatzki *et al.*, 2002). These virulence factors have different effects; for instance, pyocyanin inhibits cellular respiration (Hassett *et al.*, 1992), elastase disrupts the blood vessels and degrades the extracellular matrices of epithelial cells (Bejarano *et al.*, 1989), and rhamnolipids disrupts the cells and promote the invasion of *P. aeruginosa* (Zulianello *et al.*, 2006). The tissue damage

caused by toxins of *P. aeruginosa* affect the human immune system (Winsor *et al.*, 2005). *P. aeruginosa* utilize the arsenal of virulence factors (biofilm formation, elastase production, hemolysis, motility, pyocyanin production, and rhamnolipid production) in different stages of infection and secrete toxins to kill the host cells (Mahajan-Miklos *et al.*, 1999). In this study, we examined antibiotic resistance, biofilm formation, rhizosphere colonization, siderophores, hemolytic and cytotoxic activity, motility, elastase, rhamnolipid, pyocyanin, and PQS production of *P. aeruginosa* as virulence factors.

2.5.1 Siderophores

Iron is necessary for the growth of all bacterial species (Tsuda *et al.*, 1995) and affects *P. aeruginosa* pathogenesis and rhizosphere competence (Poole and McKay, 2003). Iron concentrations are increased in the airways of CF patients; this increase helps *P. aeruginosa* to cause persistent infections (Moreau-Marquis *et al.*, 2008). In mammalian hosts, bacteria can not acquire iron freely, and siderophores, iron chelators and iron transporters, are used by microbial pathogens (Michel *et al.*, 2005). *P. aeruginosa* uses two siderophores, pyochelin and pyoverdine to accumulate iron, and more than 30 genes to encode iron receptors (Kaneko *et al.*, 2007; Michel *et al.*, 2005).

Pyoverdine is the main siderophore of *P. aeruginosa* (Meyer *et al.*, 1996). Mutants deficient in pyoverdine synthesis are less virulent in mice (Meyer *et al.*, 1996). In *P. fluorecens,* pyoverdine deficient mutants are less competent in the rhizosphere (Mirleau *et al.*, 2000). In *P. aeruginosa,* pyoverdine production is mainly regulated by the *pvd* operon, and PvdS functions as a sigma factor in pyoverdine production (Cunliffe *et al.*, 1995). Pyochelin has a lower affinity than pyoverdine for iron but is still important for *P. aeruginosa* virulence (Poole and McKay, 2003). Pyochelin synthesis is regulated by two operons *pchDCBA* and *pchEFGHI* (Poole and

McKay, 2003). Pyochelin is also involved in the pathogenesis of *B. cepacia* (Sokol and Woods, 1988).

2.5.2 Motility

Motility is indispensable for biofilm formation and infection by *P. aeruginosa* (Van Alst *et al.*, 2007). Non-motile *Pseudomonas* mutants are severely impaired in colonization of alfalfa (Martínez-Granero *et al.*, 2006). Mortality rates for endogenous sepsis in mice are commensurate with the motility of *P. aeruginosa* strains (Hatano *et al.*, 1996). *P. aeruginosa* performs three types of motility: swimming, swarming, and twitching motility. Outside a host, the agar concentration dictates the type of motility. Swimming motility is assayed on aqueous surfaces with 0.3% agar, swarming motility is assayed on semi-solid surfaces with 0.5% agar, and twitching motility is assayed on solid surfaces with 1.0% agar (Overhage *et al.*, 2007; Rashid and Kornberg, 2000). Flagella is required for swimming and swarming motility, whereas, type IV pili are required for twitching motility (Rashid and Kornberg, 2000). Swimming motility is required for biofilm development and dispersal (Bai *et al.*, 2007). Swarming motility depends on QS, since rhamnolipid production is necessary for swarming motility, and *rhl* QS operon is required for rhamnolipid biosynthesis (Déziel *et al.*, 2003).

2.5.3 Pyocyanin

Pyocyanin is a redox-active compound that is unique for *P. aeruginosa* (Yahr and Parsek, 2006) and that is permeable to biological membranes (Lau *et al.*, 2004a). Pyocyanin inhibits cell growth in epidermal cells (Wilson *et al.*, 1988), cell respiration in mammalian and prokaryotic cells (Hassett *et al.*, 1992), and plays an important role in lung infections of mice (Lau *et al.*, 2004b) and in iron acquisition (Wilson *et al.*, 1988). Pyocyanin represses the

expression of catalase (Lau *et al.*, 2004a). Pyocyanin is also important in nematode and plant killing (Mahajan-Miklos *et al.*, 1999). Pyocyanin is synthesized by the *phz* and the *aro* pathways (Lau *et al.*, 2004a). Multidrug efflux pumps *mexGHI-opmD* operon induce pyocyanin production (Aendekerk *et al.*, 2005). A mutation in this operon represses the production of 3-oxo-C_{12}-HSL and PQS (Aendekerk *et al.*, 2005), and several studies show that the QS genes *rhlR* and *pqsR* are important for pyocyanin synthesis (Cao *et al.*, 2001; Déziel *et al.*, 2005; Evans *et al.*, 1998; Gallagher *et al.*, 2002), which reveals that pyoycanin is a QS-regulated virulence factor.

2.5.4 Elastase

Elastase is a metalloproteinase and a toxin of *P. aeruginosa*, that increases the invasiveness of *P. aeruginosa* in burn, eye, and alveolar infections and degrades the elastin of the human lung (Bejarano *et al.*, 1989; Kon *et al.*, 1999; Yanagihara *et al.*, 2003). The elastic lamina of arteries and arterioles are dissolved by elastase disrupting blood vessels (Bejarano *et al.*, 1989). Elastase also degrades the extracellular matrices at the junctions of epithelial cells (Bejarano *et al.*, 1989). Elastase increases the accumulation of lymphocytes in the lungs of mice with *P. aeruginosa* infection (Yanagihara *et al.*, 2003). In *P. aeruginosa*, the two QS genes of the *las* operon, *lasI* and *lasR,* are involved in the synthesis of elastase (Gambello and Iglewski, 1991; Passador *et al.*, 1993) and PqsR regulates elastase production (Diggle *et al.*, 2006).

2.5.5 Rhamnolipids

During the late log and stationary phases of growth, *P. aeruginosa* produces a glycolipid-type biosurfactant composed of one or two rhamnoses, called rhamnolipid (Chen *et al.*, 2007; Soberón-Chávez *et al.*, 2005; Stanghellini and Miller, 1997). Rhamnolipids are one of

the most widely used biosurfactants having potential in remediation of oil contaminated water and soil (Chen *et al.*, 2007). While, rhamnolipids can be used in industrial biotechnology, they are virulence factors of *P. aeruginosa*. Zulianello *et al.* (2006) showed that only bacteria able to secrete rhamnolipids can infiltrate the respiratory epithelium and rhamnolipids promote the invasion of rhamnolipid-deficient *P. aeruginosa* by disrupting the epithelia. In *P. aeruginosa*, rhamnolipids are involved in swarming motility and biofilm formation. A *rhlA* rhamnolipid-deficient mutant also lost its ability to swarm (Caiazza *et al.*, 2005) and altered the biofilm architecture to a thick, uniform mat of bacterial cells (Davey *et al.*, 2003). Boles *et al.* (2005) showed rhamnolipids are also required for biofilm detachment for *P. aeruginosa*. Rhamnolipids also have antifungal (Lang, 2002), antimicrobial (Abalos *et al.*, 2001), and pathogenic activities against zoosporic plant pathogens (Stanghellini and Miller, 1997). In zoospores, rhamanolipids are involved in lysis by breaking the zoospore plasma membrane (Stanghellini and Miller, 1997).

rhlA and *rhlB* encode the rhamnosyltransferase enzyme that synthesizes rhamnolipids and *rhlC* encodes a protein to synthesize di-rhamnolipids (Soberón-Chávez *et al.*, 2005). RhlR and RhlI regulate the synthesis of rhamnolipids (Stanghellini and Miller, 1997). Another QS gene, *pqsR* is involved in rhamnolipid production (Diggle *et al.*, 2006) and a multidrug efflux pump, *mexGHI-opmD* is involved in rhamnolipid production (Aendekerk *et al.*, 2002).

2.5.6 Type III secretion system

Gram-negative bacteria use a type III secretion system to transfer their toxins into host cells (Galán and Collmer, 1999). *P. aeruginosa* secretes the toxins in a two-step secretion process consisting of translocation across the inner membrane and translocation across the outer membrane (Tommassen *et al.*, 1992). Type III secretion system possesses 43 coordinately regulated genes that encode type III secretion and translocation apparatus, type III effectors and

regulators in *P. aeruginosa* (Yahr and Wolfgang, 2006). Thus far, four effector proteins, ExoS, ExoT, ExoU, and ExoY have been found in *P. aeruginosa* (Feltman *et al.*, 2001). *In vivo* contact with eukaryotic host cells induces the expression of the type III secretion system in *P. aeruginosa* (Shen *et al.*, 2008). Type III secretion causes apoptosis of epithelial cells (Hauser and Engel, 1999) and contributes to *P. aeruginosa* virulence in CF patients (Roy-Burman *et al.*, 2001).

2.6 Treatment of *P. aeruginosa* infections in cystic fibrosis patients

Cystic fibrosis (CF) is a hereditary disorder in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Döring *et al.,* 2000). Major vault protein expressed in the airway epithelium helps the clearance of *P. aeruginosa* and plays an important role in the defense against this bacterium (Kowalski *et al.,* 2007). *P. aeruginosa* is the most prevalent pathogen observed in CF patients (Döring *et al.,* 2000). The combination of tobramycin with β-lactam antibiotics was not effective to control the development of resistant strains (Döring *et al.,* 2000). Recently, Hoffman *et al.* (2007) showed that azithromycin helps to clear *P. aeruginosa* biofilms in CF mouse models infected with *P. aeruginosa* and also it has been shown that long term use of azithromycin represses the percentage of mucoid *P. aeruginosa* in sputum samples (Hansen *et al.*, 2005). However, azithromycin has a low onset of action and does not the intravenous infections (Equi *et al.*, 2002). Therefore, new drugs and therapeutic approaches are intriguing for *P. aeruginosa* infections.

CHAPTER III

PSEUDOMONAS AERUGINOSA PAO1 VIRULENCE FACTORS AND POPLAR TREE RESPONSE IN THE RHIZOSPHERE*

3.1 Overview

Whole transcriptome analysis was used here for the first time in the rhizosphere to discern the genes involved in the pathogenic response of *Pseudomonas aeruginosa* PAO1 as well as to discern the response of the poplar tree. Differential gene expression shows that 185 genes of the bacterium and 753 genes of the poplar tree were induced in the rhizosphere. Using the *P. aeruginosa* transcriptome analysis, isogenic knock-out mutants, and two novel plant assays (poplar and barley), seven novel PAO1 virulence genes were identified (PA1385, PA2146, PA2462, PA2463, PA2663, PA4150, and PA4295). The uncharacterized putative hemolysin repressor, PA2463, upon inactivation, resulted in greater poplar virulence and elevated hemolysis while this mutant remained competitive in the rhizosphere. In addition, disruption of the hemolysin gene itself (PA2462) reduced the hemolytic activity of *P. aeruginosa*, caused less cytotoxicity, and reduced barley virulence, as expected.

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*Reprinted with the permission from "*Pseudomonas aeruginosa* PAO1 virulence factors and poplar tree Response in the rhizosphere" by Can Attila, Akihiro Ueda, Suat L.G. Cirillo, Jeffrey D. Cirillo, Wilfred Chen, and Thomas K. Wood, 2008, *Microbial Biotechnology* 1: 17-29, Copyright by Wiley-Blackwell. C. Attila was responsible for poplar tree growth, DNA microarrays, poplar pathogenicity, root microscopy, crystal violet biofilm formation assays, motility assays, and bacterial rhizosphere competition experiments. Akihiro Ueda verified the knock-out mutations and performed barley germination experiments. Suat L.G. Cirillo performed the hemolysis and the cytotoxicty experiments.

Inactivating PA1385, a putative glycosyl transferase, reduced both poplar and barley virulence. Furthermore, disrupting PA2663, a putative membrane protein, reduced biofilm formation by 20-fold. Inactivation of PA3476 (*rhlI*) increased virulence with barley as well as hemolytic activity and cytoxicity, so quorum sensing is important in plant pathogenesis. Hence, this strategy is capable of elucidating virulence genes for an important pathogen.

3.2 Introduction

Greater than 99% of all bacteria are found in biofilms (Sauer *et al.*, 2004), and 80% of bacterial infections are caused by bacteria living in biofilms (Costerton, 2004); hence, discerning the genetic basis of disease caused by biofilms is important. However, discovering pathogenic genes with animal models can be expensive, time consuming, and tedious (Prithiviraj *et al.*, 2005b). In addition, animal models often do not fully resemble all aspects of human disease caused by bacteria like *Pseudomonas aeruginosa* (Rahme *et al.*, 2000) whereas the interactions of bacteria with plants are often similar to the interactions of bacteria with eukaryotes (Lugtenberg *et al.*, 2002). Therefore, plant models can provide a fast, inexpensive, and high-throughput method for discovering bacterial virulence factors (Filiatrault *et al.*, 2006; Prithiviraj *et al.*, 2005b).

P. aeruginosa with its 6.3 million base pairs and 5,570 open reading frames (ORFs) is one of the largest of the sequenced bacterial genomes (Stover *et al.*, 2000). *P. aeruginosa* is an opportunistic pathogen and causes urinary tract, respiratory tract, and skin infections (Stover *et al.*, 2000). *P. aeruginosa* primarily causes nosocomial infections, and it is frequently resistant to commonly used antibiotics and disinfectants (Stover *et al.*, 2000). Although this bacterium is well-studied, roughly one fourth of its ORFs are uncharacterized (Lewenza *et al.*, 2005).

Populus trichocarpa is a model woody plant due to its small genome size (520 Mbp)

(Tuskan *et al.*, 2004) and routine transformation system mediated by *Agrobacterium tumefaciens* (Fillatti, 1987). The poplar genome encodes more than 45,000 putative protein-coding genes (Tuskan *et al.*, 2006), and the poplar genome project is expected to help identify tree-specific characteristics such as those for wood formation, perennial crown development, and distribution of water/nutrients over long distances (Tuskan *et al.*, 2004). However, there is little known about bacterial pathogens in the poplar rhizosphere. Injury and tumor formation on poplar stems is caused by the bacterial pathogens *Xanthomonas* spp. and *Agrobacterium* spp., and enhanced resistance of poplar to these bacteria was achieved by engineering expression of the antimicrobial peptide D4E1 (Mentag *et al.*, 2003). Although determinants of disease resistance are not well studied in woody plants, poplar has diverse disease resistance proteins that typically have nucleotide binding sites and/or leucine rich repeats (LRR) in contrast to those in the model grass plants, *Arabidopsis* and rice (Tuskan *et al.*, 2006). Hence, poplar is a good model for pathogenesis research of woody plants.

The pathogenicity of *P. aeruginosa* is dependent upon more than biofilms; other virulence factors include adhesins, hemagglutinin, protein toxins, phenazine, and cyanide (Gallagher and Manoil, 2001). *P. aeruginosa* is a known colonizer of respiratory tract of cystic fibrosis patients (Potera, 1999) and causes disease in humans, animals, nematodes, and insects (Lewenza *et al.*, 2005); yet, it is not recognized as an important rhizosphere bacterium although it is frequently found in soils (Filiatrault *et al.*, 2006) and has been found to colonize cucumber roots (Lugtenberg and Dekkers, 1999), lettuce leaves (Filiatrault *et al.*, 2006), sweet basil roots (Walker *et al.*, 2004), sugar beet roots (Mark *et al.*, 2005), wheat roots (Lugtenberg and Dekkers, 1999), and *Arabidopsis* roots (Walker *et al.*, 2004). We hypothesized that not only could *P. aeruginosa* survive in the rhizosphere but that it may be pathogenic to some trees. Here we show *P. aeruginosa* is virulent to poplars (it effectively kills the tree in 48 h) and is virulent to

barley (prevents seed germination); hence, we used poplar trees and barley as model organisms to identify *P. aeruginosa* pathogenic genes (two novel virulence assays were developed). Using DNA microarrays, the whole transcriptome response of both the tree contacted with and without *P. aeruginosa* for twelve hours and the bacterium contacted with and without poplar tree roots for two days was determined. In addition, isogenic mutants were used to identify seven previously uncharacterized genes as pathogenic determinants.

3.3 Results

3.3.1 *P. aeruginosa* is a poplar pathogen

As *P. aeruginosa* is a pathogen for *Arabidopsis* (Walker *et al.*, 2004), sweet basil (Walker *et al.*, 2004), and lettuce (Filiatrault *et al.*, 2006), and given our interest in rhizoremedia tion (Shim *et al.*, 2000; Yee *et al.*, 1998), we investigated whether this strain was a pathogen for poplar trees and then developed a wilting poplar tree assay to quantify PAO1 pathogenesis. As shown in Fig. 3.1, after 48 h, *P. aeruginosa* PAO1 killed poplar trees and caused a 16 ± 12-fold increase in branch wilting. Poplar trees were contacted with both the wild-type *P. aeruginosa* PAO1 from the University of British Columbia (*P. aeruginosa* PAO1-UBC) and the wild-type strain from the University of Washington (*P. aeruginosa* PAO1-UW) as mutants from both universities were utilized (Fig. 3.1C and Fig. 3.1E, respectively). However, colonization of poplar trees with the indigenous bacterium *Pseudomonas* sp. strain Pb3-1 (Shim *et al.*, 2000) did not cause wilting in 2 days (Fig. 3.1B). In addition, poplar trees that were not inoculated with bacteria were not affected for up to ten days (a poplar tree contacted in HRP medium without bacteria after seven days is shown in Fig. 3.1A). Confocal microscopy was used to confirm the presence of *P. aeruginosa* on the growing poplar root (Fig. 3.1G). Evidence of a small number of

Fig. 3.1. Poplar tree wilting after exposure to bacteria. (A) no bacteria after 7 days, (B) indigenous *Pseudomonas* sp. strain Pb3-1 after 48 h, (C) *P. aeruginosa* PAO1-UBC after 48 h contact, (D) *P. aeruginosa* PAO1 PA2463-UBC after 48 h contact, (E) *P. aeruginosa* PAO1-UW after 48 h contact, (F) *P. aeruginosa* PAO1 PA1385-UW after 48 h contact, and (G) *P. aeruginosa* PAO1/pMRP9-1 on poplar roots after 24 h visualized with confocal microscopy (scale bar represents 50 μm).

GFP-tagged pseudomonads was also found inside root sections after 48 h. We also investigated whether *P. aeruginosa* PAO1 ot its metabolites kill the poplar trees by filter sterilizing the liquid from a flask that contained a poplar tree contacted with *P. aeruginosa* PAO1 for two days. The cell-free supernatant did not kill the poplar trees after 3 days; therefore, the *P. aeruginosa* PAO1 cells themselves kill poplar trees.

3.3.2 Differentially expressed *P. aeruginosa* genes upon infection

To determine the global transcriptome response to colonization of poplar trees by *P. aeruginosa* and to identify the genes required for poplar pathogenesis, we compared differential gene expression at 48 h for the pseudomonad on poplar roots vs. the genes required for colonization of glass wool. In this way, only the genes required for pathogenesis and interaction with poplar trees are differentially expressed while the genes required for biofilm formation are not identified since the biofilm state was used for both the pseudomonad on poplar roots and on glass wool. Colonization of *P. aeruginosa* induced 185 genes greater than 2-fold change including genes for carbon compound metabolism, a colicin immunity protein, energy metabolism, membrane protein, fatty acid, phospholipid metabolism, motility, and attachment genes (partial gene list shown in Table 3.1). Similarly, colonization of *P. aeruginosa* on poplar roots repressed 419 genes greater than 2-fold including those for adaptation, protection, amino acid biosynthesis, biosynthesis of cofactors, cell division, chemotaxis, flagella, motility, attachment, energy metabolism, membrane proteins, fatty acid, phospholipid metabolism, protein secretion, transcription, and translation regulation genes (partial gene list shown in Table 3.2).

In comparison to glass wool, colonization on poplar roots induced expression of components of the type III secretion system in *P. aeruginosa* including PA1691 (*pscT*, a homolog of *yscT* in *Yersinia pseudotuberculosis*) (Bergman *et al.*, 1994) and PA1718 (*pscE*)

Table 3.1. Partial list of *P. aeruginosa* PAO1 genes induced more than 2.5-fold with *p*-value is less than 0.05 after 48 hours of poplar root contact versus contact with glass wool. For full data, see GEO accession number GSE5887. Five important virulence genes induced 2.0- to 2.5-fold are also shown (PA1432, PA1691, PA1712, PA1718, and PA4540).

PA #	Gene name	Fold change	Function
Adaptation and protection			
PA1432	*lasI*	2.0	Autoinducer synthesis protein LasI
Carbon compound catabolism			
PA4148		2.8	Putative enzyme (probable short-chain dehydrogenase)
PA4149		3.5	Conserved hypothetical protein (acetoin catabolism protein homolog, acoX)
PA4150		4.6	Probable dehydrogenase E1 component
PA4151	*acoB*	5.7	Acetoin catabolism protein AcoB
PA4152		3.5	Probable hydrolase
PA4153		6.5	2,3-butanediol dehydrogenase
Cell wall/LPS/capsule			
PA1385		3.2	Probable glycosyl transferase
Chaperons & heat proteins			
PA0538	*dsbB*	2.6	Disulfide bond formation protein
Colicin immunity protein			
PA0984		10.6	Membrane proteins; secreted factors (toxins, enzymes, alginate)
PA0981		5.7	Hypothetical protein
Energy metabolism			
PA0519	*nirS*	2.6	Nitrite reductase precursor
PA0523	*norC*	2.6	Nitric-oxide reductase subunit C
PA0524	*norB*	2.6	Nitric-oxide reductase subunit B
Fatty acid and phospholipid metabolism			
PA3300		2.6	long-chain-fatty-acid-CoA ligase
Hypothetical, unclassified, unknown function			
PA2461		19.7	Hypothetical protein (serine/threonine protein kinase pentapeptide repeats, protein binding/voltage-gated potassium channel, heterocyst-specific glycolipids-directing protein NifU-like protein)
PA2459		7.0	Hypothetical protein (immunoglobulin heavy chain VDJ region, anti-folate binding protein, retrovirus-related like polyprotein Arabidopsis thaliana, Methenyl tetrahydrofolate cyclohydrolase
PA2146		3.5	Hypothetical protein (92% similar to hypothetical *yciG* gene product of *E. coli*)

Table 3.1. (continued)

PA #	Gene name	Fold change	Function
PA5138		2.8	Hypothetical protein (type I export signal, HisJ, ABC-type amino acid transport/signal transduction systems, periplasmic component/domain)
PA2222		2.6	Hypothetical protein, cell surface antigen, glycine cleavage, aminomethyl transferase
PA4540		2.5	Hemolysin activation and secretion protein, homolog of hxuB in *Haemophilus influenza*
Membrane proteins			
PA3235		3.7	Hypothetical protein (alternate gene name *yjcH,* predicted membrane protein)
PA2663		2.8	Hypothetical membrane protein
PA4295		3.0	Hypothetical protein (peptidase in *P. fluorescens*, arsenical-resistance protein)
PA3278		2.8	Hypothetical protein (3 predicted transmembrane helices, ABC transporter related, predicted zinc finger)
PA1960		2.8	Hypothetical protein (3 predicted transmembrane helices, integral membrane protein in *P. syringae*, cold-shock domain family protein in *Methylococcus capsulatus* str.)
Motility and attachment			
PA4549	*fimT*	4.3	Type 4 fimbrial biogenesis protein FimT
Protein secretion and export apparatus			
PA1712	*exsB*	2.3	Exoenzyme S synthesis protein B, type III secretion
PA1718	*pscE*	2.3	Type III secretion protein PscE
PA1691	*pscT*	2.1	Translocation protein in type III secretion
Transcriptional regulator			
PA0513		3.2	Energy metabolism; transcriptional regulators; biosynthesis of cofactors, prosthetic groups and carriers
Transport of small molecules			
PA0323		2.6	Probable binding protein component of ABC transporter
PA1019	*mucK*	2.6	Cis,cis-muconate transporter MucK
Virulence associated protein			
PA4674		2.8	Conserved hypothetical protein (VapI, Plasmid maintenance system antidote protein, 69% similar to virulence-associated protein in Dichelobacter nodosus, virulence associated protein in *Pseudomonas syringae pv. tomato str.* DC3000, antidote protein in *P. putida* KT2400)

Table 3.2. Partial list of *P. aeruginosa* PAO1 genes repressed more than 3-fold with *p*-value is less than 0.05 after 48 hours of poplar root contact versus contact with glass wool. For full data, see GEO accession number GSE5887.

PA #	Gene name	Fold change	Function
Adaptation and protection			
PA4671		-4.6	Probable ribosomal protein L25
Amino acid biosynthesis and metabolism			
PA1585	*sucA*	-4.3	2-oxoglutarate dehydrogenase
PA0316	*serA*	-3.2	D-3-phosphoglycerate dehydrogenase
Biosynthesis of cofactors, prosthetic groups and carriers			
PA4053	*ribE*	-4.0	6,7-dimethyl-8-ribityllumazine synthase
Cell Division			
PA4751	*ftsH*	-4.3	Cell division protein FtsH
Cell wall/LPS/capsule			
PA0764	*mucB*	-4.3	Negative regulator for alginate biosynthesis MucB
Central intermediary metabolism			
PA5549	*glmS*	-3.5	Glucosamine--fructose-6-phosphate aminotransferase
Chaperons and heat shock proteins			
PA1801	*clpP*	-4.6	ATP-dependent Clp protease proteolytic subunit
PA1802	*clpX*	-3.7	Chaperones & heat shock proteins
PA1800	*tig*	-3.2	Trigger factor
Chemotaxis, flagella, motility and attachment			
PA3361	*lecB*	-21.1	Fucose-binding lectin PA-IIL
PA1092	*fliC*	-11.3	Flagellin type B
PA1093		-9.8	Hypothetical protein (Middle portion 58% similar to flagellin protein)
PA1094	*fliD*	-7.0	Flagellar capping protein FliD
PA3351		-8.6	Hypothetical protein (61% similarity to FlgM)
PA3349		-5.7	Probable chemotaxis protein
PA2788		-6.5	Probable chemotaxis transducer
PA3385	*amrZ*	-6.1	Alginate and motility regulator Z
PA0176		-4.0	Probable chemotaxis transducer
PA1459		-3.7	Probable methyltransferase
PA1458		-3.5	Probable two-component sensor

Table 3.2. (continued)

PA #	Gene name	Fold change	Function
Cytoplasmic membrane protein			
PA1774	*cfrX*	-3.5	CfrX protein (Between two genes that are proposed to be cytoplasmic membrane associated)
DNA replication, recombination, modification, and repair			
PA4232	*ssb*	-4.6	Single-stranded DNA-binding protein
Energy metabolism			
PA1581	*sdhC*	-4.9	Succinate dehydrogenase
PA1582	*sdhH*	-4.3	Succinate dehydrogenase
PA1584	*sdhB*	-4.3	Succinate dehydrogenase
PA1588	*sucC*	-3.7	Succinyl-CoA synthetase beta chain
PA3930	*cioA*	-4.9	Cyanide insensitive terminal oxidase
PA3929	*cioB*	-3.5	Cyanide insensitive terminal oxidase
PA2624	*idh*	-4.6	Isocitrate dehydrogenase
PA1317	*cyoA*	-4.3	Cytochrome o ubiquinol oxidase subunit II
PA1318	*cyoB*	-3.7	Cytochrome o ubiquinol oxidase subunit I
PA5491		-4.0	Probable cytochrome
PA5490	*cc4*	-3.2	Cytochrome c4 precursor
PA1555		-3.5	Probable cytochrome c
Fatty acid and phospholipid metabolism			
PA1610	*fabA*	-4.3	Beta-hydroxydecanoyl-ACP dehydrase
PA1609	*fabB*	-3.2	Beta-ketoacyl-ACP synthase I
PA5174		-3.7	Probable beta-ketoacyl synthase
PA2967	*fabG*	-3.2	3-oxoacyl-[acyl-carrier-protein] reductase
Hypothetical, unclassified, unknown function			
PA0529		-7.5	Conserved hypothetical protein (60% similar to hypothetical protein YiiM *E. coli* (function unknown))
PA1096		-6.1	Hypothetical protein (67% similarity to putative protein (Orf4) *P. aeruginosa* strain DG1)
PA2567		-4.6	Hypothetical protein (49% similar to a region of c-di-GMP, phosphodiesterase A, *Acetobacter xylinus)*
PA3009		-4.3	Hypothetical protein (similar to glycosylphosphatidylinositol anchor attachment 1 protein)
PA4460		-4.3	Conserved hypothetical protein (type I export signal, 50 % similar to YhbN *E. coli*)

Table 3.2. (continued)

PA #	Gene name	Fold change	Function
PA5446		-4.0	Conserved hypothetical protein
PA0122		-3.5	Conserved hypothetical protein (58% similar to Asp hemolysin *Aspergillus fumigatus)*
PA0856		-3.5	Hypothetical protein (type I export signal)
PA1471		-3.5	Hypothetical protein (type I export signal)
PA2971		-3.5	Conserved hypothetical protein (58% similar to hypothetical gene product YceD *E.coli,* predicted metal-binding, possibly nucleic acid-binding protein)
PA3806		-3.5	Conserved hypothetical protein (gene as being in *ndk-pilF* region P. aeruginosa, 73% similar to E. coli hypothetical protein *yfgB* in *ndk-gcpE* intergenic region predicted Fe-S-cluster redox enzyme)
PA4517		-3.5	Conserved hypothetical protein (transmembrane region, 5 predicted transmembrane helices, predicted membrane-associated, metal-dependent hydrolase, sulfatase in *P. fluorescens*)
PA4940		-3.5	Conserved hypothetical protein (2 predicted transmembrane helices, 61% similar to hypothetical protein YjeT in *E. coli*)
PA1545		-3.2	Hypothetical protein (downstream gene encodes HemN, protein phosphatase)
Membrane proteins			
PA0973	*oprL*	-3.7	Peptidoglycan associated lipoprotein OprL precursor
PA1048		-3.7	Probable outer membrane protein precursor
PA4243	*secY*	-3.7	Secretion protein SecY
PA4292		-3.5	Probable phosphate transporter
Nucleotide biosynthesis and metabolism			
PA3637	*pyrG*	-4.3	CTP synthase
PA3807	*ndk*	-3.7	Nucleoside diphosphate kinase
PA5242	*ppk*	-3.5	Polyphosphate kinase
PA4758	*carA*	-3.2	Carbamoyl-phosphate synthase small chain
Putative enzymes			
PA4431		-4.3	Probable iron-sulfur protein
PA1856		-3.5	Probable cytochrome oxidase subunit
Putative peptidase			
PA3472		-4.3	Hypothetical protein (67% similar to a region of putative lipoprotein YdhO (putative peptidase) *E. coli,* cell-all-associated hydrolases)
PA5181		-3.2	Probable oxidoreductase

Table 3.2. (continued)

PA #	Gene name	Fold change	Function
Secreted Factors (toxins, enzymes, alginate)			
PA3478	*rhlB*	-4.9	Rhamnosyltransferase chain B
PA3479	*rhlA*	-4.9	Rhamnosyltransferase chain A
Transcription, RNA processing, and degradation			
PA2840		-6.5	Probable ATP-dependent RNA helicase
PA5239	*rho*	-6.1	Transcription termination factor Rho
PA4262	*rplD*	-4.9	50S ribosomal protein L4
PA4264	*rpsJ*	-4.6	30S ribosomal protein S10
PA4270	*rpoB*	-3.5	DNA-directed RNA polymerase beta chain
PA4746		-4.3	Conserved hypothetical protein (conserved location upstream of nusA, 69% similar to hypothetical protein YhbC*, E. coli*)
PA4238	*rpoA*	-4.3	DNA-directed RNA polymerase alpha chain
PA2976	*rne*	-3.7	Ribonuclease E
PA3744	*rimM*	-3.7	16S rRNA processing protein
PA0428		-3.5	Probable beta-ketoacyl synthase
PA4944	*hfq*	-3.2	Conserved hypothetical protein
Transcriptional regulators			
PA0874		-11.3	Hypothetical protein (type I export signal, next to transcriptional regulator, transcriptional regulator in *P. syringae* and *P. fluorescens*)
PA0160		-7.5	Hypothetical protein (type I export signal, next to transcriptional regulator gene)
PA0456		-4.3	Probable cold-shock protein
PA0762	*algU*	-3.5	Sigma factor AlgU
PA0576	*rpoD*	-3.5	Sigma factor RpoD
PA0547		-3.2	Probable transcriptional regulator
Translation, post-translational modification, degradation			
PA4239	*rpsD*	-4.3	30S ribosomal protein S4
PA4241	*rpsM*	-6.5	30S ribosomal protein S13
PA4242	*rpmJ*	-5.3	50S ribosomal protein L36
PA4246	*rpsE*	-5.7	30S ribosomal protein S5
PA4251	*rplE*	-4.0	50S ribosomal protein L5

Table 3.2. (continued)

PA #	Gene name	Fold change	Function
PA4252	*rplX*	-4.9	50S ribosomal protein L24
PA4253	*rplN*	-4.6	50S ribosomal protein L14
PA4254	*rpsQ*	-4.9	30S ribosomal protein S17
PA4256	*rplP*	-4.0	50S ribosomal protein L16
PA4257	*rpsC*	-4.0	30S ribosomal protein S3
PA4258	*rplV*	-5.3	50S ribosomal protein L22
PA4259	*rpsS*	-5.3	30S ribosomal protein S19
PA4260	*rplB*	-5.3	50S ribosomal protein L2
PA4261	*rplW*	-5.3	50S ribosomal protein L23
PA4263	*rplC*	-5.3	50S ribosomal protein L3
PA4265	*tufA*	-4.0	Elongation factor Tu
PA4266	*fusA1*	-4.3	Elongation factor G
PA4267	*rpsG*	-4.3	30S ribosomal protein S7
PA4268	*rpsL*	-5.3	30S ribosomal protein S12
PA4272	*rplJ*	-5.7	50S ribosomal protein L10
PA4273	*rplA*	-5.7	50S ribosomal protein L1
PA4274	*rplK*	-5.7	50S ribosomal protein L11
PA4568	*rplU*	-5.7	50S ribosomal protein L21
PA2742	*rpmL*	-5.7	50S ribosomal protein L35
PA3162	*rpsA*	-5.7	30S ribosomal protein S1
PA3656	*rpsB*	-5.3	30S ribosomal protein S2
PA4433	*rplM*	-5.3	50S ribosomal protein L13
PA2743	*infC*	-4.9	Translation initiation factor IF-3
PA4563	*rpsT*	-4.9	30S ribosomal protein S20
PA4567	*rpmA*	-3.5	50S ribosomal protein L27
PA0579	*rpsU*	-4.6	30S ribosomal protein S21
PA3745	*rpsP*	-4.6	30S ribosomal protein S16
PA2619	*infA*	-3.7	Initiation factor
PA4935	*rpsF*	-3.7	30S ribosomal protein S6
PA2741	*rplT*	-3.5	50S ribosomal protein L20

Table 3.2. (continued)

PA #	Gene name	Fold change	Function
PA3987	*leuS*	-3.5	Leucyl-tRNA synthetase
PA4741	*rpsO*	-3.5	30S ribosomal protein S15
PA0956	*proS*	-3.2	Prolyl-tRNA synthetase
PA5316	*rpmB*	-3.2	50S ribosomal protein L28
PA5569	*rnpA*	-3.2	Ribonuclease P protein component
PA5570	*rpmH*	-3.2	50S ribosomal protein L34

(Quinaud *et al.*, 2005) (Table 3.1). Additional virulence genes were up-regulated including PA1712 (*exsB*, an exoenzyme S synthesis protein secreted by the type III secretion system) (Yahr *et al.*, 1996), PA1432 (*lasI*, an autoinducer synthesis protein) (Passador *et al.*, 1993), and PA4540 (a putative hemolysin activator and homolog of *hxuB* in *Haemophilus influenzae*) (Cope *et al.*, 1995). Furthermore, PA4295 was induced in our microarray data; this gene is close to the PA4296 virulence gene that was identified in the lettuce leaf model (Wagner *et al.*, 2006b) (Table 3.1). Hence, activation of these virulent factors is necessary for infection of the poplar tree by *P. aeruginosa*.

3.3.3 Exploration of pathogenesis via knock-out mutants using poplar wilting and barley germination

To corroborate the DNA microarray results and to explore the proteins related to the differentially-expressed genes, a series of *P. aeruginosa* isogenic knock-out mutants were utilized: strains with mutations in genes PA0513, PA0984, PA1385, PA2146, PA2462, PA2463, PA2663, PA3278 (transposon mutation was inserted in the promoter region of PA3278 gene, hence, PA3278 function may not be completely disrupted), PA4150, PA4151, PA4153, PA4295, and PA4549. Two of the most induced genes (PA2461 and PA2459) are part of a putative hemolysin operon; hence, we investigated pathogenesis with isogenic mutations in PA2462 (putative hemolysin) and PA2463 (putative hemolysin regulator); note that the Affymetrix chip does not contain probes for the PA2463 gene. The other knock-out mutants were investigated since they were part of the top twenty induced genes of *P. aeruginosa* PAO1 contacted with poplar trees and mutants were readily available. Quorum sensing has a significant role in

Table 3.3. Strains and plasmids used. Cb^R and Tet^R are carbenicillin, and tetracycline resistance, respectively.

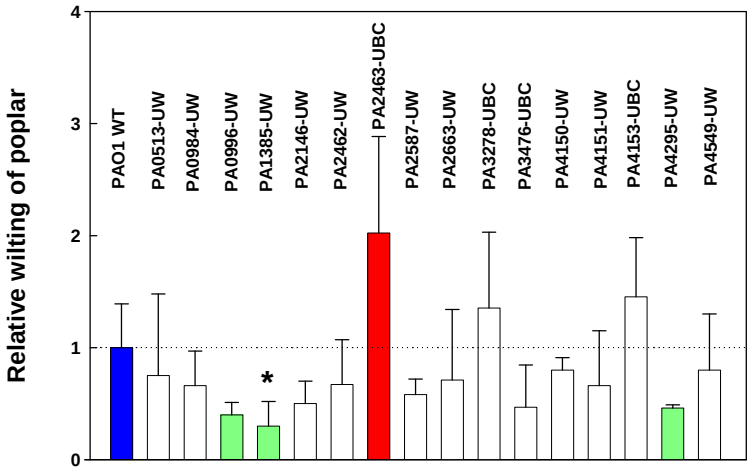
Strains and plasmids	Genotype	Source
Strains		
Pseudomonas sp. strain Pb3-1	Wild-type indigenous poplar bacterium expressing toluene *o*-monooxygenase	(Shim *et al.*, 2000; Yee *et al.*, 1998)
P. aeruginosa PAO1	Wild-type	(Stover *et al.*, 2000)
P. aeruginosa PAO1 PA0513	PA0513 Ω IS*lacZ*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 PA0984	PA0984 Ω IS*phoA*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 PA0996	PA0996 Ω IS*lacZ*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 PA1385	PA1385 Ω IS*lacZ*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 PA2146	PA2146 Ω IS*lacZ*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 PA2462	PA2462 Ω IS*lacZ*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1_lux100_C11	PA2463 Ω mini-*Tn*5-*luxCDABE,* Tet^R	(Lewenza *et al.*, 2005)
P. aeruginosa PAO1 PA2587	PA2587 Ω IS*lacZ*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 PA2663	PA2663 Ω IS*phoA*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1_lux74_F1	PA3278 Ω mini-*Tn*5-*luxCDABE,* Tet^R	(Lewenza *et al.*, 2005)
P. aeruginosa PAO1_lux19_E12	PA3476 Ω mini-*Tn*5-*luxCDABE,* Tet^R	(Lewenza *et al.*, 2005)
P. aeruginosa PAO1 PA4150	PA4150 Ω IS*phoA*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 PA4151	PA4151 Ω IS*phoA*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1_lux44_H8	PA4153 Ω mini-*Tn*5-*luxCDABE,* Tet^R	(Lewenza *et al.*, 2005)
P. aeruginosa PAO1 PA4295	PA4295 Ω IS*phoA*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 PA4549	PA4549 Ω IS*lacZ*/hah, Tet^R	(Jacobs *et al.*, 2003)
Plasmids		
pMRP9-1	Cb^R (constitutive GFP plasmid for visualizing bacteria on poplar root)	(Davies *et al.*, 1998)

bacterial colonization and virulence in bacteria (Chun *et al.*, 2004; Gallagher *et al.*, 2002); hence, strains with the PA0996 (*pqsA*), PA2587 (*pqsH*), and PA3476 (*rhlI*) inactivations were also evaluated.

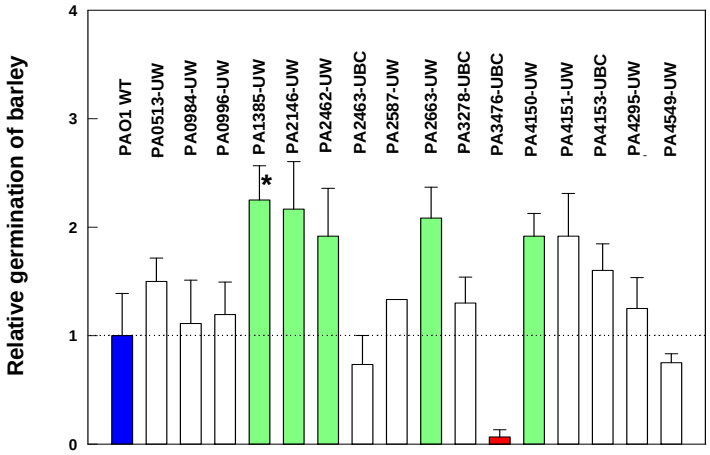
As shown in Table 3.3, knock-out mutants were obtained from both the collections of the University of British Columbia (UBC) (Lewenza *et al.*, 2005) and the University of Washington (UW) (Jacobs *et al.*, 2003); each mutation was verified using four PCR reactions for each mutant and the wild-type strain, and for all of the assays, each mutant was compared to its respective wild-type strain.

The PA2463 mutant showed over 2-fold more wilting than the wild-type strain in 48 h (Fig. 3.1D and Fig. 3.2A); hence, it is more pathogenic than the wild-type strain and appears to encode a hemolysin repressor. Conversely, the PA1385 mutant showed 3-fold less wilting in 48 hours (Fig. 3.1F) and inactivation of PA0996 caused 2.5 fold less wilting (Fig. 3.2A).

To corroborate the poplar wilting assay, we tested the ability of *P. aeruginosa* to prevent germination of barley seeds (also a novel assay for *P. aeruginosa*). Wild-type PAO1 significantly reduced the germination of barley seeds to 20% (PAO1 from UW) and 33% (PAO1 from UBC), whereas 87% of the seeds germinated in the absence of *P. aeruginosa*. Hence, wild-type *P. aeruginosa* is virulent not only to woody plants (e.g., poplar) but also is virulent to a grass (e.g., barley). The effect of the isogenic mutations on barley seed germination is shown in Fig. 3.2B. After 3 days, isogenic mutations that caused statistically less virulence included PA1385 (225% more germination), PA2146 (217%), PA2462 (192%), PA2663 (208%), and PA4150 (192%); therefore, the proteins encoded by these genes are important for plant pathogenesis. Conversely, quorum-sensing mutant PA3476 (*rhlI*) was significantly more virulent to barley because only 7% of seeds could germinate after infection with this strain (Fig. 3.2B).



A



B

Fig. 3.2. *P. aeruginosa* virulence as indicated by poplar tree wilting (A) and inhibition of barley seed germination (B). Red indicates more virulence, green indicates less virulence, and white indicates similar virulence compared to the wild-type strain based on a t-test. Poplars were grown at 22°C, and barley seeds were germinated at 25°C for 3 days (at least 45 seeds). Error bars indicate one standard deviation. Asterisk indicates the mutant showing similar trends in virulence for both poplar and barley.

3.3.4 Rhizosphere competition of the knock-out mutants

The knock-out mutants were also tested for their ability to compete simultaneously with the wild-type strain in the poplar rhizosphere. The more virulent PA2463 mutant and the PA2587 and PA4295 mutants showed statistically almost the same ability to compete with the wild-type in the rhizosphere whereas the PA4153 mutant was 100-fold less competitive (Table 3.4); hence, PA4153 encodes a protein that is very important for competition in the rhizosphere. As a control, the wild-type strain (*P. aeruginosa* PAO1-UW) with and without pMRP9-1 was inoculated on four poplar trees with two different ratios of bacteria, and after two days, the ratio of bacteria found on the roots matched the starting ratio.

3.3.5 Swimming motility, biofilm formation, and growth rates

Martínez-Granero *et al.* (2006) showed that the most severely impaired *Pseudomonas* colonization mutants appeared to be non-motile mutants, and colonization of plant tissue is a significant step in plant-bacteria interaction for pathogenesis (Lugtenberg *et al.*, 2002). Hence, swimming motility and biofilm formation were evaluated here for the isogenic mutants (Table 3.4). The PA2463 and PA3278 knock-outs exhibited 23 to 29% less motility than the wild-type strain in 18 h, respectively, whereas the PA0513, PA0984, PA1385, PA2146, PA2587, PA4151, PA4153, PA4295, and PA4549 mutants showed 50 to 83% more motility. The PA3476 mutation did not cause a difference in swimming motility which agrees with a previous report (Overhage *et al.*, 2007).

In LB glu medium at 30°C, mutations in PA3278 and PA3476 consistently enhanced biofilm formation, whereas mutations in PA0984, PA2462, PA2663, PA4151, and PA4295 decreased biofilm formation (Table 3.4). Of these, the PA2663 mutation was most significant as it reduced biofilm formation in both medium by a remarkable 15-fold. (Table 3.4). The PA1385

Table 3.4. Competition (percentage of mutant cells on poplar tree roots vs. the wild-type strain), biofilm formation (relative to wild-type), swimming motility, and specific growth rate in LB medium for the *P. aeruginosa* PAO1 mutants. UBC indicates wild-type *P. aeruginosa* from the University of British Columbia and UW indicates wild-type *P. aeruginosa* from the University of Washington. Data indicate the mean ± one standard deviation.

Strains	Competition, (%)	Relative biofilm (LB)	Relative biofilm (LB glu)	Swimming motility (cm, 5 h)	Swimming motility (cm, 18 h)	Growth rate (h^{-1})
Wild-type-UBC	NA	1.0 ± 0.3	1.0 ± 0.2	0.24 ± 0.00	1.55 ± 0.06	0.89 ± 0.04
PA2463	90 ± 30	1.0 ± 0.4	0.9 ± 0.6	0.16 ± 0.01	1.19 ± 0.08	1.03 ± 0.04
PA3278	60 ± 6	1.1 ± 0.3	1.6 ± 0.3	0.19 ± 0.01	1.1 ± 0.3	0.89 ± 0.01
PA3476	53 ± 14	1.8 ± 0.7	2.7 ± 0.7	0.19 ± 0.02	1.5 ± 0.1	0.93 ± 0.01
PA4153	0.9 ± 0.4	1.1 ± 0.3	1.3 ± 0.4	0.20 ± 0.03	1.72 ± 0.07	0.89 ± 0.02
Wild-type-UW	NA	1.0 ± 0.2	1.0 ± 0.3	0.2 ± 0.1	1.2 ± 0.2	1.02 ± 0.05
PA0513	42 ± 26	0.8 ± 0.1	0.8 ± 0.2	0.5 ± 0.1	2.1 ± 0.1	1.04 ± 0.01
PA0984	21 ± 7	0.5 ± 0.1	0.7 ± 0.1	0.32 ± 0.05	1.72 ± 0.08	1.02 ± 0.06
PA0996	67 ± 21	0.6 ± 0.1	0.7 ± 0.2	0.27 ±0.05	1.5 ± 0.2	1.02 ± 0.00
PA1385	37 ± 19	0.7 ± 0.1	0.7 ± 0.2	0.28 ± 0.07	1.8 ± 0.2	1.07 ± 0.07
PA2146	62 ± 21	0.7 ± 0.1	0.7 ± 0.2	0.36 ± 0.03	1.93 ± 0.03	1.0 ± 0.1
PA2462	39 ± 5	0.5 ± 0.1	0.6 ± 0.1	0.30 ± 0.05	1.6 ± 0.2	0.99 ± 0.01
PA2587	88 ± 36	0.6 ± 0.1	0.7 ± 0.2	0.34 ± 0.03	1.8 ± 0.2	1.14 ± 0.02
PA2663	29 ± 12	0.05 ± 0.01	0.09 ± 0.03	0.29 ± 0.08	1.8 ± 0.2	0.98 ± 0.01
PA4150	42 ± 11	0.6 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	1.7 ± 0.5	1.15 ± 0.06
PA4151	18 ± 4	0.6 ± 0.1	0.6 ± 0.2	0.37 ± 0.04	1.8 ± 0.2	1.11 ± 0.00
PA4295	84 ± 74	0.5 ± 0.1	0.7 ± 0.2	0.42 ± 0.07	1.9 ± 0.2	1.10 ± 0.04
PA4549	45 ± 21	0.7 ± 0.1	0.8 ± 0.2	0.5 ± 0.1	2.2 ± 0.1	1.11 ± 0.07

gene has been shown to encode polysaccharide biosynthesis enzymes that are important for biofilm development (Jackson *et al.*, 2004); this mutant had about 30% less biofilm formation in LB glu medium.

The knock-out mutations were not deleterious for the strains and most of the transposon insertions increased the specific growth rate slightly (Table 3.4); hence, the changes in phenotypes are not due to growth rate differences. Notably, the PA2463 mutation caused a 16 ± 7% increase in growth rate compared to the wild-type strain but this increase in growth rate is probably not the cause of its enhanced virulence as other strains (e.g., PA4150) grew more rapidly but were less virulent (Fig. 3.2).

3.3.6 Hemolysis and cytotoxicity

Since PA2463 is a putative regulator of hemolytic activity (Winsor *et al.*, 2005), we measured hemolysis with this knock-out mutant compared to the wild-type strain. *P. aeruginosa* PAO1-UBC showed around 15% hemolysis, whereas *P. aeruginosa* PAO1-UW showed around 45% hemolysis. The PA2463 mutant showed 4.7 ± 0.1 fold times more hemolytic activity than the wild-type cells; hence, the increased pathogenesis from this strain may be due to its increased hemolytic activity. Inactivation of the PA2462 putative hemolysin gene (Winsor *et al.*, 2005) itself, reduced hemolysis by 47% and cytotoxicity by 70%. Furthermore, inactivation of the PA0984 immunity protein (He *et al.*, 2004) reduced hemolysis by 41% and cytotoxicity by 67%. In contrast, inactivation of PA2663, which caused a large decrease in biofilm formation, did not affect the hemolytic activity and cytotoxicity of *P. aeruginosa*. Inactivation of PA3476 increased *P. aeruginosa* virulence for barley, and it was found to increase both hemolytic activity by 219% and cytotoxicity by 68%. Inactivation of PA1385, a putative glycosyl transferase, (Winsor *et al.*, 2005) decreased hemolytic activity by 50%. However, the

Table 3.5. Partial list of poplar tree root genes induced more than 15-fold with *p*-value is less than 0.05 after 12 hours of *P. aeruginosa* PAO1 contact versus no bacteria. For full data, see GEO accession number GSE5887. Hypothetical functions are not shown in the table.

Probe set name	Fold change	Function
Amino acid transport and metabolism		
PtpAffx.200039.1.S1_at	29.9	Amino acid transporter
PtpAffx.201320.1.S1_x_at	26.0	Glutamate-gated kainate-type ion channel receptor subunit GluR5 and related subunits
Ptp.5344.1.S1_at	18.4	Alanine aminotransferase
PtpAffx.205147.1.S1_at	18.4	Amino acid transporters
Carbohydrate transport and metabolism		
PtpAffx.200540.1.S1_s_at	42.2	Beta-glucosidase, lactase phlorizinhydrolase, and related proteins (hydrolase activity)
PtpAffx.200541.1.S1_s_at	36.8	Beta-glucosidase, lactase phlorizinhydrolase, and related proteins (hydrolase activity)
Catalytic activity		
PtpAffx.205330.1.S1_at	168.9	Catalytic activity
PtpAffx.18705.2.A1_a_at	90.5	Catalytic activity
PtpAffx.18241.1.A1_at	27.9	Catalytic activity
PtpAffx.205330.1.S1_s_at	27.9	Catalytic activity
Ptp.5002.1.S1_at	21.1	Catalytic activity
Ptp.6916.1.S1_at	17.1	Catalytic activity
Chromatin structure and dynamics		
Ptp.6635.1.S1_at	16.0	Histone H2B
Energy production and conversion genes		
Ptp.6141.1.S1_at	111.4	Mitochondrial phosphate carrier protein
Ptp.6164.1.S1_at	68.6	NAD dehydrogenase (ubiquinone)
PtpAffx.30951.1.A1_at	22.6	UDP-glucuronosyl and UDP-glucosyl transferase
PtpAffx.225249.1.S1_s_at	21.1	Proteins containing the FAD binding domain
PtpAffx.224796.1.S1_s_at	18.4	Proteins containing the FAD binding domain
PtpAffx.201142.1.S1_s_at	17.1	Kynurenine 3-monooxygenase and related flavoprotein monooxygenases
Inorganic ion transport and metabolism		
PtpAffx.213134.1.S1_x_at	45.3	Calcium transporting ATPase
Lipid transport and metabolism		
PtpAffx.224263.1.S1_s_at	45.3	Ca^{2+}-independent phospholipase A2

Table 3.5. (continued)

Probe set name	Fold change	Function
PtpAffx.211300.1.S1_at	24.3	Steroid reductase
PtpAffx.204828.1.S1_at	19.7	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies
Secondary metabolites biosynthesis, transport, and catabolism		
PtpAffx.308.5.S1_s_at	36.8	Cytochrome P450 CYP2 subfamily
PtpAffx.114367.1.A1_at	36.8	Pleiotropic drug resistance proteins (PDR1-15), ABC superfamily
PtpAffx.224778.1.S1_x_at	27.9	Cytochrome P450 CYP2 subfamily
Ptp.3277.1.S1_at	27.9	Multicopper oxidases
PtpAffx.16148.1.S1_at	26.0	Alcohol dehydrogenase, class V
PtpAffx.29753.1.A1_at	24.3	Cytochrome P450 CYP2 subfamily
Signal transduction mechanisms		
PtpAffx.217711.1.S1_at	34.3	Serine/threonine protein kinase
PtpAffx.224346.1.S1_s_at	34.3	Serine/threonine protein kinase
PtpAffx.224718.1.S1_x_at	27.9	Serine/threonine protein kinase / Replication factor C, subunit RFC4
PtpAffx.206255.1.S1_x_at	22.6	Serine/threonine protein kinase
Transcription		
PtpAffx.207223.1.S1_x_at	16.0	Transcription factor, Myb superfamily
PtpAffx.207674.1.S1_at	16.0	Regulation of transcription
PtpAffx.206330.1.S1_at	29.9	Transcription factor, activity
General function prediction only		
PtpAffx.95259.1.A1_at	22.6	Predicted transporter (ABC superfamily)
PtpAffx.201968.1.S1_at	18.4	CCCH-type Zn-finger protein
PtpAffx.202140.1.S1_at	16.0	FOG: Reverse transcriptase
Only GO Descriptions		
PtpAffx.224793.1.S1_s_at	21.1	Electron transport
PtpAffx.136046.1.A1_at	16.0	Hydrolase activity, hydrolyzing O-glycosyl compounds, carbohydrate metabolism
Ptp.6110.1.S1_at	16.0	Nucleotide binding, ATP binding
PtpAffx.135487.1.S1_at	45.3	Nutrient reservoir activity
PtpAffx.11639.1.A1_at	18.4	Nutrient reservoir activity
PtpAffx.224160.1.S1_at	26.0	Response to pathogenic fungi
PtpAffx.225394.1.S1_at	16.0	Sugar binding

Table 3.5. (continued)

Probe set name	Fold change	Function
Unknown functions, functions found with NCBI blast search		
PtpAffx.32468.1.S1_at	73.5	Pathogenesis-related protein 5
PtpAffx.120681.1.A1_at	52.0	Putative phytosulfokine peptide precursor
PtpAffx.225250.1.S1_at	52.0	Berberine bridge enzyme
PtpAffx.222980.1.S1_at	34.3	Pathogenesis-related protein 10
PtpAffx..75275.1.A1_at	32.0	Anthocyanin 5-aromatic acyltransferase
PtpAffx.212606.1.S1_s_at	26.0	Auxin response factor 1
PtpAffx.224826.1.S1_at	24.3	Tyrosine protein kinase, leucine-rich repeat receptor
PtpAffx.207301.1.S1_s_at	21.1	Ethylene induced esterase
PtpAffx.216745.1.S1_x_at	19.7	Leucine rich repeat
PtpAffx.215260.1.S1_at	18.4	NAC domain protein
PtpAffx.219632.1.S1_at	18.4	Zinc finger homeodomain protein
PtpAffx.79574.1.A1_at	18.4	Putative disease resistance gene, leucine rich repeat
PtpAffx.222211.1.S1_at	17.1	Putative *SCARECROW* gene regulator
PtpAffx.158865.1.S1_at	16.0	Flavonoid 3-hydroxylase
PtpAffx.3573.2.S1_at	16.0	Zinc finger homeodomain protein
PtpAffx.71801.1.A1_s_at	16.0	Auxin response factor 10

Table 3.6. Partial list of poplar tree root genes repressed more than 20-fold with *p*-value is less than 0.05 after 12 hours of *P. aeruginosa* PAO1 contact versus no bacteria. For full data, see GEO accession number GSE5887. Hypothetical functions are not shown in the table.

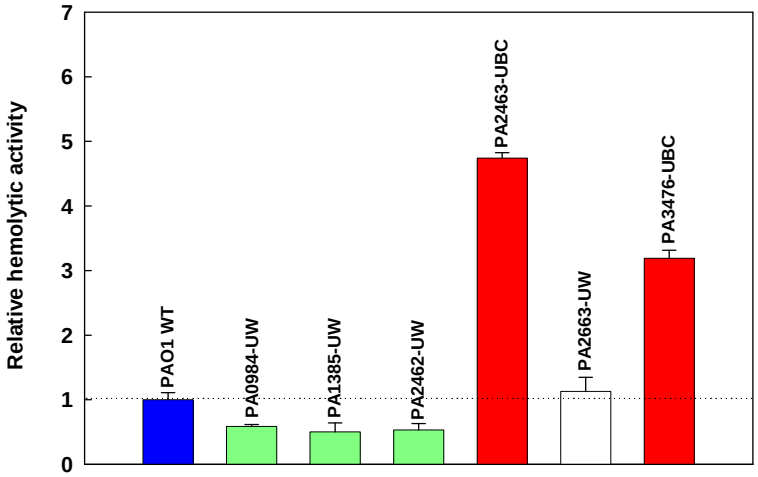
Probe set name	Fold change	Function
Amino acid transport and metabolism		
PtpAffx.31420.1.A1_s_at	-104.0	Thiamine pyrophosphate-requiring enzyme
Carbohydrate transport and metabolism		
PtpAffx.209585.1.S1_at	-26.0	Galactosyltransferases
Chromatin structure and dynamics		
PtpAffx.201059.1.S1_at	-26.0	Histone acetyltransferase (MYST family)
Coenzyme transport and metabolism		
PtpAffx.130260.1.S1_s_at	-26.0	Biotin holocarboxylase synthetase/biotin-protein ligase
Cytoskeleton		
PtpAffx.202243.1.S1_at	-36.8	Myosin class II heavy chain
Defense mechanisms		
PtpAffx.29068.1.S1_at	-32.0	DRIM (Down-regulated in metastasis)-like proteins
Energy production and conversion		
PtpAffx.214932.1.S1_s_at	-39.4	UDP-glucuronosyl and UDP-glucosyl transferase
PtpAffx.213180.1.S1_s_at	-39.4	UDP-glucuronosyl and UDP-glucosyl transferase
Inorganic ion transport and metabolism		
PtpAffx.224069.1.S1_a_at	-45.3	Metallothionein-like protein
PtpAffx.207122.1.S1_at	-21.1	Sulfate/bicarbonate/oxalate exchanger SAT-1 and related transporters (SLC26 family)
Intracellular trafficking, secretion, and vesicular transport		
Ptp.3097.3.A1_at	-26.0	Vesicle coat protein clathrin, heavy chain
Lipid transport and metabolism		
PtpAffx.128848.1.S1_at	-36.8	Ca^{2+} independent phospholipase A2
Posttranslational modification, protein turnover, chaperones		
PtpAffx.210817.1.S1_at	-97.0	20S proteasome, regulatory subunit alpha type PSMA2/PRE8
PtpAffx.35550.1.A1_at	-32.0	AAA+-type ATPase
Ptp.1275.1.S1_at	-24.3	Alkyl hydroperoxide reductase/peroxiredoxin
PtpAffx.212398.1.S1_at	-22.6	FOG: Predicted E3 ubiquitin ligase
PtpAffx.206951.1.S1_at	-21.1	Predicted metalloprotease with chaperone activity (RNAse H/HSP70 fold)

Table 3.6. (continued)

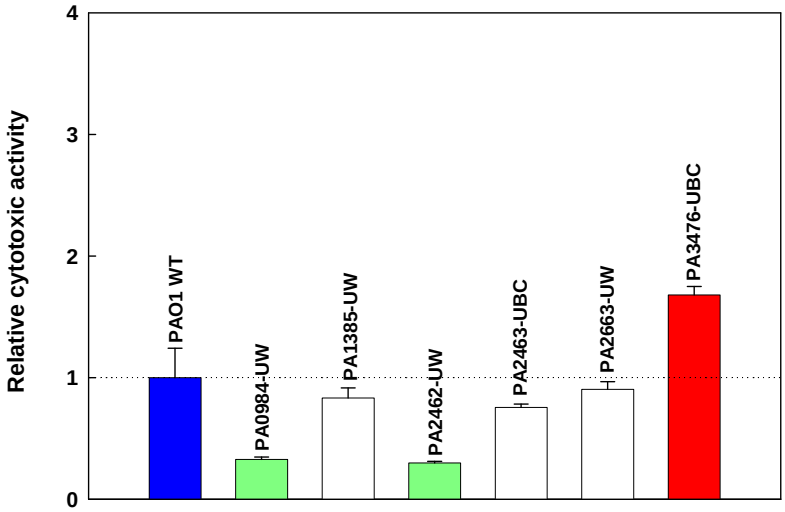
Probe set name	Fold change	Function
RNA processing and modification		
PtpAffx.220521.1.S1_at	-32.0	Protein involved in mRNA turnover and stability
Ptp.3483.1.S1_at	-22.6	Spliceosomal protein FBP11/Splicing factor PRP40
Secondary metabolites biosynthesis, transport and catabolism		
Ptp.4827.1.S1_a_at	-26.0	Iron/ascorbate family oxidoreductases
PtpAffx.64440.1.S1_at	-26.0	Cytochrome P450 CYP2 subfamily
Signal transduction mechanisms		
PtpAffx.223039.1.S1_x_at	-64.0	Apoptotic ATPase
PtpAffx.80348.1.A1_at	-27.9	Serine/threonine protein kinase
PtpAffx.201468.1.S1_at	-22.6	Two-component phosphorelay intermediate involved in MAP kinase cascade regulation
Translation, ribosomal structure and biogenesis		
PtpAffx.206914.1.S1_at	-29.9	40S ribosomal protein S20
Transcription		
PtpAffx.224153.1.S1_x_at	-45.3	Transcription factor, Myb superfamily
PtpAffx.218647.1.S1_at	-34.3	GATA-4/5/6 transcription factors
Ptp.345.1.A1_at	-24.3	RNA polymerase II, large subunit
PtpAffx.69797.1.A1_a_at	-22.6	Aryl-hydrocarbon receptor nuclear translocator
PtpAffx.221996.1.S1_at	-22.6	Transcription factor HEX, contains HOX and HALZ domains
General function prediction only		
PtpAffx.212314.1.S1_x_at	-64.0	Transposon-encoded proteins with TYA, reverse transcriptase, integrase domains in various combinations
PtpAffx.30704.1.A1_at	-39.4	Leucine rich repeat
PtpAffx.221080.1.S1_at	-39.4	Ankyrin repeat
Ptp.5665.1.S1_at	-39.4	Predicted methyltransferase
PtpAffx.211233.1.S1_at	-32.0	Reverse transcriptase
PtpAffx.32228.1.A1_at	-29.9	Endoplasmic reticulum protein EP58, contains filamin rod domain and KDEL motif
PtpAffx.217146.1.S1_x_at	-29.9	Transposon-encoded proteins with TYA, reverse transcriptase, integrase domains in various combinations
PtpAffx.213672.1.S1_x_at	-27.9	Leucine rich repeat
PtpAffx.214022.1.S1_at	-24.3	Metallopeptidase
PtpAffx.224136.1.S1_at	-24.3	Leucine rich repeat
PtpAffx.216698.1.S1_at	-24.3	Leucine rich repeat

Table 3.6. (continued)

Probe set name	Fold change	Function
PtpAffx.150443.1.S1_at	-22.6	FOG: Kelch repeat
General function prediction only and signal transduction mechanisms		
PtpAffx.221250.1.S1_s_at	-27.9	Leucine rich repeat, Serine/threonine protein kinase
Only GO Descriptions		
PtpAffx.211205.1.S1_s_at	-27.9	calcium ion binding
PtpAffx.222938.1.S1_at	-34.3	catalytic activity
PtpAffx.40216.2.S1_at	-39.4	cell wall catabolism
PtpAffx.213722.1.S1_at	-24.3	cell wall organization and biogenesis, extracellular
PtpAffx.157493.1.S1_at	-21.1	electron transporter activity, heme binding,
PtpAffx.220343.1.S1_at	-68.6	Function unknown
Ptp.5321.1.S1_at	-32.0	hydrolase activity, hydrolyzing O-glycosyl compounds, carbohydrate metabolism
PtpAffx.24322.3.A1_at	-29.9	hydrolase activity, acting on ester bonds
PtpAffx.214388.1.S1_x_at	-55.7	nucleic acid binding, zinc ion binding, nucleus
Ptp.1094.1.S1_s_at	-45.3	pectinesterase activity, cell wall modification, cell wall
PtpAffx.216901.1.S1_at	-36.8	phospholipase C activity, intracellular signaling cascade, signal transduction
PtpAffx.220343.1.S1_at	-68.6	Function unknown
Ptp.5321.1.S1_at	-32.0	hydrolase activity, hydrolyzing O-glycosyl compounds, carbohydrate metabolism
PtpAffx.24322.3.A1_at	-29.9	hydrolase activity, acting on ester bonds
PtpAffx.214388.1.S1_x_at	-55.7	nucleic acid binding, zinc ion binding, nucleus
Ptp.1094.1.S1_s_at	-45.3	pectinesterase activity, cell wall modification, cell wall
PtpAffx.216901.1.S1_at	-36.8	phospholipase C activity, intracellular signaling cascade, signal transduction



A



B

Fig. 3.3. *P. aeruginosa* hemolytic activity (A) and cytotoxic activity (B) in whole cells. Red indicates more virulence, green indicate less virulence, and white indicates similar virulence compared to the wild-type strain based on a t-test. The data for the mutants are normalized by the values for the respective wild-type strains. Error bars indicate standard deviations.

inactivation of PA1385 did not change cytotoxicity. Overall, there was good agreement with changes in hemolysis and cytotoxicity (Fig. 3.3) and virulence (Fig. 3.2).

3.3.7 Poplar genetic response to *P. aeruginosa* infection

To complement our investigation of the genes required for *P. aeruginosa* PAO1 pathogenesis of poplar, we investigated the response of the poplar roots to *P. aeruginosa* infection using an Affymetrix oligo-array that contains DNA elements for 56,055 poplar transcripts. In poplar roots, 753 genes were induced by PAO1 (1.3% of the genome, partial list Table 3.5) and 1,017 genes were repressed (1.8% of the genome, partial list Table 3.6) after 12 h of *P. aeruginosa* infection. These genes participate in a wide range of biological functions including signal transduction, pathogenesis, primary and secondary metabolism, transport, chaperoning, and transcription regulation.

Induction of expression of pathogenesis-related proteins is one of the best-known responses for grass plants and pathogenic microbes (Glazebrook *et al.*, 1996; Pinto and Ricardo, 1995). Upon *P. aeruginosa* infection in the current study, transcription of pathogenesis-related protein 5 (Glazebrook *et al.*, 1996) and PR10 (Pinto and Ricardo, 1995) increased 74-fold and 34-fold in poplar roots, respectively (Table 3.5). Transcription of the genes related to secondary metabolism was also activated, such as cytochrome P450 (37-fold), anthocyanin 5-aromatic acyltransferase (32-fold), flavonoid 3-hydroxylase (16-fold), and berberine bridge enzyme (52-fold) (Table 3.5). Berberine bridge enzyme catalyzes the reaction from reticuline to scoulerine, which is the precursor of berberine (Dittrich and Kutchan, 1991).

We also found differentially regulated genes encoding the components of signal transduction and transcription factors upon *P. aeruginosa* infection, such as nucleotide binding site-LRR disease resistance genes, *myb* transcriptional factors, AP2 domain transcription factors,

Zn-finger type transcription factors, *SCARECROW*, and the *NO APICAL MERISTEM/ATAF/CUP-SHAPED COTYLEDON* (NAC) transcription factors in poplar roots (Tables 3.5 and 3.6). Differential regulation was also observed in plant hormone-responsive genes upon *P. aeruginosa* infection, such as induction of ethylene-induced transcription factors, auxin responsive factor 1 (ARF1), and ARF10 as well as down-regulation of small auxin up RNA. In addition to plant hormone-responsive genes, the amount of phytosulfokine transcript, encoding a small peptide-growth factor in plants (Matsubayashi and Sakagami, 1996), was increased to 52-fold in poplar roots upon infection of *P. aeruginosa*.

3.4 Discussion

In this study, we demonstrate that *P. aeruginosa* PAO1 is a pathogen for poplar trees and barley. In addition, through microarrays and mutagenesis, we identified seven novel genes involved in plant pathogenesis for either the poplar or barley plant models (PA1385, PA2146, PA2462, PA2463, PA2663, PA4150, and PA4295). Mutation in PA2463 increased virulence for poplar trees (Fig. 3.2); to our knowledge, the PA2463 gene has not been studied previously. Since the function of PA2463 is predicted to be a hemolysin regulator (Winsor *et al.*, 2005), we tested this mutant and found it has more than four times elevated hemolytic activity. Given that disruption of the adjacent putative hemolysin gene PA2462 reduced by half hemolysis activity (Fig. 3.3) and reduced virulence with barley (Fig. 3.2) and given that hemolysin is a known virulence factor (Gallagher and Manoil, 2001), our findings suggest that PA2463 acts as a negative regulator of hemolysin formation, and this hemolysin is important for *P. aeruginosa* plant pathogenesis. Furthermore, disruption of PA1385 (induced 3.2-fold in the poplar rhizosphere) decreased virulence in both plant models by 3-fold; PA1385 has not been reported in previous *P. aeruginosa* DNA microarray studies (Filiatrault *et al.*, 2006; Hentzer *et al.*, 2003;

Mark *et al.*, 2005; Schuster *et al.*, 2003; Wagner *et al.*, 2003; Wagner *et al.*, 2006b). Moreover, the PA1385 mutant decreased hemolytic activity by half. PA1385 encodes a probable glycosyl transferase involved in polysaccharide formation (Jackson *et al.*, 2004). Forquin *et al.* (2007) recently showed a direct relationship between the glycosyl transferase genes and hemolysin and virulence in *Streptococcus agalactiae*.

Bacteria with disruptions in the PA2663, PA4150, and PA4295 genes were also less virulent in either the barley or poplar rhizosphere; these genes have not been identified previously in *P. aeruginosa* DNA microarray studies (Filiatrault *et al.*, 2006; Hentzer *et al.*, 2003; Mark *et al.*, 2005; Schuster *et al.*, 2003; Wagner *et al.*, 2003; Wagner *et al.*, 2006b). PA2663 encodes a hypothetical membrane protein (Winsor *et al.*, 2005), and inactivation of this gene decreased biofilm formation significantly, but did not affect cytotoxicity or hemolysis. PA4150 encodes a possible dehydrogenase (Winsor *et al.*, 2005). PA4296 is adjacent to PA4295 (identified here as induced in the poplar rhizosphere) and was shown to influence twitching, swarming, and cause virulence in the lettuce model (Wagner *et al.*, 2006b).

PA0984 encodes an immunity protein (He *et al.*, 2004), and disrupting PA0984 reduced both hemolytic activity and cytotoxicity (Fig. 3.3); this gene was identified previously as induced by root exudates from sugar beet (Mark *et al.*, 2005). Similarly, the PA2146 knock-out decreased virulence here with the barley model and was also identified previously as induced by sugar beet root exudates (Mark *et al.*, 2005); however, this is the first report of its influence on virulence. PA2146 has 92% similarity with *yciG* that controls swarming in *E. coli* (Inoue *et al.*, 2007). In addition, PA0764 (*mucB*, negative regulator of alginate synthesis), PA1458 (*cheA*, response regulator), and PA1092 (*fliC*, flagellin type B) were found here to be down-regulated in the poplar rhizosphere as seen before with sugar beet root exudates (Mark *et al.*, 2005). Mark *et al.* (2005) also showed 6 h of root exudate treatment suppressed expression of the *alg* genes

involved in alginate synthesis that is one of the essential activities for biofilm-forming bacteria. However, in our research, expression of most of the *alg* genes was induced slightly during infection of the poplar roots.

The *las* and *rhl* quorum-sensing systems control around 10% of the *P. aeruginosa* genome (Wagner *et al.*, 2006b), and PA0996 (*pqsA*) and PA2587 (*pqsH*) encode quorum-sensing genes (Gallagher *et al.*, 2002; Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003) that are up-regulated in the presence of acyl-homoserine lactone (Schuster *et al.*, 2003). In the current study, the mutation in PA0996 decreased competitiveness during poplar root colonization and decreased virulence with poplar trees. In contrast, the *rhlI* mutation (PA3476) caused a large increase in virulence with barley and increased both cytotoxicity and hemolytic activity. This is the first report that quorum-sensing as mediated by PA0996 and PA3476 is significant for virulence of *P. aeruginosa* in plants whereas loss of one of these quorum-sensing genes has been shown to decrease virulence with *Bacillus subtilis* (Park *et al.*, 2005), nematodes (Gallagher *et al.*, 2002), and mice (Gallagher *et al.*, 2002; Rumbaugh *et al.*, 1999), respectively. The *rhlI* mutant (PA3476) also increased biofilm formation in LB glu media. In contrast, Davies *et al.* (1998) reported that the *rhlI* mutant formed similar biofilms to the wild-type in flow-cells after two weeks in ERPI medium, and we observed the similar behavior in this medium (data not shown); hence, the differences in biofilm formation are due to the differences in experimental design (temperature and medium). The *pqsA* mutant (PA0996) also formed less biofilm both in LB and LB glu medium and was less competitive in the rhizosphere. Of the sixteen knocked-out genes, all but except PA2463, PA2587, and PA4295 showed less competitiveness than the wild-type strain on poplar roots. Strains that lacked PA0984, PA4151, or PA4153 were 5 to 100 times less competitive than the wild-type strain, respectively.

The approach here was successful in identifying novel virulence genes, rhizosphere

competition genes, and biofilm genes for *P. aeruginosa* PAO1 colonizing poplar roots. Nonetheless, one limitation is that the gene expression of the bacteria has only been examined for only one time (48 h after infection). Another limitation is that the gene expression of both bacteria and plants were examined on the plant roots rather than inside plant tissue; however, it is difficult to obtain sufficient bacteria for microarray experiments from bacteria inside the plant.

Although poplar is widely used as a model woody plant, global changes in its transcriptome are little known in response to bacterial infection. Here, *P. aeruginosa* infection increased expression of pathogenesis-related proteins, indicating that induction of these proteins is also a common response in woody plants like that seen in *Arabidopsis* (Glazebrook *et al.*, 1996) and *Lupinus albus* (Pinto and Ricardo, 1995). PR5 protein (induced 74-fold) is a homolog of tobacco osmotin that has antifungal activity (Yun *et al.*, 1997). Osmotin is a putative apoptosis inducer through PHO36, an osmotin receptor, in budding yeast (Narasimhan *et al.*, 2005). Although antibacterial activity of osmotin is not well-known, it might play a defensive role in poplar roots against *P. aeruginosa* infection. Moreover, we found up-regulation of the gene encoding the berberine bridge enzyme. Berberine treatment triggers apoptosis-like cell death and enhances generation of reactive oxygen species in human cells (Jantova *et al.*, 2006). Taken together with osmotin, production of berberine may also be one of the defense responses in poplar against pathogenic attack through induction of apoptosis cell death for cells infected with *P. aeruginosa*.

We also identified some poplar genes for transcriptional regulators that were differentially regulated during *P. aeruginosa* infection such as the LRR disease resistance gene, the NAC transcriptional factor, and the *myb* transcriptional factor. A typical nucleotide binding site-LRR disease resistance gene, FLS2, functions in recognition of flagellin in *Arabidopsis* and participates in activation of the defense response in the innate immune system (Gómez-Gómez

and Boller, 2000). In poplar, expression of the LRR genes indicates a diverse response to *P. aeruginosa* infection, and three of the LRRs were highly induced and seven were suppressed. Besides FLS2, some classes of NAC transcription factors in rice were controlled by flagellin perception (Fujiwara *et al.*, 2004). Furthermore, flg22, a peptide containing the most conserved domain of flagellin, induces some LRRs and the *myb* transcriptional factor in *Arabidopsis* (Navarro *et al.*, 2004). These results suggest that flagellin-mediated signal transduction may play an essential part in the early defense mechanism in poplar. Altered expression of auxin-related genes has been observed often during plant-microbe interactions (Wang *et al.*, 2005d), which suggests auxin-mediated morphological changes in plants. ARF transcriptional factors mediate auxin-dependent transcriptional activation or repression, and ARF10, participates in the development of root cap cells (Wang *et al.*, 2005a). Auxin has diverse effects on plant growth, including tissue development and tropism to light and gravity (Perrot-Rechenmann and Napier, 2005). When the pathogenic bacterium *A. tumefaciens* infects a plant, an auxin-mediated tumor is formed (Ooms *et al.*, 1981). *P. aeruginosa* also synthesizes auxin, and this may affect the auxin-mediated response in plants. It is interesting that auxin induces expression of *SCARECROW* (Gao *et al.*, 2004), which regulates cell differentiation in the root cortex/endodermis daughter cells (Di Laurenzio *et al.*, 1996). Thus, developmental regulation is often caused by auxin-related signaling in plants, and it may be one of the adaptive responses to bacterial infection.

Investigations of plant-microbe interactions in the rhizosphere are often complicated by the need to separate plant and microbe samples from soil without introducing artifacts. In this research, we used a hydroponic system to study poplar and *P. aeruginosa* interactions in the rhizosphere, and it enabled us to obtain samples from the rhizosphere without soil effects. We note that the term rhizosphere includes hydroponic growth conditions (Cramer *et al.*, 1999;

Jauert *et al.*, 2002; Morgan *et al.*, 2005; Soda *et al.*, 2007), even though it was originally defined as the soil compartment influenced by the roots (Hinsinger and Marschener, 2006). Thus, our hydroponic system is also useful for rhizosphere research, although some differences may occur in results obtained in a soil rhizosphere vs. a hydroponic rhizosphere.

Previously, *P. aeruginosa* PA14 was shown to infect disparate species by using the same virulence factors (Hendrickson *et al.*, 2001; Mahajan-Miklos *et al.*, 1999; Rahme *et al.*, 1995; Rahme *et al.*, 1997; Rahme *et al.*, 2000; Tan *et al.*, 1999b). Analogously, since some of the genes we identified through the *Pseudomonas* transcriptome analysis are significant for virulence with both plants and human models (e.g., PA1385, PA2462, PA2463, and PA3476), some virulence-related functions of *P. aeruginosa* are not limited to specific hosts. It is also clear that virulence gene expression in biofilms requires a live poplar tree as *P. aeruginosa* genes were clearly differentially expressed relative to the inert solid (glass wool). Overall, this work broadens our understanding of the genetic basis of pathogenesis in the rhizosphere (as well as biofilm formation) with *P. aeruginosa* and increases our understanding of the response of poplar trees to pathogenic bacteria; it may also help to define the interaction of this pathogenic bacterium (and others) with other eukaryotic hosts (e.g., humans).

3.5 Experimental procedures

3.5.1 Bacterial strains and growth

Strains and plasmids are listed in Table 3.3. LB medium (Sambrook *et al.*, 1989) was used to grow the bacterial strains, and HRP minimal medium (contains per liter 5.5 g of KH_2PO_4, 1.5 g of K_2HPO_4, 1 g of $(NH_4)_2SO_4$, 0.735 g of $MgCl_2 \cdot 6H_2O$, and 0.1 g of NaCl) (Huynh *et al.*, 1989) with 0.25 wt% sucrose was used during contact of bacteria with plants. The sequenced *P.*

aeruginosa PAO1 Holloway strain (Stover *et al.*, 2000) was used with the DNA microarrays. Colonization of *P. aeruginosa* PAO1 on poplar tree roots was visualized using a TCS SP5 scanning confocal laser microscope with a 63x HCX PL FLUOTAR L dry objective with correction collar and numerical aperture of 0.7 (Leica Microsystems, Mannheim, Germany) using the constitutive green fluorescent protein plasmid pMRP9-1 (Davies *et al.*, 1998). To select for the knock-out mutants, 50 μg/mL tetracycline was used.

3.5.2 Poplar trees growth

To facilitate rapid growth of plants and removal of soil without damaging fragile plant roots, poplar cuttings (DN-34 Imperial hybrid poplar, Segal Ranch, Grandview, WA) were surface sterilized with 3% hydrogen peroxide for 10 min and planted in polyethylene autoclavable bags (Fisher Scientific, Pittsburg, PA) containing 3.5 kg of autoclaved sand. Poplar trees were placed under 60 W Spot-Gro plant light bulbs (Sylvania, St. Marys, PA), illuminated for 16 h each day, and irrigated with 10% sterile Hoagland's solution every day (Shim *et al.*, 2000).

3.5.3 Bacterial RNA isolation and microarray analysis

After five weeks of growth, twenty-five poplar trees were gently removed from the autoclave bags by cutting the bag with scissors, and the fragile roots were exposed by gently rinsing with distilled water in a manner that preserved the fine root filaments. Overnight *P. aeruginosa* PAO1 cultures were resuspended in 1.5 L of 1 x HRP minimal medium with 0.25% sucrose at a turbidity of 0.6 at 600 nm, and the poplar trees were dipped in a 3 L plastic beaker containing the resuspended PAO1 culture. Since sucrose is the main carbohydrate in the young poplars (Bonicel *et al.*, 1987), it was used as a carbon source to obtain more bacteria.

Furthermore, sucrose does not effect plant root colonization (Lugtenberg *et al.*, 1999). The bacterial culture and poplar trees were shaken at 150 rpm (KS250BS1 shaker, IKA, Germany) at room temperature for 48 h and illuminated for 16 h per day. Total wet weight of poplar trees roots was approximately 20 g. In the glass wool experiment, cells were prepared with the same manner except 20 g of glass wool was used instead of poplar trees. After 48 h of contacting PAO1 with poplar roots or glass wool, the poplar roots and glass wool were washed in 200 mL of 0°C 0.85% NaCl buffer for 30 s, and the biofilm cells were removed from the poplar roots or glass wool by sonicating at 22 W (FS3 sonicator, Fisher Scientific, Pittsburg, PA) in 200 mL of 0°C 0.85% NaCl buffer. The buffer was centrifuged at 10,000 *g* for 2 min at 4°C (J2-HS centrifuge, Beckman, Palo Alto, CA). RNA isolation from *P. aeruginosa* PAO1, cDNA synthesis, fragmentation, and hybridizations were as described previously (Domka *et al.*, 2007). The absence of contamination was verified by streaking the culture contacted with poplar trees on LB agar plates and confirming the presence of green colonies as *P. aeruginosa* PAO1.

The Affymetrix Genechip *P. aeruginosa* Genome Array (Affymetrix, P/N 900339) contains 5,500 of the 5,570 ORFs of *P. aeruginosa* (Whiteley *et al.*, 2001). The reliability of induced and repressed genes was ensured with a *P*-value less than 0.05. The intensities of polyadenosine RNA control were used to monitor the labeling process. The total signal intensity was scaled to an average value of 500. Genes were identified as differentially expressed if the expression ratio was greater than 2-fold change based on standard deviations of the genes of 1.7 to 1.9 (Domka *et al.*, 2007). The expression data have been submitted to the NCBI Gene Expression Omnibus (GSE5887).

3.5.4 Poplar RNA isolation and microarray analysis

Overnight *P. aeruginosa* PAO1 cultures were re-suspended in 1.5 L of 1 x HRP + 0.25%

sucrose at turbidity at 600 nm of 0.6. Two five-week-old poplar trees with roots were gently taken from sand and washed with distilled water in a manner that preserved the fine root filaments. One poplar tree was dipped in the culture containing *P. aeruginosa* PAO1 and the other one was dipped in medium lacking *P. aeruginosa* PAO1. The trees were shaken in 250 mL Erlenmeyer flasks at 150 rpm for 12 h at room temperature, then the poplar roots were dipped in 100 mL of 0°C 0.85% NaCl buffer, the roots of the poplar tree were cut with sterile scissors, and the roots were sonicated for 2 min to remove bacteria. To avoid the effect of sonication on gene expression, sonication was also performed for the poplar tree not non-contacted with *P. aeruginosa* PAO1. RNA was isolated from poplar roots as described previously (Brunner *et al.*, 2004) with minor modifications by grinding roots to a powder with a pestle in a mortar containing liquid nitrogen. The remaining steps of RNA isolation were performed according to the Qiagen RNeasy Plant Mini kit protocol. Subsequent steps consisting of cDNA synthesis, biotin labeling of cDNA, fragmentation, hybridization, and scanning were performed at the Center for Functional Genomics at the State University of New York, University at Albany.

The Genechip Poplar Genome Array (Affymetrix, P/N 900728) contains 61,251 poplar probe sets representing 56,055 transcripts. Induced and repressed genes were identified as differentially expressed if the *P*-value was less than 0.05 and the expression ratio was greater than 4 based on the standard deviations of 4.2 for both arrays (Domka *et al.*, 2007). Data quality was assessed by the hybridization controls and scaling factors. The expression data have been deposited in the NCBI Gene Expression Omnibus (GSE5887).

3.5.5 Verification of knock-outs

The *P. aeruginosa* transposon mutants were obtained from UBC (Lewenza *et al.*, 2005)

or UW (Jacobs *et al.*, 2003); the UBC library was constructed with a mini-Tn*5*-*luxCDABE* (promoter trap), and the UW library was constructed using IS*phoA*/hah or IS*lacZ*/hah (contain internal promoters). Insertion of mini-*Tn5*-*luxCDABE* transposon in PA2463 locus was confirmed by a PCR-based method with chromosomal DNA purified from both wild-type PAO1 and PA2463 mutant (McPhee *et al.*, 2003) (see Table 3.7 for primer sequences). The PA2463-F primer and PA2463-R primer were used to confirm the presence of the transposon insertion in PA2463 locus by amplifying 450 bp of the partial PA2463 gene. In addition, the Tn5-out primer and PA2463-R were used to amplify 700 bp fragment corresponding to the end of the transposon and its flanking region of PA2463 gene. Similarly, all of the knock-out mutants used in this study were verified by PCR.

3.5.6 Poplar pathogenicity wilting assay

The degree of wilting of poplar tree branches was devised by us to indicate poplar tree health. To minimize contamination, poplar trees were dipped in bacterial cultures in 1 x HRP medium containing kanamycin 50 (μg/mL); kanamycin at this concentration does not alter tree viability (poplar trees were viable for more than seven days in 1 x HRP medium with kanamycin without bacteria). The change in branch angle was measured during the poplar tree-bacteria contact. To measure pathogenicity of mutants and wild-type strain, angles were measured every 24 h from the vertical axis (clockwise) and the stem of poplar was considered to be origin. For each strain, at least three separate trees were used. The data for wilting experiments were analyzed with a Student t-test, and those with the *p*-value less than or equal to 0.05 were chosen as significant (Ross, 2004).

Table 3.7. Primers used for verifying the *P. aeruginosa* knock-out mutations.

Primer Name	Nucleotide sequence (5' to 3')	Primer Name	Nucleotide sequence (5' to 3')
UW mutants			
PA0513-F	GCAGGTAATGGCTGATCCTC	PA0513-R	TGGCCATACAACCTGTTCTG
PA0981-F	AACTGCGATCCAATTGAAGG	PA0981-R	GCAGAGTCACACTCGGATGA
PA0984-F	TTGGATACAGGTCCGGAGAG	PA0984-R	AGTTGGCTTGTGGGTATTGC
PA0985-F	GGAGCGATGAAGGTTTTTCA	PA0985-R	TAGACAAGCAGATGATGGCG
PA0996-F	CGCCTCGAACTGTGAGATTT	PA0996-R	GAGAAATCGTCGAGCAAAGG
PA1385-F	TCGATGCCGTGTTCAGTTAC	PA1385-R	ACCTGGAAACTCACATTCCG
PA1432-F	GGGAGAAGGAAGTGTTGCAG	PA1432-R	ATCATCATCTTCTCCACGCC
PA2146-F	TAATTAAGTGCCTCCGCCTG	PA2146-R	CAGGGTGTTCTGGTTATCGG
PA2459-F	CCTCCCTGCTCAGAAATCAG	PA2459-R	GAAAAACCGCTCTGCAAAAG
PA2462-F	GATTGTTCAGTGTGTTGGCG	PA2462-R	TAGCAAGGGCGATACACACA
PA2587-F	AGACGGTGACTACCACGACC	PA2587-R	CGCGCGAGATGTATGTCTAC
PA2663-F	AGGAAGCTCAGTTGCAGCAC	PA2663-R	AGCAGGTCGAGCACTTCTTC
PA4150-F	GACTGCATCGCCTCCACC	PA4150-R	CCATGATGGTTCTCCTCGTT
PA4151-F	AAGGGCCTCTACCACCAGTT	PA4151-R	ATCTTGTCGGTCTCCACGTC
PA4549-F	CTCGATCGGCACTTCCTTC	PA4549-R	AGGAGCCTTTCCTTCTTTGC
HaH minus 138	CGGGTGCAGTAATATCGCCCT	LacZ 148	GGGTAACGCCAGGGTTTTCC
UBC mutants			
PA2463-F	GGGTCAGGCTGTACTTGTCG	PA2463-R	TCCGACAGCCAGAGCCTGTT
PA3278-F	ATCAAGCCGGAGAACAAGGA	PA3278-R	GCAGGAAACCAGCAGGTAGG
PA3476-F	TGCTCTCTGAATCGCTGGAA	PA3476-R	GAAACGGCTGACGACCTCAC
PA4153-F	GTTGCTGGACAACCTGTTCG	PA4153-R	CATGCCGGCAGTAGTAGCAG
Tn5-out	GATCCCCGGGTACCGAGCTCGAATTC	Tn5-out2	CAGAACATATCCATCGCGTCCGCC

3.5.7 Root microscopy

To observe *P. aeruginosa* cells inside roots, poplar trees were infected with *P. aeruginosa* PAO1-UW tagged with pMRP9-1 by placing trees in 250 mL flasks. After 48 h, poplar root tips were embedded in optimum cutting temperature compound (Tissue-Tek, SAKURA Finetechnical, Tokyo, Japan), sectioned at 20 μm thickness by a cryostat microtome (JUNG CM 1800, Leica), and observed using scanning confocal laser microscope.

3.5.8 Barley seed pathogenicity assay

Barley seeds (cultivar Belford) were purchased from Stover Seed Company (Los Angeles, CA) and were surface-sterilized in 1% sodium hypochlorite solution for 30 min, then washed with sterilized distilled water ten times. *P. aeruginosa*, grown in LB medium at 30°C, was harvested at turbidity at 600 nm of 1. *P. aeruginosa* cells were washed once with sterilized distilled water and twice with 1 x Hoagland solution, and then resuspended to turbidity at 600 nm of 1.00 ± 0.03. Fifteen barley seeds were germinated in 10 mL of 1 x Hoagland solution without (control), with PAO1, or with each mutant at 25ºC with gentle shaking. After 3 days, the number of germinated seeds was counted compared to that of wild-type PAO1 treatment. All experiments were repeated at least three times (45 seeds). The data for barley germination experiments were analyzed with a Student t-test and those with a *p*-value less than or equal to 0.05 were chosen as significant (Ross, 2004).

3.5.9 Crystal violet biofilm assay

Biofilm formation was quantified in 96-well polystyrene plates as described previously (Ren *et al.*, 2005a). Overnight *P. aeruginosa* PAO1 cultures were diluted in LB and LB supplemented with 0.2 wt% glucose to a turbidity of 0.05 of 600 nm. Diluted cultures were

inoculated into the plates and were grown at 30°C without shaking. Before measuring the biofilm mass, the growth of the cells was quantified using turbidity at 620 nm. Ten replicate wells were averaged to obtain each data point. Two independent cultures were used.

3.5.10 Swimming motility assay

Agar plates containing 1% tryptone, 0.25% NaCl, and 0.3% agar were used to assay motility in plates as described previously (González-Barrios *et al.*, 2006b). Motility halos were measured at 5 and 18 h. Six plates were used to evaluate motility in each strain. Two independent cultures were tested for each strain.

3.5.11 Bacterial rhizosphere competition assay

One mL of overnight-grown *P. aeruginosa* PAO1 and the knock-out mutants were inoculated each into 25 mL of LB and were grown to a turbidity of 1.6 at 600 nm. Once, the cells reached this OD, they were washed with HRP minimal media at 5,500 *g* for 5 min at 4°C, and resuspended in 7.5 mL of HRP minimal media. The OD of the mutants and wild-type were measured after resuspension. The same amount of mutant and wild-type cells was added to a total of 150 mL of HRP minimal media in a 250 mL of Erlenmeyer flask. One poplar tree was placed into the culture containing *P. aeruginosa* PAO1 and the mutant was contacted for 48 h, the root tip was cut with sterilized scissors, sonicated in 100 mL of 0.85% NaCl buffer, then plated with appropriate dilutions on LB agar containing kanamycin 50 µg/mL to determine the total number of *P. aeruginosa* cells as well as LB containing kanamycin 50 µg/mL and tetracycline 50 µg/mL to determine the number of *P. aeruginosa* knock-out cells. Bacterial concentrations were expressed as colony forming units per gram of dry root weight. All experiments were repeated at least twice. The data for crystal violet biofilm assay, swimming

motility, and bacterial rhizosphere competition experiments were analyzed with a Student t-test, and those with a *p*-value less than or equal to 0.05 were chosen as significant (Ross, 2004).

3.5.12 Hemolysis and cytotoxicity assays

Hemolysis assays to determine the hemolytic activity were carried out as described previously (Blocker *et al.*, 1999) with some modifications. Basically, bacteria were grown overnight at 37°C and inoculated into fresh medium until they reached turbidity at 600 nm of approximately 1. Bacteria were then pelleted at 1,000 *g* for 10 min and suspended at equal concentrations in 2 mL of saline. The whole cells were then aliquoted into 96-well plates in 200 µL final volume at a concentration equivalent to 10^8 colony forming units and diluted 1:2 in saline six times. Eighty microliter of 2% human red blood cells and 80 µL of each bacterial preparation were then added to a fresh 96-well plate and incubated at 37°C for two hours. The plate was centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant was transferred to a fresh 96-well plate and the OD_{450} was determined. Controls include saline (negative) and 0.02% Tween-20 (positive) in all experiments. The positive control was set as 100% hemolysis, and the negative control was set as 0% hemolysis. The value for red blood cells without Tween and bacteria was used to subtract the background in the spectrophotometer readings. Each sample was prepared in quadruplicate.

Standard lactate dehydrogenase release cytotoxicity assay was used in these studies (Behl *et al.*, 1994; Brander *et al.*, 1993) as described previously (Cirillo *et al.*, 2001). The procedure used was essentially as recommended by the manufacturer of the CytoTox96 Non-Radioactive Cytotoxicity Assay system (Promega). Serial dilutions were made of each bacterial strain at multiplicity of infections of 500, 250, 100, and 10 in a final volume of 100 µL for each assay using 4 x 10^4 human peripheral blood monocytic cells (PBMCs). Appropriate numbers of

cells for CytoTox96 assays were determined as suggested by the manufacturer (Promega). As a positive control for 100% cytotoxicity, the cells are lysed with 9% v/v Triton X-100 (Promega). The cells were incubated with the bacteria for 4 h at 37°C + 5% CO_2. Cytotoxicity readings were taken using an ELISA plate reader at 450 nm. Percent cytotoxicity was calculated as recommended by the manufacturer and corrected for small differences in the inocula used. PBMCs were isolated from 50 mL of human blood obtained from healthy volunteers. The mononuclear cell fraction was purified by centrifugation in Ficoll at 700 *g* for 30 min at room temperature. The PBMC containing band was removed, washed twice in Hanks balanced salt solution (Gibco), and suspended in Roswell Park Memorial Institute medium with 0.1% heat-inactivated human serum to a concentration of 10^6 cells/mL.

CHAPTER IV

PA2663 (PpyR) INCREASES BIOFILM FORMATION IN *PSEUDOMONAS AERUGINOSA* PAO1 THROUGH THE *PSL* OPERON AND STIMULATES VIRULENCE AND QUORUM-SENSING PHENOTYPES*

4.1 Overview

Previously, we identified the uncharacterized predicted membrane protein PA2663 of *Pseudomonas aeruginosa* PAO1 as a virulence factor using a poplar tree model; PA2663 was induced in the poplar rhizosphere, and upon inactivation, it caused 20-fold lower biofilm formation (Attila *et al.*, 2008a). Here, we confirmed PA2663 is related to biofilm formation by restoring the wild-type phenotype by complementing the PA2663 mutation in *trans* and investigated the genetic basis of its influence on biofilm formation through whole-transcriptome and phenotype studies. Upon inactivating PA2663 by transposon insertion, the *psl* operon that encodes a galactose- and mannose-rich exopolysaccharide was highly repressed (verified by RT-PCR). The inactivation of PA2663 also repressed 13 pyoverdine genes, which eliminated the production of the virulence factor pyoverdine in *P. aeruginosa*. The inactivation of PA2663 also affected other quorum-sensing-related phenotypes in that it repressed the *Pseudomonas* quinolone signal (PQS) genes, which abolished PQS production, and repressed *lasB*, which

*Reprinted with the permission from "PA2663 (PpyR) increases biofilm formation in *Pseudomonas aeruginosa* PAO1 through the *psl* operon and stimulates virulence and quorum-sensing phenotypes" by Can Attila, Akihiro Ueda, and Thomas K. Wood, 2008, *Applied Microbiology and Biotechnology* 78: 293-307, Copyright by Springer. C. Attila was responsible for all the experiments except the construction of the pMMB207-PA2663 plasmid.

decreased elastase activity sevenfold. Genes were also induced for motility and attachment (PA0499, PA0993, PA2130, and PA4549), and for small molecule transport (PA0326, PA1541, PA1632, PA1971, PA2214, PA2215, PA2678, and PA3407). Phenotype arrays also showed that PA2663 represses growth on *D*-gluconic acid, *D*-mannitol, and *N*-phthaloyl-*L*-glutamic acid. Hence, the PA2663 gene product increases biofilm formation by increasing *psl*-operon-derived exopolysaccharides and increases pyoverdine synthesis, PQS production, and elastase activity while reducing swarming and swimming motility. We speculate PA2663 performs these myriad functions as a novel membrane sensor.

4.2 Introduction

Eighty percent of bacterial infections are caused by bacteria in biofilms (Costerton, 2004), and *P. aeruginosa* is notorious for biofilm formation (Sauer *et al.*, 2004); hence, it is a model organism for biofilm formation (Caiazza *et al.*, 2007). *P. aeruginosa* is a Gram-negative, opportunistic bacterium that causes several acute and chronic infections (Filiatrault *et al.*, 2006) in humans, animals, insects, nematodes, and plants (Lewenza *et al.*, 2005). Its resistance to antibiotics and disinfectants makes it an important pathogen (Stover *et al.*, 2000). With more than 150 predicted outer membrane proteins and four chemotaxis systems, *P. aeruginosa* is also an important bacterium for the transport and efflux of compounds (Stover *et al.*, 2000).

The extracellular matrix of biofilms encloses bacterial cells and is comprised of polymeric substances such as nucleic acids, proteins, and polysaccharides (Friedman and Kolter, 2004a); this biofilm matrix protects cells from host defenses (Fux *et al.*, 2005). In *P. aeruginosa* PAO1, two operons, *pel* and *psl*, are important for biofilm formation. The *pel* operon affects biofilm maturation, and the *psl* operon affects biofilm initiation (Ma *et al.*, 2006). The *psl* operon was identified by three different research groups (Friedman and Kolter, 2004a; Jackson *et*

al., 2004; Matsukawa and Greenberg, 2004), and the polysaccharide encoded by this operon is essential for preserving the biofilm structure of *P. aeruginosa* (Ma *et al.*, 2006). The *psl* operon is not found in all *P. aeruginosa* strains; for example it is absent in *P. aeruginosa* PA14 (Kuchma *et al.*, 2007).

Other biofilm factors for *P. aeruginosa* include GacA, a response regulator (Parkins *et al.*, 2001), which increases biofilm formation and antibiotic resistance (Parkins *et al.*, 2001). Surface attachment is also important as *sadB* mutation impairs irreversible attachment of *P. aeruginosa* (Caiazza and O'Toole, 2004). Another important biofilm determinant is iron, as it promotes both biofilm formation and pathogenicity since iron is essential for growth and is present at low concentrations (10^{-18} M) (Kaneko *et al.*, 2007). *P. aeruginosa* has over 30 genes targeting iron (Kaneko *et al.*, 2007) and is bound to iron-carriers (siderophores) to increase availability for bacterial cells (Michel *et al.*, 2005). Pyoverdine (Merriman *et al.*, 1995) and pyochelin (Michel *et al.*, 2005) are the two siderophores produced by *P. aeruginosa*. In *P. aeruginosa,* pyoverdine controls three virulence factors, endotoxin A, elastase, and itself (Lamont *et al.*, 2002); hence, pyoverdine is important for the pathogenicity of *P. aeruginosa*.

Another virulence factor of *P. aeruginosa* is *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone), a quorum-sensing signal that controls the production of pyocyanin, rhamnolipids, and elastase (Diggle *et al.*, 2006). PQS regulation is intertwined with the other two quorum-sensing systems, Rhl and Las (Diggle *et al.*, 2006).

Previously, we identified PA2663 as induced in a rhizosphere biofilm of *P. aeruginosa* formed on poplar trees vs. a biofilm formed on inert glass wool (Attila *et al.*, 2008a). We showed that this mutant formed 20-fold and 11-fold less biofilm in LB and LB glu medium in 24 h, respectively, and that the inactivation of PA2663 reduced the virulence of *P. aeruginosa* toward barley (Attila *et al.*, 2008a). PA2663 is a putative membrane protein (Winsor *et al.*,

2005). To investigate the genetic basis of this biofilm inhibition and reduction in virulence, DNA microarrays were used here to study the differential gene expression of *P. aeruginosa* PAO1 PA2663 vs. the wild-type strain in biofilms. Phenotype arrays were also utilized to study the effect of inactivation of PA2663 on metabolism. We found PA2663 encodes a protein that influences the *psl* biofilm locus, pyoverdine formation, elastase activity, and PQS production; hence, we identified the first regulatory protein for the *psl* operon and another regulatory protein for quorum-sensing and virulence phenotypes.

4.3 Results

4.3.1 Differential gene expression in biofilms upon inactivating PA2663

To explore the mechanism by which PA2663 induces biofilm formation in *P. aeruginosa*, differential gene expression in biofilms was performed. The most striking changes in gene expression were found in the genes repressed upon inactivating PA2663, and these are shown in Table 4.1 (there were 71 genes repressed with a 50-fold cutoff and 49 genes induced with a 4-fold cutoff). Clearly the repression of transcription was dominant; hence, PA2663 is an activator of gene expression.

Of the repressed genes, fifteen genes of the *psl* operon that play an important role in biofilm formation in *P. aeruginosa* (Ma *et al.*, 2006) were repressed consistently from 6- to 512-fold; for example, *pslA* and *pslB*, which encode proteins that are important in biofilm initiation (Jackson *et al.*, 2004), were repressed by 79-fold and 512-fold, respectively. *pslD*, which encodes a secretion protein necessary for biofilm formation (Campisano *et al.*, 2006), was also repressed by 194-fold change. Hence, the PA2663 protein is critical for induction of the *psl* operon. Thirteen pyoverdine genes were also repressed from 9- to 832-fold (Table 4.1). For

Table 4.1. Partial list of repressed genes for *P. aeruginosa* PAO1 PA2663 versus *P. aeruginosa* PAO1 in biofilms formed on glass wool in LB at 30°C after 7 h. Complete analysis deposited at the NCBI Gene Expression Omnibus (GSE9255).

PA #	Gene name	Fold change	Descriptions
Pyoverdine genes			
PA2385	*pvdQ*	-13.9	Adaptation, protection, protein related to penicillin acylase
PA2386	*pvdA*	-831.7	Adaptation, protection, *L*-ornithine N^5-oxygenase
PA2393		-111.4	Central intermediary metabolism, probable dipeptide precursor
PA2394	*pvdN*	-97.0	Selenocysteine lyase, pyoverdine synthesis
PA2395	*pvdO*	-13.9	Pyoverdine synthesis
PA2396	*pvdF*	-36.8	Pyoverdine synthetase, folate-dependent phosphoribosylglycinamide formyltransferase PurN
PA2397	*pvdE*	-42.2	Transport of small molecules, membrane protein, pyoverdine biosynthesis protein
PA2398	*fpvA*	-119.4	Transport of small molecules, ferripyoverdine receptor, outer membrane receptor for ferric coprogen and ferric-rhodotorulic acid
PA2399	*pvdD*	-9.2	Secreted factors, pyoverdine synthetase D
PA2400	*pvdJ*	-104.0	Non-ribosomal peptide synthetase modules and related proteins
PA2413	*pvdH*	-45.3	Adaptation, protection, *L*-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, pyoverdine synthesis
PA2424	*pvdL*	-84.4	Adaptation, protection, non-ribosomal peptide synthetase modules and related proteins, pyoverdine synthesis
PA2425	*pvdG*	-84.4	Predicted thioesterase involved in non-ribosomal peptide biosynthesis, pyoverdine synthesis
PA2426	*pvdS*	-55.7	Transcriptional regulator, sigma factor PvdS
***psl* genes**			
PA2231	*pslA*	-78.8	Probable glycosyl transferase, 69% similar to colonic acid UDP-glucose lipid carrier, WcaJ in *E. coli*
PA2232	*pslB*	-512.0	Probable phosphomannose isomerase/GDP-mannose pyrophosphorylase
PA2233	*pslC*	-111.4	Probable glycosyl transferase
PA2234	*pslD*	-194.0	Transport of small molecules, probable exopolysaccharide transporter
PA2235	*pslE*	-59.7	Uncharacterized protein involved in exopolysaccharide biosynthesis

Table 4.1. (continued)

PA #	Gene name	Fold change	Descriptions
PA2236	*pslF*	-36.8	Glycosyltransferase
PA2237	*pslG*	-222.9	Probable glycosyl hydrolase, β-xylosidase
PA2238	*pslH*	-104.0	Glycosyltransferase
PA2239	*pslI*	-97.0	Glycosyltransferase
PA2240	*pslJ*	-84.4	Part of *pslABCDEFGHIJKLMNO* cluster involved in biofilm development
PA2241	*pslK*	-5.7	Uncharacterized membrane protein, putative virulence factor
PA2242	*pslL*	-17.1	Fucose 4-*O*-acetylase and related acetyltransferases
PA2243	*pslM*	-17.1	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit
PA2244	*pslN*	-9.8	Topoisomerase IB
PA2245	*pslO*	-168.9	Hypothetical protein
PA2246	*bkdR*	-11.3	Transcriptional regulator
PA2247	*bkdA1*	-831.7	2-oxoisovalerate dehydrogenase
PA2248	*bkdA2*	-97.0	2-oxoisovalerate dehydrogenase
PA2249	*bkdB*	-73.5	Branched-chain alpha-keto acid dehydrogenase
PA2250	*lpdV*	-97.0	Amino acid biosynthesis and metabolism, energy metabolism, pyruvate 2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component
Biosynthesis of cofactors, prosthetic groups and carriers			
PA0996	*pqsA*	-3.0	Probable coenzyme A ligase, part of *pqsABCDE* operon
PA0997	*pqsB*	-4.3	Homologous to beta-keto-acyl carrier protein synthase, part of *pqsABCDE* operon
PA0998	*pqsC*	-3.5	Homologous to beta-keto-acyl carrier protein synthase, part of *pqsABCDE* operon
PA0999	*pqsD*	-2.8	3-oxoacyl synthase III, part of *pqsABCDE* operon
PA1000	*pqsE*	-2.1	Quinolone signal response protein, part of *pqsABCDE* operon
PA2587	*pqsH*	-2.6	Probable FAD-dependent monooxygenase,

Table 4.1. (continued)

PA #	Gene name	Fold change	Descriptions
PA2512	*antA*	-415.9	Anthranilate dioxygenase large subunit
PA2513	*antB*	-168.9	Anthranilate dioxygenase small subunit
PA2514	*antC*	-90.5	Anthranilate dioxygenase reductase
Carbon compound metabolism			
PA2265		-78.8	Gluconate dehydrogenase, this family of proteins bind FAD as a cofactor
PA2290	*gcd*	-157.6	Energy metabolism, glucose dehydrogenase
Antibiotic resistance and susceptibility			
PA2491		-111.4	Probable oxidoreductase
PA2492	*mexT*	-6.1	Transcriptional regulator
PA2493	*mexE*	-6.5	Transport of small molecules, resistance-nodulation-cell division (RND) multidrug efflux membrane fusion protein MexE
PA2494	*mexF*	-55.7	Transport of small molecules, membrane protein, resistance-nodulation-cell civision, multidrug efflux transporter MexF
PA2495	*oprN*	-4.0	Transport of small molecules, membrane protein, multidrug efflux outer membrane protein OprN precursor
PA2496		-59.7	Hypothetical protein
Amino acid biosynthesis and metabolism			
PA2443	*sdaA*	-45.3	*L*-serine dehydratase
PA2444	*glyA2*	-955.4	Serine hydroxymethyltransferase
PA2445	*gcvP2*	-137.2	Central intermediary metabolism, glycine cleavage system protein P2
PA2446	*gcvH2*	-84.4	Glycine cleavage system protein H2
Secreted factors			
PA3724	*lasB*	-4.0	Elastase LasB

Table 4.1. (continued)

PA #	Gene name	Fold change	Descriptions
Transport of small molecules			
PA2276		-59.7	Transcriptional regulator, AraC-type DNA-binding domain-containing proteins
PA2277	*arsR*	-5.7	Transcriptional regulator
PA2278	*arsB*	-6.5	Membrane proteins, transport of small molecules, Na^+/H^+ antiporter NhaD and related arsenite permeases
PA2279	*arsC*	-64.0	Adaptation, protection, protein phosphatase
Hypothetical, unclassified, unknown function			
PA2303		-52.0	Hypothetical protein, probable taurine catabolism dioxygenase
PA2304		-24.3	Hypothetical protein, probable taurine catabolism dioxygenase
PA2305		-48.5	Probable non-ribosomal peptide synthetase
PA2306		-52.0	Membrane protein, putative threonine efflux protein
PA2330		-137.2	Hyothetical protein, Acyl-CoA dehydrogenases
PA2331		-238.9	Membrane protein
PA2363		-157.6	Hypothetical protein
PA2365		-207.9	Hypothetical protein, 71% similar to putative 19.5 kDa protein Eip20 in *Edwardsiella ictaluri*
PA2366		-1024.0	Hypothetical protein, 76% similar to putative 19.5 kDa protein Eip55 in *Edwardsiella ictaluri*
PA2367		-724.1	Hypothetical protein, hemolysin-coregulated protein
PA2368		-238.9	Hypothetical protein
PA2369		-27.9	Membrane protein
PA2370		-119.4	Hypothetical protein
PA2371		-27.9	Translation, post-translational modification, degradation, ATPases with chaperone activity
PA2372		-147.0	Hypothetical protein
PA2373		-39.4	Hypothetical protein, 46% similar to VgrG protein in Escherichia coli
PA2374		-52.0	Hypothetical protein
PA2375		-238.9	Membrane protein

Table 4.1. (continued)

PA #	Gene name	Fold change	Descriptions
PA2376		-5.7	Transcriptional regulator, response regulator containing a CheY-like receiver and a DNA-binding domain
PA2377		-4.9	Hypothetical protein, ABC-type Fe3+ transport system, periplasmic component
PA2378		-64.0	Probable aldehyde dehydrogenase, aerobic-type carbon monoxide dehydrogenase, CoxL/CutL homologs
PA2379		-9.2	Probable oxidoreductase
PA2380		-128.0	Hypothetical protein
PA2381		-362.0	Hypothetical protein
PA2382	*lldA*	-6.5	Energy metabolism, *L*-lactate dehydrogenase
PA2383		-78.8	Probable transcriptional regulator, 50% similar to positive regulator of *gcv* operon GcvA in *Escherichia coli*
PA2384		-111.4	Hypothetical protein
PA2403		-52.0	Membrane proteins, uncharacterized iron-regulated membrane protein
PA2404		-415.9	Membrane proteins
PA2405		-256.0	Type I export signal computationally predicted by LipoP v.1.0
PA2406		-18.4	Hypothetical protein
PA2407		-29.9	Motility and attachment, probable adhesion protein, ABC-type metal ion transport system
PA2408		-128.0	Transport of small molecules, probable ATP-binding component of ABC transporter
PA2409		-17.1	Membrane proteins, transport of small molecules, probable permease of ABC transporter
PA2410		-24.3	Hypothetical protein
PA2411		-73.5	Probable thioesterase, predicted thioesterase involved in non-ribosomal peptide biosynthesis
PA2412		-512.0	Hypothetical protein
PA2414		-22.6	Carbon compound metabolism, *L*-sorbosone dehydrogenase
PA2415		-32.0	Membrane proteins
PA2416	*treA*	-13.0	Carbon compound catabolism, periplasmic trehalase precursor
PA2417		-18.4	Transcriptional regulator
PA2418		-147.0	Hypothetical protein, pirin-related protein

Table 4.1. (continued)

PA #	Gene name	Fold change	Descriptions
PA2419		-24.3	Probable hydrolase, amidases related to nicotinamidase
PA2420		-24.3	Membrane proteins, transport of small molecules, probable porin
PA2423		-111.4	Hypothetical protein
PA2433		-147.0	Hypothetical protein
PA2449		-52.0	Probable transcriptional regulator
PA2452		-274.4	Hypothetical protein
PA2453		-97.0	Hypothetical protein
PA2454		-39.4	2-polyprenyl-3-methyl-5-hydroxy-6-metoxy-1,4-benzoquinol methylase
PA2455		-32.0	Predicted acetyltransferase
PA2456		-168.9	Hypothetical protein
PA2501		-1910.9	Membrane protein

example, *pvdA* was the most repressed pyoverdine gene (832-fold); PvdA (*L*-ornithine N^5-oxygenase) converts ornithine into N^5-formyl-N^5-hydroxyornithine (Lamont and Martin, 2003; Visca *et al.*, 1994). These data show PA2663 is also critical for pyoverdine synthesis in *P. aeruginosa.*

Three anthranilate dioxygenase genes (*antABC*) were also repressed 91- to 416-fold upon inactivating PA2663. Anthranilate is a precursor for signal 2-heptyl-3-hydroxy-4-quinolone (PQS) (Urata *et al.*, 2004) that controls the *rhl* quorum sensing pathway, virulence, and biofilm formation (Diggle *et al.*, 2006). Three arsenite resistance (*arsB*, *arsC*, and *arsR*) genes were also repressed 6- to 64-fold; arsenite induces the oxidative stress response in *P. aeruginosa* (Parvatiyar *et al.*, 2005). In addition, *bkdA1*, *bkdA2*, *bkdB*, and *lpdV* which belong to a dehydrogenase operon (Winsor *et al.*, 2005) were repressed in the biofilm formed by the PA2663 mutant by 74- to 832-fold. Previously, this gene cluster was induced in developing biofilms (Waite *et al.*, 2006). Two more dehydrogenase genes, PA2265 (gluconate dehydrogenase) (Winsor *et al.*, 2005) and PA2290 (glucose dehydrogenase) (Winsor *et al.*, 2005), were also repressed by 79- and 158-fold, respectively.

Of the 49 induced genes (Table 4.2), seven small molecule transport genes including a porin precursor gene *oprP* (Winsor *et al.*, 2005) were induced by 4.3-fold. OprP controls the transport of phosphate ions in *P. aeruginosa* (Moraes *et al.*, 2007). The *braZ* gene, which encodes a Na^+ -coupled transporter of branched-chain amino acids, was induced 4-fold. Two ATP–binding cassette transporter genes, PA0326 and PA2678 (Winsor *et al.*, 2005), and four motility and attachment genes (PA0499, PA0993, PA2130, and PA4549) were also induced.

4.3.2 Verification of repression of *pslAB* by RT-PCR

To corroborate the DNA microarrays which indicated severe and consistent repression

Table 4.2. Partial list of induced genes for *P. aeruginosa* PAO1 PA2663 versus *P. aeruginosa* PAO1 in biofilms formed on glass wool in LB at 30°C after 7 h. Complete analysis deposited at the NCBI Gene Expression Omnibus (GSE9255).

PA #	Gene name	Fold change	Descriptions
Amino acid biosynthesis and metabolism			
PA0288	*gpuA*	4.0	3-guanidinopropionase, arginase/agmatinase/formimionoglutamate hydrolase, arginase family
PA1393	*cysC*	4.6	Central intermediary metabolism, nucleotide biosynthesis and metabolism, adenosine 5'-phosphosulfate kinase
Biosynthesis of cofactors, prosthetic groups and carriers			
PA4891	*ureE*	4.3	Urease accessory protein UreE
Carbon compound catabolism			
PA1384	*galE*	5.7	Central intermediary metabolism, Nucleotide biosynthesis and metabolism, UDP-glucose 4-epimerase
PA1983	*exaB*	5.7	Energy metabolism, cytochrome c550
PA2086		4.0	Probable epoxide hydrolase
PA2093		4.0	Probable sigma-70 factor, ECF
PA4150		4.0	Probable dehydrogenase E1 component
PA4151	*acoB*	6.1	Acetoin catabolism protein AcoB, pyruvate/2-oxoglutarate dehydrogenase complex
PA4152		3.7	Probable hydrolase
PA4153		4.9	2,3-butanediol dehydrogenase
PA5417	*soxD*	4.0	Sarcosine oxidase delta subunit
Cell wall, LPS, capsule			
PA1385		5.3	Probable glycosyltransferase
Motility and attachment			
PA0993	*cupC2*	4.0	Chaperones and heat shock proteins, P pilus assembly protein, chaperone PapD
PA2130	*cupA3*	4.0	Usher CupA3, P pilus assembly protein, porin PapC
PA4549	*fimT*	4.0	Type 4 fimbrial biogenesis protein FimT

Table 4.2. (continued)

PA #	Gene name	Fold change	Descriptions
Hypothetical, unclassified, unknown function			
PA0497		4.3	Hypothetical protein
PA0498		4.0	Hypothetical protein
PA0499		4.3	Motility and attachment, chaperones and heat shock proteins, P pilus assembly protein, chaperone PapD, 47% similar to filamentous hemagglutinin in *Bordetella pertussis*
PA0697		4.3	Hypothetical protein
PA0823		4.9	Hypothetical protein
PA0825		4.3	Hypothetical protein
PA0874		4.9	Hypothetical protein
PA0939		2.5	Rof, transcriptional antiterminator, 57% similar to YaeO (pleiotropic multicopy suppressor of Ts mutations; ribosome-associated; growth-rate regulated) in *E. coli*
PA0977		4.6	Hypothetical protein
PA0978		4.3	Related to phage, transposon, or plasmid, transposase and inactivated derivatives
PA0979		4.0	Related to phage, transposon, or plasmid, transposase and inactivated derivatives
PA1501		4.0	Hydroxypyruvate isomerase
PA1936		4.6	Hypothetical protein
PA1953		4.9	Predicted double-glycine peptidase
PA2209		4.0	Hypothetical protein
PA2935		7.0	Hypothetical protein
PA3412		4.3	Hypothetical, unclassified, unknown
PA3713	*spdH*	4.0	Spermidine dehydrogenase
PA4683		6.1	Hypothetical protein
PA4881		4.6	Hypothetical protein
PA5385		4.9	Hypothetical protein

Table 4.2. (continued)

PA #	Gene name	Fold change	Descriptions
Protein secretion, export apparatus			
PA1701		4.0	Conserved hypothetical protein in type III secretion
PA1856		4.3	Probable cytochrome oxidase subunit, 74% similar to the *ctyN* gene product of *Azospirillum brasilense*
Transcriptional regulator and transport of small molecules			
PA0885		4.0	Probable C_4-dicarboxylate transporter
Transport of small molecules			
PA0326		4.0	Probable ATP-binding component of ABC transporter
PA1541		4.6	Membrane protein, Membrane transporters of cations and cationic drugs
PA1632	*kpdF*	4.9	KpdF protein
PA1971	*braZ*	4.0	Branched chain amino acid transporter BraZ
PA2214		4.3	Probable major facilitator superfamily (MFS) transporter, sugar phosphate permease
PA2215		4.6	Hypothetical protein, *L*-alanine-*D*,*L*-glutamate epimerase and related enzymes of enolase superfamily
PA2678		4.3	Membrane proteins, probable permease of ABC-2 transporter
PA3279	*oprP*	4.3	Phosphate-specific outer membrane porin OprP precursor
PA3407	*hasAp*	2.6	Heme acquisition protein

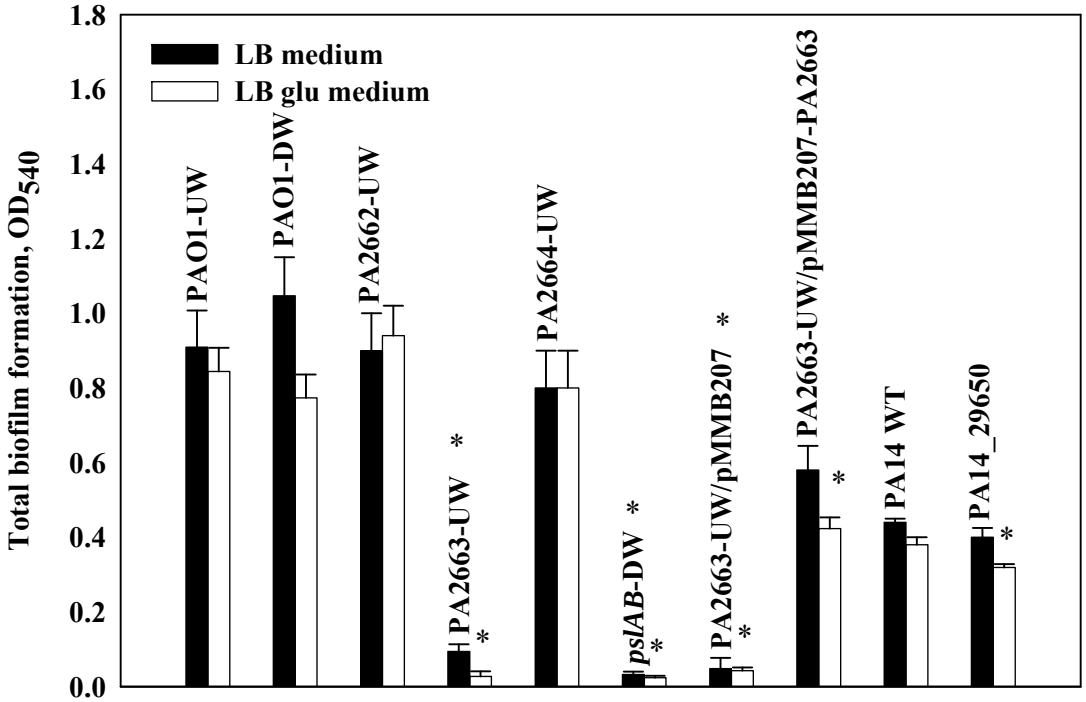


Fig. 4.1. Effect of the PA2663, PA2662, PA2664, and *pslAB* mutations on biofilm formation at 30°C after 7 h in 96-well plates with LB medium (black graphs) and LB glu medium (white graphs) supplemented with 0.2 wt% glucose. At least two independent experiments were conducted (total of 12 wells), and error bars indicate one standard deviation. Asterisks indicate significant differences as estimated by Student's t-test ($p < 0.05$). PA2663-UW/pMMB207-PA2663 and PA14_29650 were different than the wild-type strain in LB glu medium. PAO1-UW indicates the wild-type strain *P. aeruginosa* PAO1 from the University of Washington and PAO1-DW indicates wild-type *P. aeruginosa* PAO1 from Prof. Daniel J. Wozniak.

of the *psl* operon upon inactivation of PA2663 (e.g, *pslA* and *pslB* repressed 79- and 512-fold, respectively), RT-PCR was performed. We observed no amplification for these two genes in the RT-PCR reaction with PA2663 RNA while amplification was clear for the wild-type strain (template specificity was ensured by using melt-curve analysis). Therefore, these two genes are not expressed upon inactivating PA2663, which agrees with the DNA microarray analysis.

4.3.3 Biofilm formation of the PA2663 and *pslAB* mutants and complementation of PA2663

To corroborate the microarray studies and the link between reduced biofilm formation in the inactivated PA2663 mutant (20-fold biofilm reduction in 24 h in LB medium) (Attila *et al.*, 2008a), and repression of the *psl* operon, we investigated biofilm formation of the *pslAB* mutant (Jackson *et al.*, 2004). The inactivation of PA2663 and *pslAB* decreased biofilm formation by 30-fold after 7 h in LB and LB glu media (Fig. 4.1). Hence, the *pslAB* and PA2663 mutations reduce biofilm formation to nearly the same extent in rich medium. In minimal glucose medium, only the *pslAB* mutant failed to form biofilm (35 ± 8-fold less than the wild type strain at 7 h). Biofilm reduction was not the result of a growth defect since the specific growth rate of PA2663 and the wild-type strain in LB medium are nearly identical (Attila *et al.*, 2008a), and the specific growth rate of the *pslAB* mutant was only 25% less than its wild type strain.

To confirm that the decrease in biofilm formation is due to the mutation in PA2663, the mutant strain was complemented by measuring biofilm formation in LB and LB glu with pMMB207-PA2663. As expected, PA2663 expression increased biofilm formation of the PA2663 mutant in both media (6.4-fold in LB and 15.7-fold in LB glu) (Fig. 4.1).

To further investigate the relation of PA2663 and biofilm formation, we examined biofilm formation of the PA2663 mutant homolog in *P. aeruginosa* PA14 strain (*P. aeruginosa* PA14_29650), which does not possess the *psl* operon (Kuchma *et al.*, 2007). As expected, the

inactivation of the PA2663 homolog in *P. aeruginosa* PA14 does not inhibit biofilm formation in LB or LB glu (Fig. 4.1). This result confirms that PA2663 controls biofilm formation through the *psl* operon. We also examined the biofilm formation of the PA2662 and PA2664 mutants, which belong to the same operon as the PA2663 gene (Winsor *et al.*, 2005). Mutation on these two genes did not cause any defect in biofilm formation (Fig. 4.1).

4.3.4 Swimming and swarming motility

Previously, we found that the PA2663 mutation increased 1.5-fold swimming motility after 18 h (Attila *et al.*, 2008a); hence, we investigated the impact of this mutation on swarming. Compared to the wild-type strain, inactivating PA2663 increased swarming motility 2.1 ± 0.3-fold after 48 h. The *pslAB* mutant also induced swimming motility 1.3 ± 0.1-fold but repressed swarming motility 2.4 ± 0.4-fold. As expected, the PA2663 mutant ortholog in *P. aeruginosa* PA14 (which lacks *psl*) did not show any difference in swimming or swarming motility.

4.3.5 PA2663 stimulates pyoverdine production

Since the DNA microarrays indicated thirteen pyoverdine genes were repressed, we measured pyoverdine synthesis. Upon inactivation of PA2663 in PAO1, pyoverdine production normalized by cell density (OD_{405}/OD_{600}) decreased up to 15-fold (Fig. 4.2). Therefore, as indicated by the whole transcriptome analysis, inactivation of PA2663 eradicates pyoverdine synthesis. As a positive control, pyoverdine synthesis with the unrelated rhizosphere mutant *P. aeruginosa* PA2463-UBC was also checked, and no difference between the wild-type and PA2463 was observed (data not shown). In addition, the negative control, a *pvdF* did not produce pyoverdine.

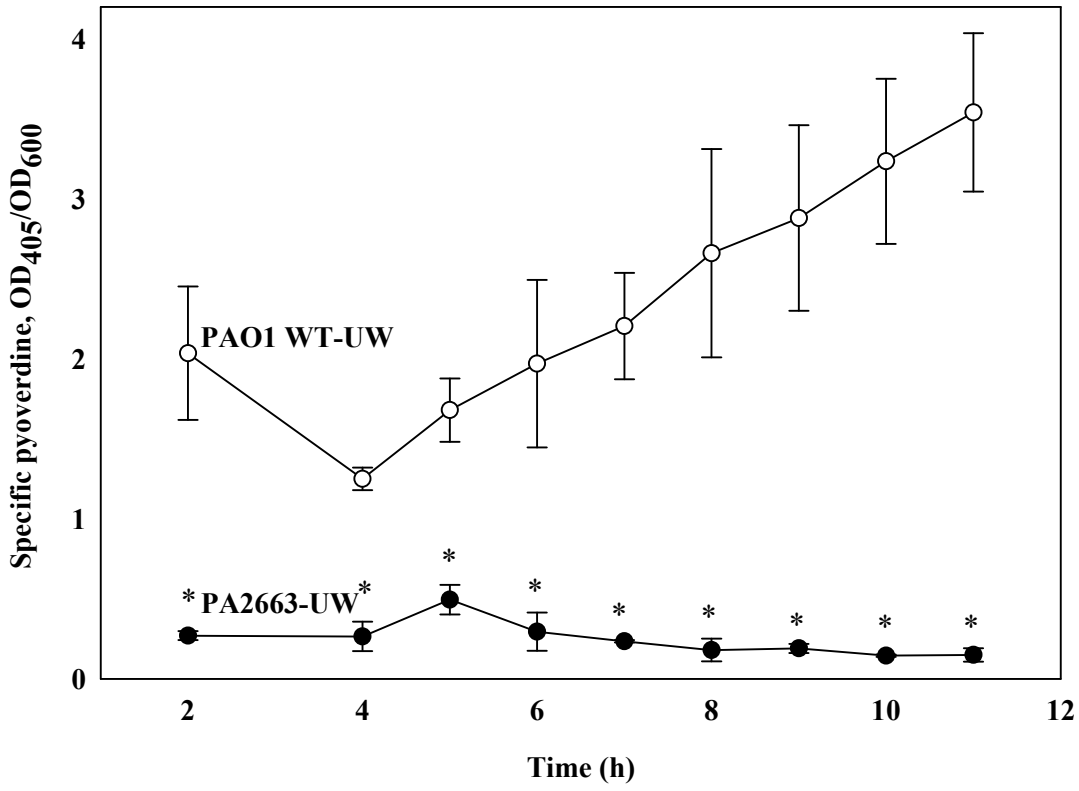


Fig. 4.2. Effect of the PA2663 mutation on cell density-normalized pyoverdine production. Pyoverdine production was normalized by measuring OD at 405 nm as explained in details and normalized by dividing to the growth OD at 600 nm. The data are collected at 2, 4, 6, 7, 8, 9, 10, and 11th hours. Error bars indicate one standard deviation. Asterisks indicate significant differences as estimated by Student's t-test ($p < 0.05$).

4.3.6 PA2663 stimulates PQS and elastase production

The DNA microarrays show that *pqsABCDEH* genes were repressed (Table 4.1). Therefore, we examined the PQS production in PA2663 mutant. Inactivation of PA2663 abolished PQS production compared to the wild-type PAO1 strain. The PA0996-UW (*pqsA*) and PA2587-UW (*pqsH*) mutants were used as negative controls for PQS production. Furthermore, the DNA microarrays showed that *lasB* was repressed fourfold upon inactivating PA2663 (Table 4.3); therefore, we examined the elastase activity of the PA2663 mutant and found that the PA2663 mutant synthesized 7.0 ± 0.7-fold less elastase activity compared to the wild-type PAO1 strain. A *P. aeruginosa* PA14 *lasB* mutant was used as the negative control for elastase activity. However, the PA2663 mutation did not alter rhamnolipid production. A *P. aeruginosa* PA14 *rhlR* mutant was used as a negative control for rhamnolipid production. Therefore, PA2663 is a positive effector for PQS and elastase.

4.3.7 PA2663 affects cell phenotypes

Since the regulation of 129 genes was impacted by inactivating PA2663 as shown by the DNA microarrays (partial list shown in Tables 4.1 and 4.2), we explored the impact of inactivating PA2663 on 756 phenotypes using the phenotype arrays (Table 4.3). The phenotype arrays showed that inactivating PA2663 severely suppressed growth on many carbon sources (e.g., *D*-gluconic acid, *D*-mannitol, *D*-trehalose, glycine, *L*-leucine, *L*-isoleucine, and tyramine), severely suppressed growth with many nitrogen sources (e.g., glycine, *L*-isoleucine, *L*-leucine, *L*-valine, *N*-phthaloyl-*L*-glutamic acid, parabanic acid, tyramine, uracil, and uridine), and severely suppressed growth with phosphorus and sulfur sources (e.g., *D*-3-phospho-glyceric acid, glutathione, *L*-methionine sulfone, *N*-acetyl-*D*, *L*-methionine, and *S*-methyl-*L*-cysteine). The lack of growth of the PA2663 mutant on *D*-mannitol was confirmed using M9 mannitol

Table 4.3. Metabolic phenotype based on Biolog arrays upon inactivating PA2663 versus *P. aeruginosa* PAO1 WT at 24 h at 30°C.

Compound	OD_{590} PA2663 /OD_{590} wild type	Physiological role	Pathway
D-gluconic acid	0.01	Carbon source	Pentose phosphate pathway
D-mannitol	0.01	Carbon source	Fructose and mannose metabolism, ABC transporter
D-3-phospho-glyceric acid	0	Phosphorus and sulfur source	
D-psicose	7.24	Carbon source	
D-trehalose	0.09	Carbon source	Starch and sucrose metabolism
Glycine	0	Carbon source	Glycine, serine, and threonine metabolism
	0.12	Nitrogen source	
Glutathione	0.26	Phosphorus and sulfur source	Glutathione metabolism
L-cysteine	9.15	Nitrogen source	Cysteine metabolism, methionine metabolism, taurine and hypotaurine metabolism, thiamine metabolism, pantothenate and CoA biosynthesis, and sulfur metabolism
L-isoleucine	0	Carbon source	Valine, leucine, isoleucine biosynthesis and degradation
	0.33	Nitrogen source	
L-leucine	0.01	Carbon source	Valine, leucine, isoleucine biosynthesis and degradation
	0	Nitrogen source	
L-valine	0.16	Nitrogen source	Valine, leucine, isoleucine biosynthesis and degradation
L-methionine sulfone	0	Phosphorus and sulfur source	
L-pyroglutamic acid	16.0	Carbon source	
N-acetyl-*D*-glucosamine	7.8	Carbon source	
N-acetyl-*D,L*-methionine	0.13	Phosphorus and sulfur source	
N-phtaloyl-*L*-glutamic acid	0	Nitrogen source	
Parabanic acid	0	Nitrogen source	
S-methyl-*L*-cysteine	0.09	Phosphorus and sulfur source	
Tyramine	0.02	Carbon source	Tyrosine metabolism, alkaloid biosynthesis
	0	Nitrogen source	

Table 4.3. (continued)

Compound	OD_{590} PA2663 /OD_{590} wild type	Physiological role	Pathway
Uridine	0.35	Nitrogen source	Pyrimidine metabolism
Ala-Thr	0	Peptide nitrogen source	
Ala-Val	0	Peptide nitrogen source	
Gly-Gly	0	Peptide nitrogen source	
Gly-Gly-Gly	0	Peptide nitrogen source	
Gly-Gly-Ile	0	Peptide nitrogen source	
Gly-Gly-Leu	0	Peptide nitrogen source	
Gly-Phe-Phe	0	Peptide nitrogen source	
Gly-Val	0	Peptide nitrogen source	
Gly-Pro	0.44	Peptide nitrogen source	
Gly-Ser	0.18	Peptide nitrogen source	
Ile-Gly	0	Peptide nitrogen source	
Ile-Ser	0.15	Peptide nitrogen source	
Leu-Glu	0	Peptide nitrogen source	
Leu-Gly	0	Peptide nitrogen source	
Leu-Gly-Gly	0	Peptide nitrogen source	
Leu-Ile	0	Peptide nitrogen source	
Leu-Trp	0	Peptide nitrogen source	
Leu-Val	0.09	Peptide nitrogen source	
Phe-Gly-Gly	0	Peptide nitrogen source	
Pro-Val	0.06	Peptide nitrogen source	
Trp-Trp	0.07	Peptide nitrogen source	
Val-Leu	0	Peptide nitrogen source	
Val-Gly	0	Peptide nitrogen source	
Val-Tyr-Val	0.12	Peptide nitrogen source	

medium; since both the wild-type PAO1 strain and the PAO1 *pslAB* mutant grew well in this medium, the PA2663 mutation prevents growth on *D*-mannitol.

Several of these compounds identified by the phenotype arrays are related to biofilm polysaccharide formation; for example, bacterial cellulose production in *Acetobacter xylinum* is enhanced by the inhibition of gluconic acid (Keshk and Sameshima, 2006). The composition of the polysaccharide produced by the *psl* locus in *P. aeruginosa* has been investigated by two groups and found to consist mainly of mannose, rhamnose, and glucose as well as contains *N*-acetyl quinovosamine, *N*-acetylglucosamine, and 3-deoxy-*D*-manno-octulosonic acid (Friedman and Kolter, 2004a) or consists chiefly of galactose, mannose, glucose, xylose, rhamnose and *N*-acetylglucosamine (Ma *et al.*, 2006); hence, mannose is important for this polysaccharide. Also, the polysaccharides of other Gram-negative bacteria (e.g., *E. coli* and *Klebsiella pneumoniae*) are rich in mannose (Yokochi *et al.*, 1990). Adding mannitol to the medium reduced both capsular polysaccharide and extracellular polysaccharide production in *Rhizobium leguminosarum* (Breedveld *et al.*, 1991), and mannitol increases extracellular polysaccharide secretion by *Thiobacillus versutus* mutants (Claassen *et al.*, 1986); hence, the repression of the *psl* locus in the PA2663 mutant is probably related to the lack of growth on mannitol by this mutant, and the strain may normally convert mannitol into mannose for polysaccharide synthesis but this conversion is altered by the PA2663 mutation.

Other compounds identified by the phenotype arrays such as glutathione, glycine, isoleucine, leucine, valine (Cho *et al.*, 2006), and trehalose are also biofilm related. In biofilm cells, glutathione is involved in potassium efflux (Gillam *et al.*, 2005); hence, the membrane protein PA2663 may affect potassium transport. Also, trehalose increases the exopolysaccharide production (Nicolaus *et al.*, 2002).

The phenotype arrays also revealed that the PA2663 mutation induced growth on *D*-

psicose, *L*-cysteine, *L*-pyroglutamic acid, and *N*-acetylglucosamine (Table 4.3). Since the polysaccharide matrix of *Actinobacillus pleuropneumoniae* (Izano *et al.*, 2007) and *Bacillus anthracis* (Ezzell *et al.*, 1990) consists of *N*-acetylglucosamine, and *N*-acetylglucosamine was found in the *psl* exopolysaccahrides (Friedman and Kolter, 2004a; Ma *et al.*, 2007) it appears inactivation of PA2663 alters the metabolism of this compound. Also, poly-*N*-acetylglucosamine is necessary for *Staphylococcus aureus* virulence (Kropec *et al.*, 2005) so the changes in barley virulence of the PA2663 mutant may be related to altered metabolism of this compound.

The inactivation of PA2663 also repressed severely growth on many peptide mixtures as nitrogen sources (Table 4.3). These results imply that PA2663 mutation affects amino acid synthesis or transport.

4.4 Discussion

In this study, by investigating the changes that occur upon inactivating PA2663 (which encodes a putative transmembrane protein) in *P. aeruginosa* PAO1, we demonstrated that the PA2663 gene product increases production of the virulence factor pyoverdine by activating 13 genes of the pyoverdine synthesis locus and increases biofilm formation by activating 15 genes of the *psl* locus. Along with the DNA microarrays, the other lines of evidence linking PA2663 to the *psl* operon include (i) RT-PCR showing transcription of PA2663 is abolished upon inactivation of PA2663, (ii) the similarities in biofilm formation of the PA2663 mutant and the *pslAB* mutants, (iii) the lack of a change in biofilm formation in *P. aeruginosa* PA14 when the homolog of PAO1 PA2663 is inactivated since PA14 lacks a *psl* operon, and (iv) the lack growth of the PA2663 mutant on mannitol as found through the phenotype arrays (*psl*-encoded polysaccharide is mannose-rich and both mannose and mannitol are related to biofilm

formation). The phenotype arrays also revealed other building blocks for exopolysaccharides (e.g., *D*-gluconic acid) were no longer good carbon sources and growth on another exopolysaccharide building block, *N*-acetylglucosamine, was increased; hence, the PA2663 protein appears to be related directly to exopolysaccharide synthesis via its stimulation of the *psl* operon and via sugar utilization, and PA2663 influences pyoverdine production. Therefore we suggest *ppyR* for *psl* and pyoverdine operon regulator as the new name for this gene.

The inactivation of PA2663 repressed the transcription of 71 genes; hence, it appears that PA2663 (85 aa) is part of a signaling regulon as an activator. Sharing an operon with PA2663, the flanking genes PA2662 and PA2664 are both associated with the signal nitric oxide (NO). PA2664 is a flavohemoglobin protein that detoxifies NO to nitrate, and its gene is induced the most in response to NO (Firoved *et al.*, 2004), and PA2662 is predicted to respond to NO (Winsor *et al.*, 2005). NO is a reactive nitrogen intermediate that reduces bacterial attachment, enhances motility, and induces dispersal in *P. aeruginosa* (Barraud *et al.*, 2006). Therefore, it appears that PA2663 is also part of the NO regulon since it reduces biofilm formation, and its role needs to be elucidated. Most transporters have six or more transmembrane regions, and many bacterial membrane signals have two transmembrane regions (von Heijne, 2006). Since PA2663 has only two transmembrane regions, 14-IGHGLLAAGLALLVAGVIAAYFL-36 and 45-LVFSHALVILGPTLLKIGYVMRL-67 as predicted by SOSUI (Hirokawa *et al.*, 1998), PA2663 is unlikely to be a transporter and is probably a novel type of membrane sensor that regulates exopolysaccharide and pyoverdine production perhaps via NO.

It is also evident that PA2663 works through the PQS and *las* quorum sensing systems. PQS genes (*pqsABCDEH)* were repressed in our microarray analysis (Table 4.1), and the inactivation of PA2663 reduced PQS production (note that the transcription of *antABC* was also

controlled). In addition, *lasB* was repressed (Table 4.1) and elastase activity was controlled by the PA2663. Pyoverdine is also controlled by the Las system (Stintzi *et al.*, 1998), and its production was markedly reduced. Hence, along with the stimulation of biofilm formation, PA2663 controls quorum-sensing phenotypes including pyoverdine production. Since the knowledge of specific *P. aeruginosa* genes that are required for biofilm formation may lead to novel therapeutic agents (Junker and Clardy, 2007), it is interesting that another protein (encoded by PA2663) that enhances biofilm formation and pyoverdine production has been identified.

4.5 Experimental procedures

4.5.1 Bacterial strains, media, and growth conditions

Strains and plasmids are listed in Table 4.4. *P. aeruginosa* was grown at 30°C. LB medium (Sambrook *et al.*, 1989) was used for the crystal violet biofilm experiment, the glass wool biofilm DNA microarrays, PQS assays, elastase assays, rhamnolipid experiments, and the swarming motility experiments. Minimal succinate medium (Ren *et al.*, 2005b) was used to measure pyoverdine production, and minimal M9 medium (Rodriguez and Tait, 1983) with 0.4% *D*-mannitol was used to evaluate growth on mannitol. Tetracycline (50 μg/mL) was used to select *P. aeruginosa* PAO1 transposon mutants, gentamycin (15 and 100 μg/mL) was used to select the *pslAB* mutant and *P. aeruginosa* PA14 transposon mutants, and chloramphenicol (200 μg/mL) was used to maintain plasmid-bearing strains.

4.5.2 Confirmation of PA2663 inactivation

Four polymerase chain reactions (PCR) were used to verify the insertion of the IS*phoA*/hah transposon (4.83 kb) to deactivate PA2663. The IS*phoA*/hah transposon was

Table 4.4. *P. aeruginosa* PAO1 strains used in this study. Chl^R, Gen^R, and Tet^R are chloramphenicol, gentamycin, and tetracycline resistance, respectively. UBC indicates *P. aeruginosa* strains obtained from the University of British Columbia, UW indicates *P. aeruginosa* strains obtained from the University of Washington, and DW indicates the strains obtained from Dr. Daniel Wozniak.

Strains and plasmids	Genotype	Source
Strains		
P. aeruginosa PAO1 WT-UW	Wild type strain from University of Washington	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 WT-DW	Wild type strain from Daniel Wozniak	(Jackson *et al.*, 2004)
P. aeruginosa PAO1 WFPA60-DW	*ΔpslAB* mutant, Gen^R	(Jackson *et al.*, 2004)
P. aeruginosa PAO1 PA2463-UBC	PA2463 Ω mini-*Tn*5-*luxCDABE,* Tet^R	(Lewenza *et al.*, 2005)
P. aeruginosa PAO1 PA2662-UW	PA2662 Ω IS*phoA*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 PA2663-UW	PA2663 Ω IS*phoA*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PAO1 PA2664-UW	PA2664 Ω IS*lacZ*/hah, Tet^R	(Jacobs *et al.*, 2003)
P. aeruginosa PA14 WT	Wild type PA14 strain	(Liberati *et al.*, 2006)
P. aeruginosa PA14_16250	*lasB* mutant, PA14_16250 Ω Mar2xT7 Gen^R	(Liberati *et al.*, 2006)
P. aeruginosa PA14_19120	*rhlR* mutant, PA14_19120 Ω Mar2xT7 Gen^R	(Liberati *et al.*, 2006)
P. aeruginosa PA14_29650	PA2663 homolog in PA14 strain, PA14_29650 Ω Mar2xT7 Gen^R	(Liberati *et al.*, 2006)
P. aeruginosa PA14_33700	*pvdF* mutant, PA14_33700 Ω Mar2xT7 Gen^R	(Liberati *et al.*, 2006)
Plasmids		
pMMB207	Chl^R, $lacI^q$	(de Lorenzo *et al.*, 1993)
pMMB207-PA2663	Chl^R, *ptac*::PA2663 in pMMB207	This study

Table 4.5. Primers used for PCR amplification, RT-PCR, and sequence verification used in this study.

Primer Name	Nucleotide sequence (5' to 3')	Primer Name	Nucleotide sequence (5' to 3')
PA2663-F	AGGAAGCTCAGTTGCAGCAC	PA2663-R	AGCAGGTCGAGCACTTCTTC
PA2663-Forward	GCCCCGAATTCTTCGGAAAAAGGAGATCGAGA	PA2663-Reverse	GCCCCAAGCTTAGGAAGAACGGGCGGAAG
29650-F	CACGAGCAGGTCGAGCACTT	29650-F	ATTTCGTGACGATGCCAGGC
GB3a	TACAGTTTACGAACCGAACAGGC	R1	ATCGACCCAAGTACCGCCAC
pslA-Forward	GCAAGCTGGTGATCTTCTGGTTCA	*pslA*-Reverse	TGTCGTGGTTGCGTACCAGGTATT
pslB-Forward	TTCAAGATCAAGCGCATCGTGGTG	*pslB*-Reverse	AGGTGGATTCGTTGGTGTTGAGGA
Seq-PA2663	CCGCTTCTGCGTTCTGATTT	HaH minus 138	CGGGTGCAGTAATATCGCCCT

inserted 51 bp from the start codon of the PA2663 gene. With the PA2663-F and PA2663-R primers that amplify 914 bp of the PA2663 gene (Table 4.5), PCR was used to confirm the insertion of the transposon into the PA2663 mutant because these primers produced a 914 bp band for the PAO1 wild-type but did not produce a band for the PA2663 mutant due to short elongation time (1 min). The HaH minus 138 and PA2663-R primers were used to verify the transposon insertion in the PA2663 mutant, but not in the PAO1 wild-type by amplifying 484 bp of the PCR product corresponding to the 3' end of the IS*phoA*/hah and a part of the PA2663 gene. The direction of the IS*phoA*/hah transposon was opposite to that of the PA2663 gene transcription. Similarly, the 29650-F, 29650-R, GB3a, and R1 primers (Table 4.5) were used to confirm *MAR2xT7* transposon insertion and direction in the PA2663 gene homolog in *P. aeruginosa* PA14.

4.5.3 Construction of pMMB207-PA2663

PA2663 was amplified by Pfu DNA polymerase with the PA2663-Forward and PA2663-Reverse primers (Table 4.5) that create *Eco*RI and *Hind*III restriction enzyme sites flanking 20 bp upstream of the start codon and 60 bp downstream of the stop codon of PA2663, respectively. The 360 bp PCR product was digested with *Eco*RI and *Hind*III and then ligated into pMMB207, which encodes chloramphenicol resistance (de Lorenzo *et al.*, 1993). The PA2663 gene and 118 bp upstream of the resulting plasmid, pMMB207-PA2663, were sequenced with primer Seq-PA2663 (Table 4.5) to confirm the presence of PA2663 using the ABI BigDye Terminator Cycle Sequencing Kit.

4.5.4 Crystal violet biofilm assay

Biofilm formation in 96-well polystyrene plates was performed as indicated previously

(Pratt and Kolter, 1998) with 300 µL of crystal violet per well, and the absorbance measured at 540 nm. Briefly, overnight cultures were inoculated with an initial turbidity of 0.05 at 600 nm at 30°C in LB and in LB supplemented with 0.2 % (wt/vol) glucose medium for 7 and 24 h without shaking. The biofilm at the liquid-plastic interface and the total biofilm were measured by using crystal violet staining. The dye staining the biofilms was dissolved in 95% ethanol, and the total biofilm formation was measured at an optical density of 540 nm. Twelve replicates (6 wells from each of two independent cultures) were averaged to obtain each data point.

To complement the PA2663 mutation during biofilm formation, PA2663 expression was induced in *P. aeruginosa* PAO1 PA2663-UW via pMMB207-PA2663 by adding 0 to 100 µM isopropyl-β-D-thiogalactopyranoside (IPTG). The negative control was *P. aeruginosa* PAO1 PA2663-UW containing the empty pMMB207 plasmid.

4.5.5 Swimming and swarming motility assays

Swimming motility experiments were performed as explained previously (Sperandio *et al.*, 2002) by measuring halos on agar plates after 5 and 18 h. For swarming motility, LB supplemented with 0.5% (wt/vol) agar plates was used. Briefly, overnight *P. aeruginosa* cultures were re-grown to a turbidity of 1 at 600 nm, and halos were measured at 24 h and 48 h. Ten replicates (5 plates from each of two independent cultures) were used to evaluate motility in each strain.

4.5.6 Biofilm RNA isolation for DNA microarrays

P. aeruginosa PAO1 and the PA2663 transposon mutant were grown overnight in LB (wild-type strain) and LB with 50 µg/mL tetracycline (PA2663 mutant). One milliliter of the overnight cultures (optical density 7.5 at 600 nm) was inoculated into 250 mL of fresh LB

medium with 10 g of glass wool (Corning Glass Works, Corning, NY) (Ren *et al.*, 2004b). The cultures were incubated for 7 h at 30°C with shaking, then the glass wool was removed quickly and washed in 200 mL of 0°C 0.85% NaCl buffer for 30 sec. The cells were removed from the glass wool by sonicating at 22 W (FS3 sonicator, Fisher Scientific, Pittsburg, PA) for 2 min in 200 mL of 0.85% NaCl buffer at 0°C. The buffer was centrifuged at 10,000 *g* for 2 min at 4°C (J2-HS centrifuge, Beckman, Palo Alto, CA) to obtain the cell pellets, and total RNA was isolated and checked as described previously (Ren *et al.*, 2005a). To prevent mRNA decay, bacterial beadbeater vials for the bacterial cell pellets were kept at -80°C. All instruments to isolate RNA were wiped with RNaseZap (Ambion, Austin, TX). In addition, the Qiagen Kit used to isolate RNA (Cat #74104) contained buffer RLT, which contains guanidine isothiocyanate and β-mercaptoethanol to inactivate RNases.

4.5.7 DNA microarrays

The *P. aeruginosa* Genechip Genome Array (Affymetrix, P/N 900339) which contains 5,500 of the 5,570 ORFs of *P. aeruginosa* (Whiteley *et al.*, 2001), was used to analyze the *P. aeruginosa* transcriptome. cDNA synthesis, fragmentation, and hybridization were performed as described previously (Attila *et al.*, 2008a). The data were obtained by using GeneChip operating software (Affymetrix). A gene was considered differentially expressed when the *p* value for comparing two microarrays was <0.05 (to assure that the change in gene expression was statistically significant, i.e., there are no more than 5% false positives). In addition, for each comparison of two genes, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 13 probe pairs (p value < 0.05), these genes were discarded. The intensities of polyadenosine RNA control were used to monitor the labeling process. Total signal intensity was scaled to an average value of 500. The probe array images were inspected

for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and they were close to each other. Therefore, the data of the two microarrays were comparable. The gene functions were obtained from the *Pseudomonas* Genome Database (http://v2.pseudomonas.com) (Winsor *et al.*, 2005). The expression data have been deposited in the NCBI Gene Expression Omnibus (GSE9255) (Edgar *et al.*, 2002).

4.5.8 Real time polymerase chain reaction

Real-time, reverse transcription polymerase chain reaction (RT-PCR) was performed to corroborate the microarray data as described previously (Lee *et al.*, 2007a). The primers were designed using PrimerQuest^{SM} online software (http://www.idtdna.com) (Table 4.5). The RT-PCR was performed using an ABsolute^{TM} MAX QRT-PCR SYBR Green Mixes Kit (ABgene House, UK) in a MyiQ^{TM} Single Color Real-Time PCR Detection System (Bio-Rad Laboratories).

4.5.9 Pyoverdine production

P. aeruginosa strains were grown in minimal succinate medium (Ren *et al.*, 2005b), then the cells were diluted to a turbidity of 0.05 at 600 nm in fresh minimal succinate medium and were grown at 30°C. The experiment was performed as described previously (Ren *et al.*, 2005b). Briefly, one milliliter of culture was centrifuged at 16,000 *g* for 1 min, and the supernatant was used to determine the pyoverdine concentration by measuring the absorption at 405 nm (Stintzi *et al.*, 1998); these values were normalized by cell density.

4.5.10 PQS production

To assay PQS production, overnight cultures were diluted to a turbidity of 0.02 at 660

nm, and PQS was extracted as previously described (Gallagher *et al.*, 2002; Pesci *et al.*, 1999). Briefly, cells were grown for 24 h at 30°C and 300 µL of the culture was extracted with acidified ethyl acetate (0.1 mL of glacial acetic acid in 1 L of ethyl acetate). The organic layer was transferred to a microcentrifuge tube and evaporated with a stream of nitrogen. Dried extracts were resuspended in 40 µL of 1:1 mixture of acidified ethyl acetate - acetonitrile. Synthetic PQS was used as a standard and 10 µL of each extract was placed on a Silica Gel 60 F_{254} (VWR, 5729-6) plates. The solvent used for TLC was 19:1 mixture of dichloromethane – methanol. The PQS levels were determined and photographed using a Bio-Rad VersaDoc 3000 imaging system (Bio-RAD, Hercules, CA).

4.5.11 Elastase activity assay and rhamnolipid assay

Elastase activity assay was performed as described previously (Ohman *et al.*, 1980). Briefly, overnight cultures were regrown to an optical density of 1.5 at 600 nm. One mL of the bacterial cells was harvested and 100 µL of bacterial supernatant was incubated with 900 µL of Tris-HCl buffer and 20 mg of elastin-Congo Red (MP Biomedicals, 101637) for three hours. The reaction was terminated by adding 350 µL of sodium phosphate buffer. The insoluble elastin-Congo Red was removed by centrifuging and the supernatant was measured at optical density at 405 nm to determine the elastase activity. Rhamnolipid assay was performed as described previously (Wilhelm *et al.*, 2007).

4.5.12 Phenotype microarray

Phenotype PM1-8 microarray plates (12111, 12112, 12121, 12131, 12141, 12181, 12182, 12183) (Biolog, Hayward, CA) were used to investigate 756 different phenotypes. Briefly, overnight cells (30°C) were removed from BUG+B agar plates with a sterile swab and

placed into IF-O base buffer, and the cell turbidity at 600 nm was adjusted to 0.065. Bacterial inocula were prepared, and 100 µL of each cell suspension was inoculated on the plates.

CHAPTER V

INDOLE AND 7-HYDROXYINDOLE DIMINISH *PSEUDOMONAS AERUGINOSA* VIRULENCE*

5.1 Overview

Indole is an extracellular biofilm signal for *E. coli,* and many bacterial oxygenases readily convert indole to various oxidized compounds including 7-hydroxyindole (7HI). Here we investigate the impact of indole and 7HI on *Pseudomonas aeruginosa* PAO1 virulence and quorum sensing (QS)-regulated phenotypes; this strain does not synthesize these compounds but degrades them rapidly. Indole and 7HI both altered extensively gene expression in a manner opposite that of acylhomoserine lactones; the most-repressed genes encode the *mexGHI-opmD* multidrug efflux pump and genes involved in the synthesis of QS-regulated virulence factors including pyocyanin (*phz* operon), 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) signal (*pqs* operon), pyochelin (*pch* operon), and pyoverdine (*pvd* operon). Corroborating these microarray results, indole and 7HI decreased production of pyocyanin, rhamnolipid, PQS, and pyoverdine and enhanced antibiotic resistance. In addition, indole affected the utilization of carbon, nitrogen, and phosphorus, and 7HI abolished swarming motility. Furthermore, 7HI reduced

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* Reprinted with permission from "Indole and 7-hydroxyindole diminish *Pseudomonas aeruginosa* virulence" by Jintae Lee, Can Attila, Suat L. G. Cirillo, Jeffrey D. Cirillo, and Thomas K. Wood, 2008, *Microbial Biotechnology*, in press, Copyright Wiley-Blackwell. C. Attila was responsible for all the quorum sensing experiments and the phenotype microarrays. J. Lee was responsible for all the other experiments except the *P. aeruginosa* guinea pig infection and the cytotoxicity assays. Suat L. G. Cirillo performed the *P. aeruginosa* guinea pig infection and the cytotoxicity assays.

pulmonary colonization of *P. aeruginosa* in guinea pigs and increased clearance in lungs. Hence, indole-related compounds have potential as a novel anti-virulence approach for the recalcitrant pathogen *P. aeruginosa*.

5.2 Introduction

Cell signals can be promiscuous. For example, *Escherichia coli* senses the quorum-sensing (QS) signal autoinducer-2 (AI-2) that is produced by *V. harveyi* to assess changes in its cell population (Xavier and Bassler, 2005a), and *Pseudomonas aeruginosa* responds to AI-2 and modulates its gene expression pattern including pathogenicity, although it does not itself produce AI-2 (Duan *et al.*, 2003). In addition, *Salmonella enterica* and *E. coli* detect acylhomoserine lactones (AHLs) via SdiA, although they do not synthesize AHLs (Michael *et al.*, 2001), and *E. coli* decreases biofilm formation in the presence of AHLs through SdiA (Lee *et al.*, 2007b). *S. enterica* enhances drug resistance in response to indole, although it does not produce indole (Nikaido *et al.*, 2008), and we found that biofilm formation of *P. aeruginosa* and *P. fluorescens* increases in the presence of indole, even though these pseudomonads do not produce indole (Lee *et al.*, 2007b). Furthermore, the large quantities of indole secreted by *E. coli* (up to 0.6 mM in Luria-Bertani medium) (Domka *et al.*, 2006) may be hydroxylated by other bacteria such as *Burkholderia cepacia* G4 (Rui *et al.*, 2005) to form hydroxyindoles that both increase and decrease biofilm formation in *E. coli* O15:H7 (Lee *et al.*, 2007a).

A variety of bacteria produce indole from *L*-tryptophan such as *E. coli* (Crawford and Yanofsky, 1958), *Vibrio vulnificus* (Dalsgaard *et al.*, 1999), *Haemophilus influenzae* (Stull *et al.*, 1995), *Pasteurella multocida* (Clemons and Gadberry, 1982), *Klebsiella oxytoca* (Liu *et al.*, 1997), and *Proteus vulgaris* (DeMoss and Moser, 1969); a NCBI BLAST search shows more than 27 genera utilize tryptophanase (*tnaA*) to convert tryptophan into indole, pyruvate, and

ammonia (Stewart and Yanofsky, 1985). Indole is an extracellular *E. coli* signal (Wang *et al.*, 2001) that inhibits biofilms (Lee *et al.*, 2007b), and it works in a quorum sensing fashion (Lee *et al.*, 2007a) primarily at temperatures less than 37^{o}C in *E. coli* (Lee *et al.*, 2008). Indole in *E. coli* also influences motility, acid resistance, chemotaxis, and attachment to epithelial cells (Bansal *et al.*, 2007; Domka *et al.*, 2006; Lee *et al.*, 2007a; Lee *et al.*, 2007b). It also controls plasmid stability by delaying cell division in *E. coli* (Chant and Summers, 2007), induces the expression of multidrug exporter genes, and increases drug resistance (Hirakawa *et al.*, 2005). Hence, indole is wide-spread in the environment and may be encountered by other bacteria.

P. aeruginosa is virulent to a variety of hosts including man, plants, and invertebrates (Lewenza *et al.*, 2005). *P. aeruginosa* captures iron with two endogenous siderophores, pyoverdine and pyochelin (Michel *et al.*, 2005), and both contribute to its virulence (Cox, 1982; Meyer *et al.*, 1996). RhlR-regulated rhamnolipids are glycolipids with biosurfactant properties that are involved in bacterial virulence (Zulianello *et al.*, 2006). Pyocyanin from *P. aeruginosa* has antibiotic activity toward competing bacteria including indole-producing *E. coli* (Hassett *et al.*, 1992), and PQS is structurally similar to pyocyanin and possesses antimicrobial activity (Mashburn and Whiteley, 2005). Therefore, *P. aeruginosa* utilizes several virulence factors.

It is likely that *P. aeruginosa* encounters indole since (i) *P. aeruginosa* is ubiquitous (Stover *et al.*, 2000), (ii) is found in the gut where *E. coli* is dominant (Wang *et al.*, 2006) and produces gut-derived generalized sepsis (Matsumoto *et al.*, 1999), and (iii) *P. aeruginosa* may also encounter *E. coli* outside the human body as *E. coli* exists outside the human host as well. Hence, it is possible *Pseudomonas* encounters indole and 7-hydroxyindole (7HI).

In this study, we used both DNA microarrays and phenotype arrays to investigate the impact of the *E. coli* signal indole and hydroxylated indole (7HI) on global gene expression and QS-regulated phenotypes of *P. aeruginosa* PAO1. Indole and 7HI altered extensively gene

expression, decreased the production of various virulence factors, decreased swarming motility, and increased antibiotic resistance in *P. aeruginosa*. Furthermore, 7HI reduced colonization of *P. aeruginosa* in guinea pig lungs and increased clearance in the gastrointestinal region.

5.3 Results

5.3.1 Indole and 7HI are nontoxic

To test the toxicity with *P. aeruginosa*, the specific growth rate was measured with indole, 7HI, and plant hormone indole-3-acetic acid (IAA, structurally-similar plant hormone which served as a negative control). In the absence of indole compounds, the specific growth rate was 1.5 ± 0.1/h whereas the growth rate was 1.51 ± 0.06/h with 1.0 mM indole, 1.89 ± 0.01/h with 0.5 mM 7HI, and 1.64 ± 0.04/h with 1.0 mM IAA. Hence, indole, 7HI, and IAA do not decrease the specific growth rate of *P. aeruginosa* at these concentrations, and the effects of indole and 7HI on *P. aeruginosa* are not due to toxicity. However, higher concentrations (2 mM) decreased the specific growth by 14% for indole (1.31 ± 0.01/h) and by 47% for 7HI (0.8 ± 0.2/h).

5.3.2 Indole and 7HI stimulate biofilm formation

Since indole and 7HI increased *P. aeruginosa* biofilm formation at 30°C (Lee *et al.*, 2007b), we tested different concentrations of indole, 7HI, and IAA for their ability to affect *P. aeruginosa* biofilm formation in 96-well plates after 24 h at 37°C (Fig. 5.1). Indole increased *P. aeruginosa* biofilm formation up to 1.8 ± 0.6-fold (0 to 1 mM) compared to no indole addition and did not affect cell growth. 7HI also increased biofilm formation 2.5 ± 0.8-fold in a dose-

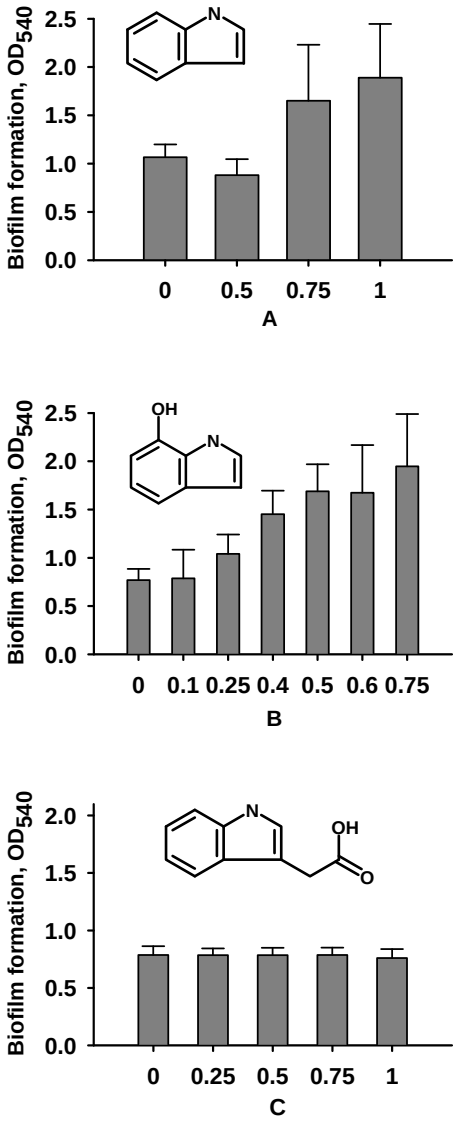


Fig. 5.1. Indole and 7HI increase *P. aeruginosa* biofilm formation. Biofilm formation in LB after 24 h in 96-well plates with indole (A), 7HI (B), and IAA (negative control) (C). Each experiment was performed two to four times with 6 wells each, and one standard deviation is shown. Structures of compounds are indicated as insets.

response manner (0 to 0.75 mM) compared to no 7HI addition without affecting cell growth. Notably, 7HI (0.5 mM) increased the liquid/solid (bottom) biofilm formation 6-fold (0.60 ± 0.21 vs. 0.10 ± 0.07). Therefore, indole and 7HI increase *P. aeruginosa* biofilm formation although *P. aeruginosa* does not synthesize these compounds. However, IAA did not influence the biofilm formation of *P. aeruginosa* (Fig. 5.1).

5.3.3 Indole and 7HI are global regulators

To investigate the genetic mechanism of biofilm enhancement by indole and 7HI on a global basis, we performed three sets of microarray experiments with 7 h *P. aeruginosa* biofilm cells: (i) a comparison of cells with and without 1.0 mM indole, (ii) a comparison of cells with and without 0.5 mM 7HI, and (iii) a comparison of cells with and without 1.0 mM IAA (negative control). The expression data for the biofilm samples are summarized in Table 5.1.

Overall, we found the addition of indole regulates significantly 532 genes more than 2-fold in biofilm cells at 7 h; 88 genes were induced and 444 genes were repressed. Similarly, the addition of 7HI regulates significantly 733 genes more than 2-fold in biofilm cells at 7 h; 25 genes were induced and 708 genes were repressed. In contrast, the addition of IAA altered expression of 10-fold fewer genes (only 71 genes more than 2-fold); 57 genes were induced and 14 genes were repressed. Therefore, indole and 7HI primarily repress genes in *P. aeruginosa*. To confirm the presence of indole and 7HI in the microarray samples, the extracellular indole and 7HI were measured using HPLC. The level of indole decreased from1.0 mM to 0.68 mM in 7 h (the negative control without indole addition showed zero indole). Also, the level of extracellular 7HI decreased from 0.5 mM to 0.154 mM after 7 h.

Table 5.1. Indole and 7HI regulate quorum sensing genes in *P. aeruginosa* PAO1. Partial list of differentially-expressed genes in LB medium after 7 h for (i) biofilm cells grown with 1.0 mM indole versus no indole, (ii) biofilm cells grown with 0.5 mM 7-hydroxyindole (7HI) vs. no 7HI, and (iii) biofilm cells grown with 1.0 mM indole-3-acetic acid (IAA, control compound) vs. no IAA. Most significant changes are shown in bold.

		Fold change			
PA#	**Gene**	**indole vs. no indole**	**7HI vs. no 7HI**	**IAA vs. no IAA**	**Description**
Multidrug efflux transporter					
PA4205	*mexG*	**-13.0**	**-7.0**	-1.1	Hypothetical protein, part of the *mexGHI-opmD* cluster
PA4206	*mexH*	**-11.3**	**-5.7**	-1.3	Efflux membrane fusion protein precursor
PA4207	*mexI*	**-6.1**	**-4.0**	-1.3	Efflux transporter
PA4208	*opmD*	**-7.5**	**-4.6**	-1.1	Probable outer membrane efflux protein
Phenazine (pyocyanin) synthesis					
PA1901	*phzC2*	**-2.5**	**-2.1**	-1.2	Phenazine biosynthesis protein PhzC
PA1902	*phzD2*	**-3.2**	**-3.0**	-1.1	Phenazine biosynthesis protein PhzD
PA1903	*phzE2*	**-3.5**	**-2.6**	-1.2	Phenazine biosynthesis protein PhzE
PA1904	*phzF2*	**-4.0**	**-4.3**	-1.1	Phenazine biosynthesis protein
PA1905	*phzG2*	**-3.2**	**-3.2**	-1.2	Pyridoxamine 5'-phosphate oxidase
PA2274		**-4.9**	**-3.2**	-1.1	Possible monooxygenase, involved in phenazine biosynthesis
PA4210	*phzA1*	**-2.5**	**-3.7**	-1.1	Phenazine biosynthesis protein
PA4211	*phzB1*	**-2.3**	**-2.3**	-1.3	Phenazine biosynthesis protein
PA4217	*phzS*	**-2.8**	**-2.8**	-1.1	Flavin-containing monooxygenase
PQS synthesis					
PA0996	*pqsA*	-1.9	**-2.0**	-1.1	PqsA, probable coenzyme A ligase
PA0997	*pqsB*	**-2.3**	**-2.5**	-1.1	PqsB, homologous to beta-keto-acyl-acyl-carrier protein synthase
PA0998	*pqsC*	-1.7	-1.9	-1.1	PqsC, homologous to beta-keto-acyl-acyl-carrier protein synthase

Table 5.1. (continued)

PA#	Gene	Fold change			Description
		indole vs. no indole	7HI vs. no 7HI	IAA vs. no IAA	
PA0999	*pqsD*	-1.3	-1.9	-1.1	PqsD, 3-oxoacyl-[acyl-carrier-protein] synthase III
PA1000	*pqsE*	**-2.0**	**-2.1**	-1.1	PqsE, quinolone signal response protein
PA1003	*mvfR*	1.3	-1.1	-1.1	MvfR (PqsR), PQS transcriptional regulator
Pyochelin synthesis					
PA4225	*pchF*	**-2.0**	-1.1	1.3	Pyochelin synthetase
PA4226	*pchE*	**-2.8**	-1.3	1.2	Dihydroaeruginoic acid synthetase
PA4227	*pchR*	-1.5	-1.2	1.1	Transcriptional regulator PchR
PA4228	*pchD*	**-3.2**	-1.3	1.2	Pyochelin biosynthesis protein PchD
PA4229	*pchC*	**-2.3**	-1.1	1.2	Pyochelin biosynthetic protein PchC
PA4230	*pchB*	**-2.0**	-1.2	1.2	Salicylate biosynthesis protein PchB
PA4231	*pchA*	**-2.1**	-1.3	1.2	Salicylate biosynthesis isochorismate synthase
Pyoverdine synthesis					
PA2385	*pvdQ*	**-2.1**	-1.6	1.1	pyoverdine biosynthesis protein PvdQ
PA2393	*pvdM*	**-2.5**	-1.1	**2.1**	pyoverdine biosynthesis protein PvdM
PA2394	*pvdN*	**-2.5**	-1.2	1.3	pyoverdine biosynthesis protein PvdN
PA2395	*pvdO*	**-2.0**	-1.1	1.5	pyoverdine biosynthesis protein PvdO
PA2397	*pvdE*	**-2.0**	-1.4	1.1	pyoverdine biosynthesis protein PvdE
PA2424	*pvdL*	**-2.3**	-1.3	1.9	pyoverdine biosynthesis protein PvdV
PA2426	*pvdS*	**-6.1**	**-2.6**	**2.0**	Sigma factor PvdS, transcriptional regulator
Metabolism					
PA0283	*sbp*	**-8.6**	-1.7	1.3	Sulfate-binding protein precursor
PA1838	*cysI*	**-6.1**	**-2.1**	1.2	Sulfite reductase

Table 5.1. (continued)

PA#	Gene	Fold change			Description
		indole vs. no indole	7HI vs. no 7HI	IAA vs. no IAA	
PA3441	*ssuF*	**-7.5**	**-5.7**	1.2	SsuF, part of the *ssu* locus,
PA3442	*ssuB*	**-4.6**	**-2.6**	1.3	SsuB, part of the *ssu* locus
PA3443	*ssuC*	**-3.2**	**-2.6**	1.3	SsuC, part of the *ssu* locus
PA3444	*ssuD*	**-6.1**	**-2.1**	1.1	SsuD, part of the *ssu* locus
PA3445	*ssuA*	**-6.5**	**-2.8**	**2.8**	SsuA, part of the *ssu* locus
PA3446	*ssuE*	**-6.5**	-1.6	1.5	SsuE, part of the *ssu* locus
PA4442	*cysN*	**-4.6**	**-2.0**	-1.1	ATP sulfurylase GTP-binding subunit
PA4443	*cysD*	**-4.9**	-1.7	1.2	ATP sulfurylase small subunit
PA5025	*metY*	**-2.6**	-1.3	-1.1	Homocysteine synthase
PA5427	*adhA*	**2.1**	**2.6**	-1.1	Alcohol dehydrogenase
Fimbriae and pili					
PA2131		-1.9	**-2.6**	1.0	Probable pili assembly chaperone
PA2570	*pa1L*	1.6	**-2.8**	1.1	PA-I galactophilic lectin
PA4084	*cupB3*	**-2.8**	**-4.0**	1.5	Probable fimbrial biogenesis usher protein
PA4085	*cupB2*	**-3.0**	**-2.1**	1.1	Probable pili assembly chaperone
PA4297	*tadG*	**2.3**	**3.5**	1.7	TadG
PA4298		1.6	**-3.2**	1.2	Hypothetical protein
PA4299	*tadD*	1.7	**-2.6**	1.1	Flp pilus assembly protein TadD
PA4300	*tadC*	1.7	**-2.3**	1.3	Flp pilus assembly protein TadC
PA4301	*tadB*	1.6	**-3.0**	1.5	Flp pilus assembly protein TadB
PA4302	*tadA*	1.9	**-2.5**	1.2	Flp pilus assembly protein, ATPase CpaF
PA4303	*tadZ*	1.7	**-2.3**	1.2	Flp pilus assembly protein, ATPase CpaE

Table 5.1. (continued)

PA#	Gene	Fold change: indole vs. no indole	Fold change: 7HI vs. no 7HI	Fold change: IAA vs. no IAA	Description
PA4304	*rcpA*	**2.0**	-1.9	1.0	Flp pilus assembly protein, secretin CpaC
PA4305	*rcpC*	1.6	**-2.5**	1.1	Flp pilus assembly protein CpaB
PA4306	*flp*	1.5	**-2.8**	1.1	Flp pilus assembly protein, pilin Flp
PA4651		**2.0**	**-3.5**	1.3	Probable pili assembly chaperone
Transport of small molecules					
PA1108		-1.9	**-5.7**	1.5	Probable MFS transporter
PA2092		**-2.6**	**-4.0**	1.4	Probable MFS transporter
PA2204		**-9.8**	-1.9	1.1	Binding protein component of ABC transporter
PA2328		**-6.1**	**-5.3**	1.1	Hypothetical protein in the cluster of ABC transporter
PA2329		**-5.7**	**-5.7**	1.0	Probable ATP-binding component of ABC transporter
PA2330		**-4.9**	**-4.3**	1.1	Hypothetical protein in the cluster of ABC transporter
PA2331		**-5.7**	**-4.0**	-1.1	Hypothetical protein in the cluster of ABC transporter
PA3531	*bfrB*	**3.2**	**2.3**	-1.2	Bacterioferritin, transport of small molecules
PA3926		-1.7	**-2.0**	1.3	Probable MFS transporter
PA4514	*piuA*	**-4.3**	**-2.5**	1.1	Probable outer membrane receptor for iron transport
Oxygenases					
PA2512	*antA*	**5.3**	**-2.0**	**9.2**	Anthranilate dioxygenase large subunit
PA2513	*antB*	**6.1**	**-2.6**	1.6	Anthranilate dioxygenase small subunit
PA2514	*antC*	**3.0**	**-2.8**	1.6	Anthranilate dioxygenase reductase
PA0106	*coxA*	**3.2**	1.4	1.0	Cytochrome c oxidase, subunit I
PA0107		**2.8**	1.3	-1.1	Predicted cytochrome oxidase assembly factor
PA0108	*coIII*	**3.2**	1.2	-1.1	Cytochrome c oxidase, subunit III
PA0109		**2.0**	-1.1	1.1	Hypothetical protein in the cluster of oxidase

Table 5.1. (continued)

PA#	Gene	Fold change			Description
		indole vs. no indole	7HI vs. no 7HI	IAA vs. no IAA	
PA0110		**2.6**	1.4	1.1	Hypothetical protein in the cluster of oxidase
PA0111		**3.0**	1.3	1.1	Hypothetical protein in the cluster of oxidase
PA0112		**3.0**	1.1	1.1	Hypothetical protein in the cluster of oxidase
PA0113		**3.0**	1.4	-1.3	Probable cytochrome c oxidase assembly
Others					
PA0715		**3.7**	1.4	**-2.0**	Probable bacteriophage protein
PA0716		**5.7**	**7.0**	-1.3	Probable bacteriophage protein
PA0740	*sdsA1*	**-8.6**	**-3.0**	1.1	Probable beta-lactamase
PA1109		**-2.6**	**-5.7**	**2.5**	Transcriptional regulator
PA2258	*ptxR*	-1.6	**-3.2**	1.9	Transcriptional regulator PtxR
PA3153	*wzx*	**2.6**	1.0	-1.4	O-antigen translocase
PA3154	*wzy*	**3.5**	1.0	-1.3	B-band O-antigen polymerase
PA3155	*wbpE*	**2.8**	1.5	-1.2	Probable aminotransferase WbpE
PA3156	*wbpD*	**2.3**	1.4	-1.1	Probable acetyltransferase
PA3234	*yjcG*	**4.3**	**2.0**	-1.1	Probable sodium:solute symporter
PA3337	*rfaD*	**2.0**	**2.5**	1.1	ADP-L-glycero-D-mannoheptose 6-epimerase
PA3450	*lsfA*	**-6.1**	-1.4	1.1	Probable antioxidant protein
PA3721	*nalC*	**3.0**	-1.1	1.6	Transcriptional regulator NalC, induced by MvfR
PA4563	*rpsT*	**2.8**	**2.8**	-1.1	30S ribosomal protein S20
Hypothetical proteins					
PA0284		**-13.9**	-1.6	1.2	Hypothetical protein, induced by MvfR
PA0492		**-4.3**	**-3.5**	1.9	Hypothetical protein
PA0696		**-4.3**	**-2.5**	1.3	Hypothetical protein
PA0939		-1.1	**-5.3**	1.6	Hypothetical protein

Table 5.1. (continued)

PA#	Gene	Fold change			Description
		indole vs. no indole	7HI vs. no 7HI	IAA vs. no IAA	
PA1190		**5.7**	1.6	1.2	Hypothetical protein
PA1837		**-4.6**	**-2.1**	1.1	Hypothetical protein
PA1914		1.1	**-6.1**	1.1	Hypothetical protein
PA1953		**-3.2**	**-6.1**	1.5	Hypothetical protein
PA2036		**-2.0**	**-7.0**	1.1	Hypothetical protein
PA2078		**-3.0**	**-5.7**	1.6	Hypothetical protein
PA2419		-1.6	**6.1**	1.5	Hypothetical protein
PA2805		**2.3**	**2.1**	1.1	Hypothetical protein
PA3080		**3.2**	1.1	-1.1	Hypothetical protein
PA3235		**4.0**	**2.5**	1.1	Hypothetical protein
PA3237		-1.9	**-6.1**	1.9	Hypothetical protein
PA3572		**2.0**	**2.5**	1.1	Hypothetical protein
PA3719		**4.6**	1.3	1.3	Hypothetical protein
PA3720		**2.6**	1.2	1.6	Hypothetical protein
PA3931		**-5.3**	-1.7	1.0	Hypothetical protein
PA4087		**-2.5**	**-3.5**	1.0	Hypothetical protein
PA4359		**-4.0**	-1.1	1.3	Hypothetical protein
PA4377		**3.7**	1.7	1.4	Hypothetical protein
PA4614		**3.5**	1.4	1.1	Hypothetical protein
PA4638		**6.5**	-1.6	-1.2	Hypothetical protein
PA4683		**-2.8**	-1.3	1.1	Hypothetical protein

5.3.4 Indole represses the *mexGHI-opmD* multidrug efflux genes and QS-regulated genes

The most noticeable change of gene expression with indole was that the *mexGHI-opmD* multidrug efflux genes were highly repressed (6- to 13-fold), and many genes involved in the synthesis of QS-regulated virulence factors were repressed (Table 5.1). Specifically, the phenazine synthesis operon (*phz*) which is involved in the pyocyanin biosynthesis, the PQS synthesis operon (*pqs*), the pyochelin synthesis operon (*pch*), and the pyoverdine synthesis operon (*pvd*) were repressed (2- to 6-fold) by indole. Also, several genes involved in the transport of small molecules were repressed by indole. Among genes involved in metabolism, genes involved in sulfur metabolism, such as *sbp*, the whole *ssuFBCDAE* locus that plays a key role in organosulfur uptake (Kahnert *et al.*, 2000), and *cysI* and *cysND*, were significantly repressed (3- to 8-fold) by indole (Table 5.1). This suggests that indole down-regulates sulfur uptake of *P. aeruginosa*. In contrast, several oxidase genes and *antABC* encoding anthranilate dioxygenase genes were induced (3- to 6-fold) by indole, while 7HI repressed the *antABC* operon. However, the transcriptional level of the QS signal regulators, such as RhlRI, LasRI, and MvfR (PqsR), were not altered by indole or 7HI.

5.3.5 7HI represses the *mexGHI-opmD* multidrug efflux genes and QS-regulated genes

Like indole, 7HI repressed (4- to 7-fold) the four *mexGHI-opmD* multidrug efflux genes and genes involved in the synthesis of QS-regulated virulence factors (e.g., *phz* operon, *pqs* operon, and *pvdS*) (Table 5.1). Unlike indole, 7HI did not change the expression of pyochelin synthesis genes.

Note, the control IAA did not change the transcription of nearly all the genes involved in virulence (Table 5.1); therefore, the impact on virulence is specific for indole and 7HI. The most induced gene (9.2-fold) in the presence of IAA was *antA* (encodes the anthranilate dioxygenase

large subunit). Also induced were six genes involved in metabolism (2- to 3-fold) and twelve genes involved in the transport of small molecules (2- to 8-fold).

5.3.6 Verification of DNA microarray results

Real-time reverse-transcription polymerase chain reaction (RT-PCR) was used to verify gene expression for several of the highly differentially-expressed loci in the two sets of the DNA microarray experiments (response to indole and 7HI). Using independent cultures, RT-PCR showed differential changes in expression comparable to the DNA microarrays. For the indole experiments, both techniques (RT-PCR vs. microarray) showed the genes were repressed in a similar manner: 14.1- vs. 13.0-fold for *mexG*, 26.4- vs. 11.3-fold for *mexH*, 1.9- vs. 6.0-fold for *mexI*, 5.6- vs. 6.1-fold for *pvdS,* 10.0- vs. 7.5-fold for *ssuF*, and 12.7- vs. 13.6-fold for PA0284. Similarly, for the 7HI experiments, both techniques (RT-PCR vs. microarray) showed the genes were repressed in a similar manner: 54.7- vs. 7.0-fold for *mexG*, 4.0- vs. 5.7-fold for *mexH*, 2.8- vs. 4.0-fold for *mexI*, 3.8- vs. 2.6-fold for *pvdS,* 3.0- vs. 5.7-fold for *ssuF*, and 2.6- vs. 1.6-fold for PA0284.

5.3.7 Indole and 7HI decrease QS-regulated virulence phenotypes

Since the microarray data showed that indole and 7HI repressed genes involved in the synthesis of QS-regulated virulence factors (Table 5.1), we assayed phenotypes related to the three QS systems of *P. aeruginosa* including the RhlR-regulated phenotypes (pyocyanin (Gallagher *et al.*, 2002), rhamnolipid production (Ochsner and Reiser, 1995), and swarming motility (Déziel *et al.*, 2003)), LasR- and RhlR-regulated PQS production (Diggle *et al.*, 2006), and LasR-regulated elastase production (Gambello and Iglewski, 1991). Production of the siderophore pyoverdine, a virulence factor in *P. aeruginosa* (Lamont and Martin, 2003), was

also measured since indole and 7HI repressed the pyoverdine synthesis genes and a transcriptional regulator *pvdS* (Table 5.1).

As expected, indole and 7HI decreased PQS production by 5.0 ± 2-fold and 10 ± 5-fold, decreased pyocyanin production by 7.1 ± 0.6 -fold and 11 ± 3-fold, decreased rhamnolipid production by 2.2 ± 0.7-fold and 2.0 ± 0.5-fold, and decreased pyoverdine production by 1.7 ± 0.3-fold and 3.2 ± 0.3-fold, respectively (Fig. 5.2A and Fig. 5.3 for PQS production); however, indole and 7HI did not alter elastase production. Therefore, the decreased production of these QS-regulated virulence factors upon addition of indole and 7HI corroborated the microarray data (Table 5.1). Notably, the impact of 7HI on the production of PQS and pyoverdine was more significant than that with indole. In contrast, IAA did not significantly change the production of the four virulence factors; hence the changes in the regulated phenotypes are specific for indole and 7HI.

5.3.8 7HI abolishes swarming

The impact of indole and 7HI on the swimming, swarming, and twitching motility of *P. aeruginosa* was tested since swarming motility plays an important role in *P. aeruginosa* biofilm formation (Overhage *et al.*, 2007) and swarming is related to quorum sensing (Köhler *et al.*, 2000), and (iii) our microarray data showed that quorum sensing genes (e.g., *phz* operon, *pqs* operon, and *pvdS*) were repressed by 7HI (Table 5.1). Indole and 7HI both decreased slightly swimming motility; the swimming halo diameters at 24 h were 3.24 ± 0.08 cm (no addition), 2.75 ± 0.01 cm (indole), and 2.28 ± 0.01 cm (7HI). Twitching motility was not significantly changed with indole and 7HI**.** However, indole significantly decreased swarming motility, and 7HI completely abolished swarming motility (Fig. 5.4).

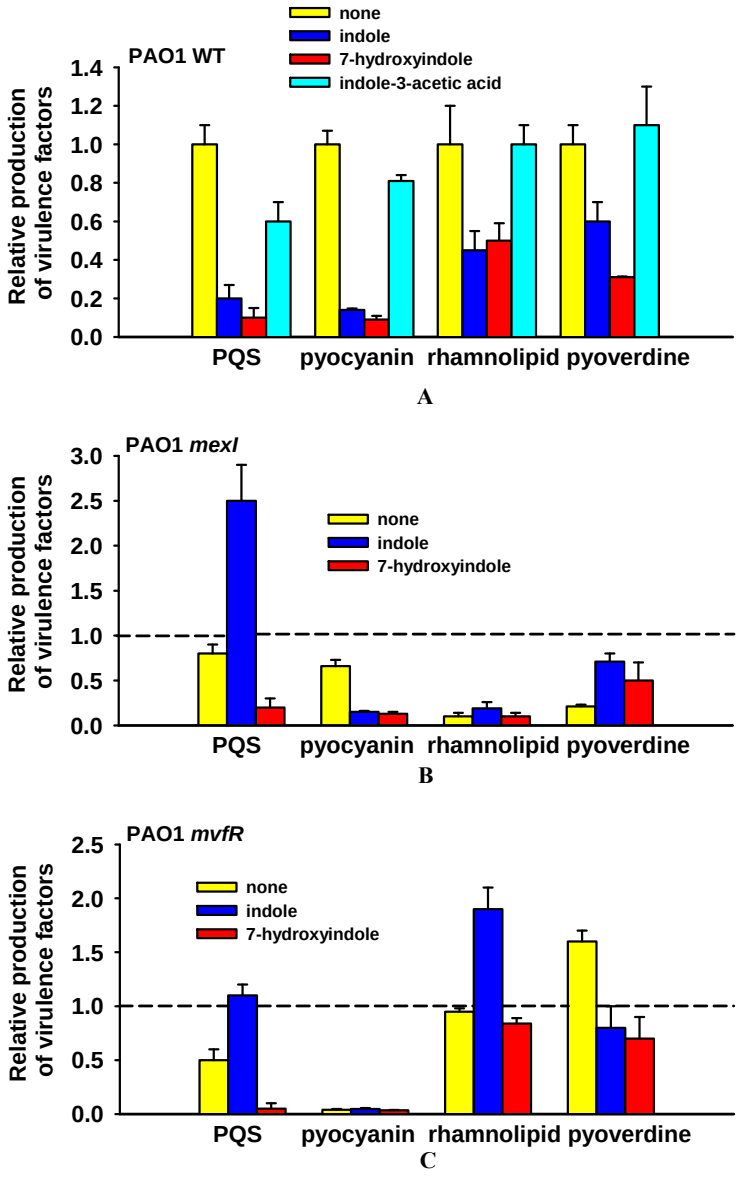


Fig. 5.2. Reduction of virulence factors by indole and 7HI. Production of virulence factors with 1.0 mM indole, 0.5 mM 7HI, and 1.0 mM IAA (negative control) with *P. aeruginosa* PAO1 (A), with *P. aeruginosa* PAO1 *mexI* (B), and *P. aeruginosa* PAO1 *mvfR* (C). For clarity, wild-type values are not shown in panels B and C so bars indicate the relative amount of each compound made compared to the wild-type strain without indole or 7HI added. Each experiment was performed with at least two independent cultures. Data show the average of the replicates, and one standard deviation is shown.

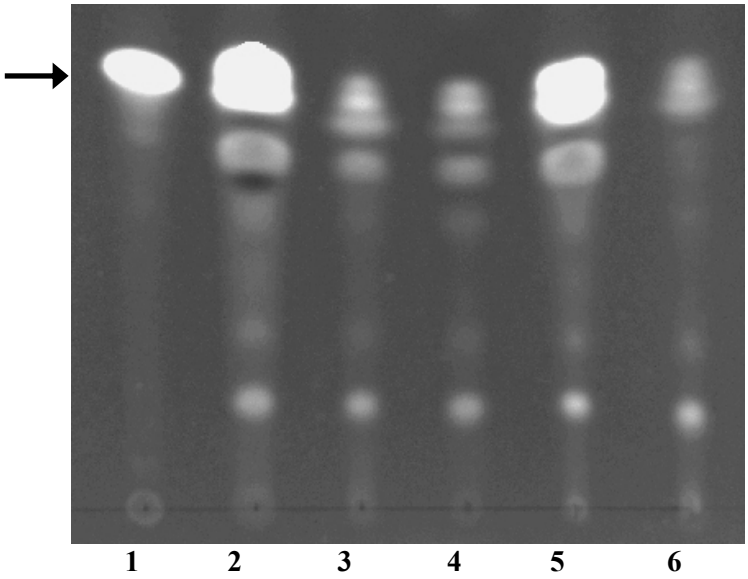


Fig. 5.3. Indole and 7HI decrease PQS production. Culture components were purified and analyzed by thin layer chromatography. Included on the TLC plate are 500 ng of chemically synthesized PQS (arrow at lane 1), extract of *P. aeruginosa* wild-type culture (2), extract of *P. aeruginosa* wild-type culture with indole (1.0 mM) (3), extract of *P. aeruginosa* wild-type with 7HI (0.5 mM) (4), extract of *P. aeruginosa mexI* culture (5), and extract of *P. aeruginosa* PA14 *pqsA* as a negative control (6).

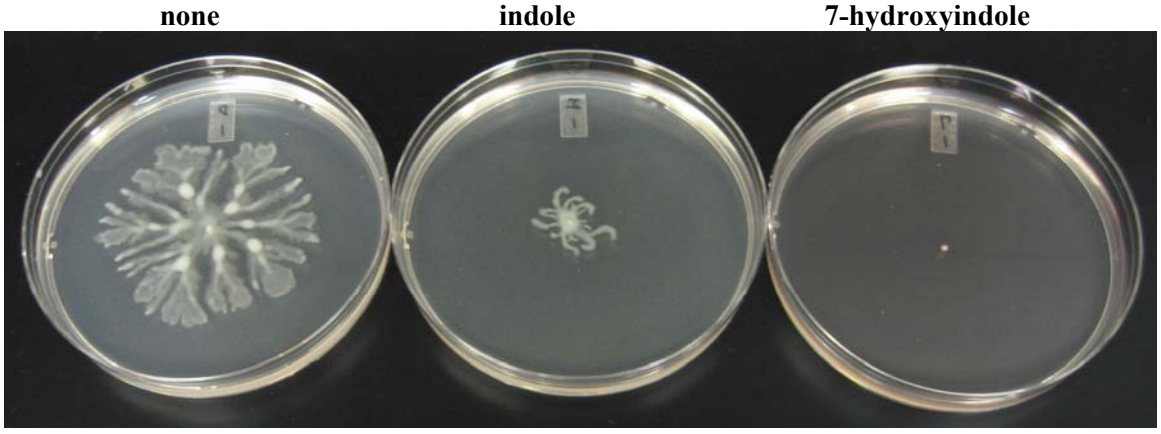


Fig. 5.4. Inhibition of swarming motility by indole and 7HI. Swarming motility of *P. aeruginosa* on BM2 medium with 0.5% agar with 1.0 mM indole and 0.5 mM 7HI after 28 h. Each experiment was performed with at least two independent cultures and one representative data set is shown.

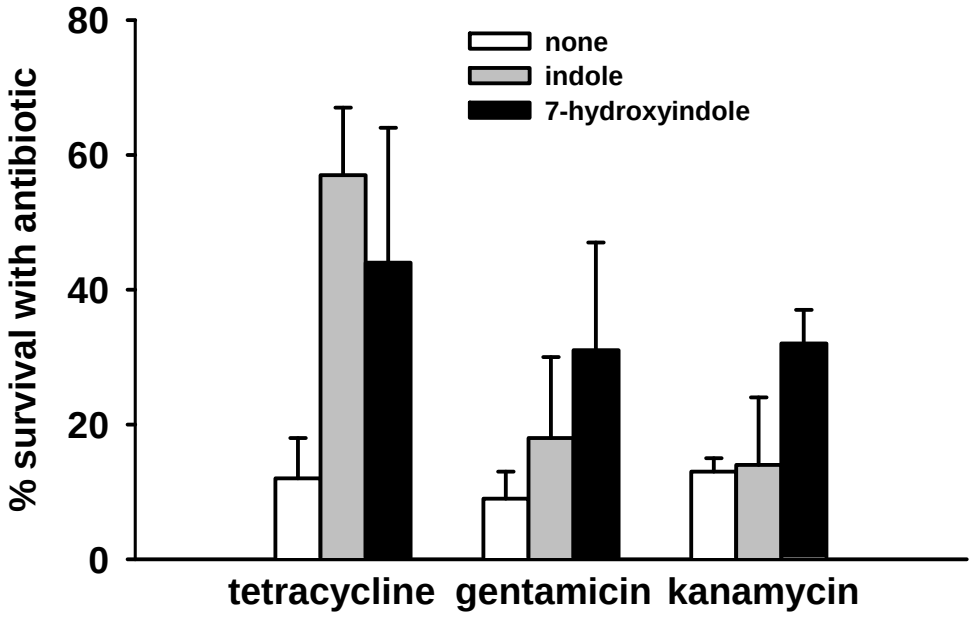


Fig. 5.5. Indole and 7HI increase *P. aeruginosa* antibiotic resistance. Antibiotic resistance with 1.0 mM indole and 0.5 mM 7HI. Final concentrations were 0.4 mg/mL tetracycline, 0.06 mg/mL gentamicin, and 10 mg/mL kanamycin. Each experiment was performed with at least two independent cultures. Data show the average of the replicates, and one standard deviation is shown.

5.3.9 Indole and 7HI increase antibiotic resistance

Since MexGHI-OmpD efflux pump mutants of *P. aeruginosa* show enhanced antibiotic resistance (Aendekerk *et al.*, 2005) and since our microarray data showed that these efflux genes were the most repressed genes by indole and 7HI (Table 5.1), we assayed the antibiotic resistance of *P. aeruginosa* upon addition of indole and 7HI with five antibiotics. Four antibiotics (tetracycline, gentamicin, kanamycin, and vanadyl sulfate) were investigated by measuring the survival rate, and ampicillin was investigated by using a minimal inhibition concentration (MIC) method. Indole and 7HI increased tetracycline resistance by 5 ± 3-fold and 4 ± 3-fold, respectively, and increased gentamicin resistance by 2 ± 2-fold and 3 ± 2-fold, respectively, and 7HI increased kanamycin resistance by 2.5 ± 0.5-fold (Fig. 5.5). Hence, our results agreed well with previous results using *mexI* and *ompD* mutants with kanamycin and tetracycline (Aendekerk *et al.*, 2005) in that repression of MexGHI-OmpD by indole and 7HI enhanced antibiotic resistance consistently. However, indole and 7HI did not change the survival rates (~ 55%) in the presence of 5 mM vanadyl sulfate (Aendekerk et al., 2002). Similarly, the MIC of ampicillin was increased in the presence of indole and 7HI (300 μg/mL for no indole or 7HI, 500 μg/mL with indole, and 600 μg/mL with 7HI). Therefore, indole and 7HI enhance *P. aeruginosa* antibiotic resistance.

5.3.10 Indole affects many cell phenotypes

Since the regulation of 532 genes were altered by adding indole as shown by the DNA microarrays (Table 5.1), we explored the impact of adding indole to *P. aeruginosa* on 756 phenotypes using phenotype arrays (Table 5.2). The phenotype arrays showed that the addition of indole severely suppressed growth with many carbon sources (e.g., *D*-saccharic acid, *L*-alanine, glucuronamide, *M*-tartaric acid, *D*-aspartic acid, and α-methyl-*D*-galactoside), many

Table 5.2. Indole influences *P. aeruginosa* metabolism. Metabolic phenotype based on Biolog arrays upon adding 1.0 mM indole versus no indole addition at 24 h. Optical density at 590 nm (OD_{590}) indicates growth under these conditions.

Compound	OD_{590} indole/OD_{590} no indole	Physiological role	Pathway
α-Hydroxy glutaric acid-γ-lactone	9.2	Carbon Source	
Glycolic Acid	2.8	Carbon Source	
L-Sorbose	Infinity	Carbon Source	Fructose and mannose metabolism
Inulin	Infinity	Carbon Source	
β-Methyl-*D*-xyloside	Infinity	Carbon Source	
L-Tartaric Acid	Infinity	Carbon Source	Glyoxylate and dicarboxylate metabolism
β-Methyl-*D*-galactoside	Infinity	Carbon Source	ABC transporter
D-Saccharic Acid	0.1	Carbon Source	
L-Alanine	0.2	Carbon Source	Alanine and aspartate metabolism, aminoacyl-tRNA biosynthesis
Glucuronamide	0.0	Carbon Source	
M-Tartaric acid	0.0	Carbon Source	Glyoxylate and dicarboxylate metabolism
D-Tartaric acid	0.3	Carbon Source	
D-Aspartic acid	0.0	Carbon Source	Urea cycle and metabolism of amino groups
α-Methyl-*D*-galactoside	0.0	Carbon Source	
Hydroxyl-*L*-proline	0.4	Carbon Source	
N-Acetyl-*D*-mannosamine	2.6	Nitrogen Source	Amino sugars metabolism
L-pyroglutamic acid	2.4	Nitrogen Source	
L-leucine	4.6	Nitrogen Source	Valine, leucine, isoleucine biosynthesis and degradation
Formamide	3.7	Nitrogen Source	
D, *L*-Lactamide	33.8	Nitrogen Source	
D-serine	2.2	Nitrogen Source	Glycine, serine, and threonine metabolism
Thymine	2.2	Nitrogen Source	Pyrimidine metabolism
Ethylenediamine	Infinity	Nitrogen Source	
Xanthosine	0.0	Nitrogen Source	
Tyramine	0.3	Nitrogen Source	Tyrosine metabolism, alkaloid biosynthesis, pyrimidine metabolism
Cytidine	0.4	Nitrogen Source	
Cytosine	0.2	Nitrogen Source	Pyrimidine metabolism

Table 5.2. (continued)

Compound	OD_{590} indole/OD_{590} no indole	Physiological role	Pathway
Guanine	0.3	Nitrogen Source	Purine metabolism
D-Lysine	0.0	Nitrogen Source	Lysine biosynthesis
Met-Ala	0.0	Nitrogen Source	
Phospho-glycolic acid	2.5	Phosphorus Source	
Adenosine-5-monophosphate	2.5	Phosphorus Source	
D-mannose-1-phosphate	0.3	Phosphorus Source	
D-2-Phospho-glyceric Acid	0.3	Phosphorus Source	
Guanosine-2-monophosphate	0.4	Phosphorus Source	
Cytidine-2-monophosphate	0.3	Phosphorus Source	
Guanosine-5-monophosphate	0.4	Phosphorus Source	
Guanosine-2,3-cyclic monophosphate	0.4	Phosphorus Source	
Adenosine-3,5-cyclic monophosphate	0.3	Phosphorus Source	
Uridine-5-monophosphate	0.3	Phosphorus Source	
L-methionine	2.2	Sulfur Source	Methionine metabolism, aminoacyl-tRNA biosynthesis
Lanthionine	0.4	Sulfur Source	
Glutathione	0.2	Sulfur Source	Glutathione metabolism
L-cysteine	0.4	Sulfur Source	Taurine and hypotaurine metabolism
Gly-Val	0.3	Nitrogen Source	
Ala-Leu	0.4	Nitrogen Source	
Gly-Phe	0.2	Nitrogen Source	
His-Trp	0.1	Nitrogen Source	
Tyr-Trp	0.0	Nitrogen Source	
Tyr-Tyr	0.1	Nitrogen Source	
Val-Arg	0.4	Nitrogen Source	
Pro-Ala	0.3	Nitrogen Source	
Lys-Tyr	0.3	Nitrogen Source	
Pro-Gly	0.2	Nitrogen Source	
Val-Tyr	0.2	Nitrogen Source	
Phe-Ile	0.0	Nitrogen Source	

Table 5.2. (continued)

Compound	OD_{590} indole/OD_{590} no indole	Physiological role	Pathway
Val-Ser	0.2	Nitrogen Source	
Thr-Ser	0.1	Nitrogen Source	
Gly-*D*-Ala	0.0	Nitrogen Source	
Tyr-Ile	0.0	Nitrogen Source	
β-Ala-His	0.2	Nitrogen Source	
Tyr-Val	0.0	Nitrogen Source	
Pro-Val	0.1	Nitrogen Source	
D-Ala-Gly	0.1	Nitrogen Source	
Leu- *β*-Ala	0.2	Nitrogen Source	
Phe-Val	0.0	Nitrogen Source	
Val-Phe	0.0	Nitrogen Source	
Phe-Gly-Gly	0.2	Nitrogen Source	
Lys-Asp	0.1	Nitrogen Source	
Thr-Gly	0.2	Nitrogen Source	
Tyr-Gly-Gly	0.3	Nitrogen Source	
Gly-Val	0.3	Nitrogen Source	

nitrogen sources (e.g., xanthosine, tyramine, cytidine, cytosine, guanine, and *D*-lysine), many phosphorus sources (e.g., *D*-mannose-1-phosphate, *D*-2-phospho-glyceric acid, and cytidine-2-monophosphate), and three sulfur sources (lanthionine, glutathione, and *L*-cysteine). The reduced cell growth in the presence of indole with three sulfur sources corroborates our microarray data in that indole repressed genes involved in sulfur uptake (*sbp*, *ssuFBCDAE*, *cysI*, and *cysND*) (Table 5.1), which suggests that indole decreases sulfur uptake in *P. aeruginosa*. Overall, indole alters the carbon, nitrogen, sulfur, and phosphorus metabolism of *P. aeruginosa*.

5.3.11 *P. aeruginosa* degrades indole and 7HI

In the microarray samples of *P. aeruginosa*, the concentration of indole and 7HI decreased significantly after 7 h (1.0 mM indole decreased to 0.68 mM and 0.5 mM 7HI decreased to 0.154 mM); hence, the degradation of indole and 7HI was quantified by measuring the concentration of extracellular indole and 7HI using HPLC. It was found that *P. aeruginosa* degrades indole in LB medium at 1.04 ± 0.04 nmol/(min mg protein) and degrades 7HI in LB medium at 2.6 ± 0.1 nmol/(min mg protein) while there was no significant change of indole and 7HI concentrations with the negative control (dead cells) (Fig. 5.6A and 5.6B). Furthermore, *P. aeruginosa* PAO1 does not utilize either indole (1 or 2 mM) or 7HI (0.5 or 2 mM) as a sole source of carbon and energy (data not shown) although *P. aeruginosa* Gs isolated from mangrove sediments (Yin *et al.*, 2005) and *Pseudomonas* sp. ST-200 from soil (Doukyu and Aono, 1997) grow on indole.

In the whole transcriptome analysis, *antABC* was the most-induced locus upon indole addition (Table 5.1); hence, we investigated if anthranilate dioxygenase is necessary for indole degradation. We found that the *P. aeruginosa* PAO1 *antA* mutant degraded indole to the same extent as the wild-type strain; therefore, indole degradation is not related to anthranilate

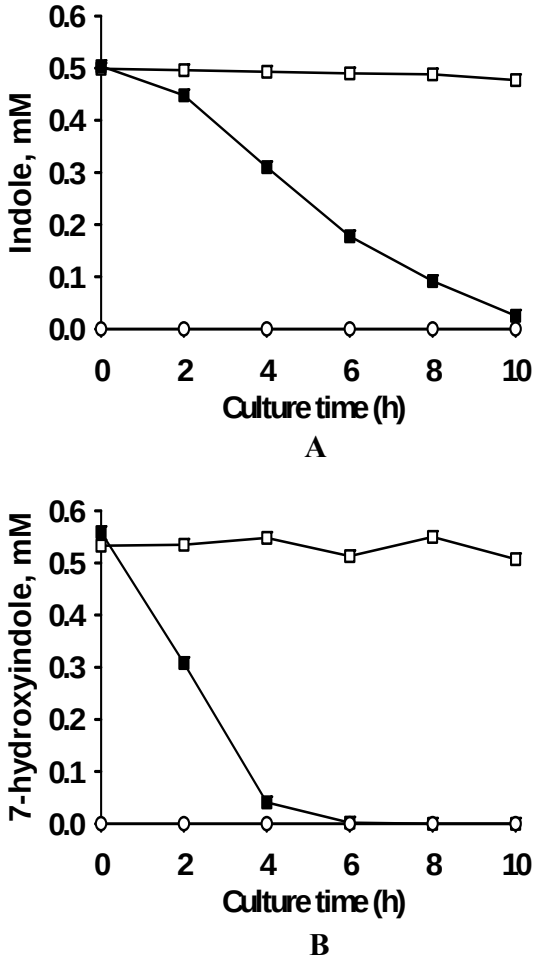


Fig. 5.6. Degradation of indole and 7HI by *P. aeruginosa*. *P. aeruginosa* degrades 0.5 mM indole (A) and 0.5 mM 7HI (B) in LB. The initial turbidity of cells was 1.0 at 600 nm. Closed square data (■) are from live cells, open square data (□) are from autoclaved cells (dead cell control), and open circle data (○) are from live cells that lack added indole or 7HI. Each experiment was performed using two independent cultures, and one representative data set is shown.

metabolism. Furthermore, since addition of the negative control IAA highly induced *antA* (Table 5.1), it appears both IAA and indole induce the *ant* locus due to some non-specific interaction with a regulatory protein rather as substrates.

5.3.12 Indole and 7HI are less effective in the *mexI* and *mvfR* mutants

Since the most highly-repressed locus in the presence of indole and 7HI was *mexGHI-opmD* (Table 5.1) and since mutation of *mexI* decreased the production of rhamnolipid and pyoverdine (Aendekerk *et al.*, 2002), the effect of indole and 7HI was further investigated with *P. aeruginosa* PAO1 *mexI*. Consistent with a previous report (Aendekerk *et al.*, 2002), the *mexI* mutation reduced rhamnolipid and pyoverdine by 10-fold and 5-fold, respectively (Fig. 5.2B). However, unlike with the wild-type strain, indole did not decrease the production of PQS, rhamnolipid, and pyoverdine in the *mexI* strain (Fig. 5.2B), and 7HI also did not decrease the production rhamnolipid and pyoverdine in the *mexI* strain. These results suggest *mexI* is involved in a complex manner with the decrease of virulence factors with indole and 7HI. In contrast, indole and 7HI significantly decreased pyocyanin production in the *mexI* strain probably due to the presence of two different pathways for pyocyanin synthesis, the *aro* pathway and the *phz* pathway (Lau *et al.*, 2004a).

Since the transcriptional regulator MvfR is required for full *P. aeruginosa* virulence and induces both *mexGHI-opmD* and phenazine transcription (*phz* operon that is involved in pyocyanin biosynthesis) (Déziel *et al.*, 2005), the effect of indole and 7HI was also investigated with the *P. aeruginosa mvfR* mutant. As expected, PQS and pyocyanin were decreased in the *mvfR* mutant compared to the wild-type strain (Fig. 5.2C). However, unlike with the wild-type

strain, the addition of indole did not decrease the production of PQS, rhamnolipid, and pyocyanin. These results suggest that indole requires MvfR to fully reduce virulence factors.

In addition, the *P. aeruginosa* PAO1 *antA* mutant was investigated since indole diminished PQS production (Fig. 5.2A) while inducing the *antABC* (Table 5.1); *antABC* encode proteins involved in the degradation of anthranilate, a precursor of PQS. As expected, the *antA* mutant produced about 1.8-fold more PQS (Fig. 5.7) (since there is a larger pool of anthranilate for PQS). However, the response of the *antA* mutant upon addition of indole was similar to that of the wild-type strain. Hence, *antA* is not directly involved in the indole mechanism to control virulence factors.

5.3.13 7HI reduces *P. aeruginosa* virulence in guinea pigs

Since 7HI diminished virulence factors in *P. aeruginosa* more than indole (Table 5.1 and Fig. 5.2) and since 7HI abolished swarming motility (Fig. 5.4), we evaluated the ability of *P. aeruginosa* pre-treated with 7HI or diluent dimethylformamide (DMF) as a control to colonize guinea pig lungs after infection. Pulmonary colonization was examined immediately after infection until 48 h post-infection (Fig. 5.8). Colonization of the lung by *P. aeruginosa* treated with 7HI was reduced by 25%, and 7HI-treated bacteria are cleared more easily from the lungs for the first four hours post infection, leading to a greater than 50% reduction in pulmonary bacteria by this time point. In the acute model, both the 7HI and DMF treated *P. aeruginosa* continue to be expelled from the lungs, resulting in nearly complete clearance of the bacterial pneumonia by 48 h. Since it was not possible to maintain 7HI levels with the bacteria *in vivo*, the differences between DMF- and 7HI-treated bacteria decrease over the course of this experiment, as expected.

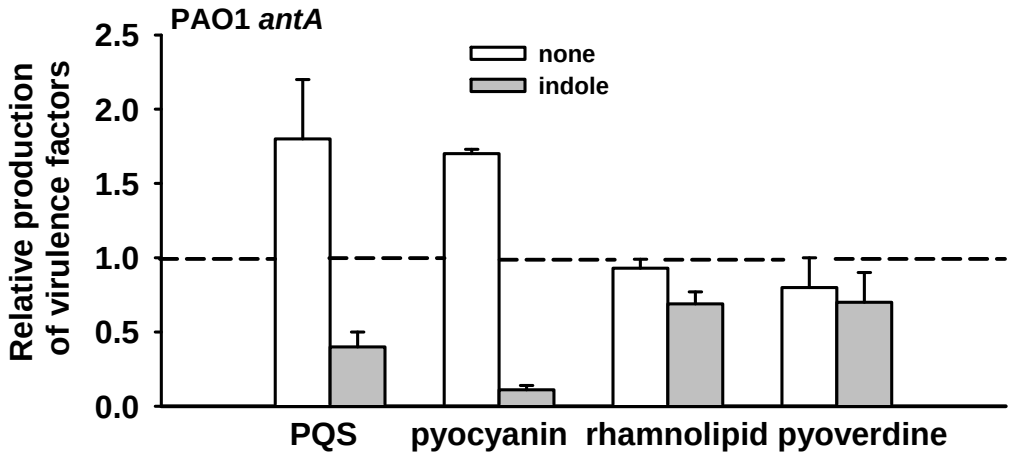
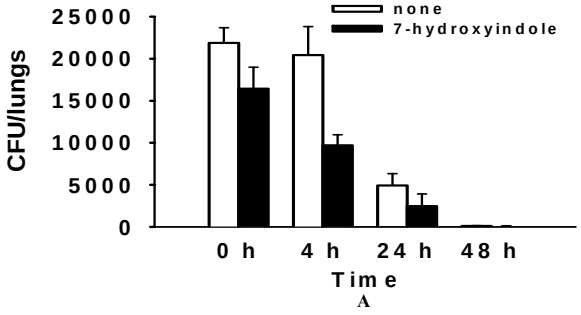
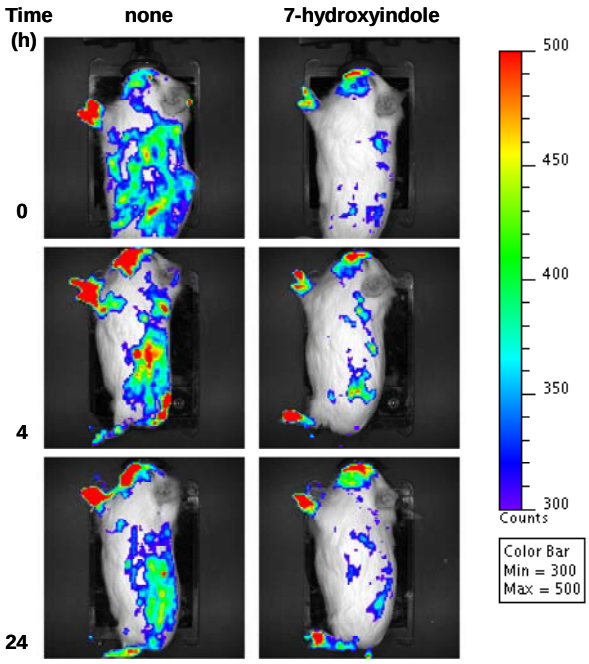


Fig. 5.7. The *ant* locus is not responsible for indole signaling. Production of virulence factors with 1.0 mM indole with *P. aeruginosa* PAO1 *antA*. For clarity, values of the wild-type strain are not shown so bars indicate the relative amount of each compound made compared to no indole addition to the wild-type strain. Each experiment was performed with at least two independent cultures. Data show the average of the replicates and one standard deviation is shown.



A



B

Fig. 5.8. Reduction of virulence of *P. aeruginosa* in guinea pigs. Colonization and clearance of *P. aeruginosa* pre-treated with 7HI or solvent (DMF) prior to infection of guinea pigs by aerosol with ~2 x 10^5 cfu. Average of five replicates, and one standard deviation is shown (A). Real-time analysis of *P. aeruginosa* pre-treated with 7HI or solvent (DMF) in the acute guinea pig infection model (representative guinea pigs are shown for each group and are imaged laterally) using the Xenogen IVIS CCD camera (B). Color bar represents the intensity of luminescent signal in photons/sec/cm^2 from low (blue) to high (red).

We used *in vivo* bioluminescence imaging to obtain a more comprehensive picture of the tissue colonization by DMF- and 7HI-treated *P. aeruginosa* and to confirm differences in pulmonary colonization in guinea pigs (Fig. 5.8B). Guinea pigs infected by aerosol were colonized in the nasopharynx, lungs, liver, and gastrointestinal area (Fig. 5.8B), similar to previous studies in mice (DiGiandomenico *et al.*, 2007). Since 7HI did not affect bioluminescence and since equal amounts of *P. aeruginosa* cells was used to infect the guinea pigs, the difference in colonization of *P. aeruginosa* was due to the treatment of 7HI. Similar to data obtained by counting cfu in the guinea pig lungs, pulmonary clearance occurs rapidly for 7HI-treated *P. aeruginosa*, with low bioluminescence signal observed in the pulmonary region by four hours in this group ($2.05 \pm 0.04 \times 10^5$ photons/sec/cm^2) as compared to the DMF-treated group ($3.59 \pm 0.47 \times 10^5$ photons/sec/cm^2). Lower colonization ($P < 0.03$) of all organs was observed for animals infected with 7HI-treated bacteria ($3.5 \pm 0.1 \times 10^6$ photons/sec/cm^2) as compared to DMF-treated bacteria ($4.19 \pm 0.08 \times 10^6$ photons/sec/cm^2). In addition, regional lymph nodes do not appear to be significantly involved, with primarily colonization of the gastrointestinal region. These observations could indicate that dissemination of *P. aeruginosa* to extrapulmonary and extra-intestinal sites is also reduced, in addition to initial colonization. Further studies are needed to definitively identify the sites and tissues infected, despite these intriguing observations. Therefore, both cfu and bioluminescence imaging confirm that 7HI pre-treatment of *P. aeruginosa* decreases colonization of guinea pig tissues.

Additionally, indole and 7HI-treated *P. aeruginosa* decreased cytotoxicity in human red blood cells by 40% and 57%, respectively. These results imply that indole and 7HI made *P. aeruginosa* less virulent to red blood cells.

5.4 Discussion

QS is related to virulence as has been shown by whole-transcriptome studies of *P. aeruginosa* with 3O-C_{12}-HSL and C_4-HSL (Schuster *et al.*, 2003; Wagner *et al.*, 2003) and PQS (Bredenbruch *et al.*, 2006). Here, the addition of indole and 7HI resulted in an opposite pattern of gene expression of the *mexGHI-opmD* multidrug efflux genes and many virulence genes compared to gene expression with the two exogenous AHLs. For example, the addition of the AHLs consistently induced the *mexGHI-opmD* genes, genes involved in phenazine synthesis and PQS synthesis, and the *flp-tad-rcp* gene cluster (Schuster *et al.*, 2003; Wagner *et al.*, 2003), while indole and 7HI repressed these genes (Table 5.1). Similarly, the addition of PQS most significantly induced virulence genes involved in pyochelin synthesis (*pch* operon) (Bredenbruch *et al.*, 2006) whereas indole repressed the *pch* operon. Genes involved in small molecule transport (PA2328, PA2329, PA2330, and PA2331) were also induced by AHLs (Schuster *et al.*, 2003), while indole repressed them. Furthermore, the addition of pyocyanin, a physiological terminal signal molecule for the up-regulation of QS-controlled genes (Dietrich *et al.*, 2006), induced *mexGHI-opmD* and a possible regulator/putative monooxygenase for pyocyanin synthesis, PA2274 (Dietrich *et al.*, 2006), while indole and 7HI repressed PA2274 5-fold (Table 5.1). In addition to the whole-transcriptome data, our virulence factor assays (Fig. 5.2) clearly show that indole and 7HI inhibit QS-controlled virulence factors. Taken together, indole and 7HI decrease the production of antimicrobial compounds and virulence factors in *P. aeruginosa*. A conceptual model of the interaction of indole and 7HI on cellular phenotypes of *P. aeruginosa* is shown in Fig. 5.9.

Genes encoding the MexGHI-OmpD efflux pump were the most-highly repressed genes in *P. aeruginosa* upon addition of indole and 7HI (Table 5.1), and antibiotic resistance was clearly enhanced in the presence of indole and 7HI (Fig. 5.5). With *P. aeruginosa*, inactivation

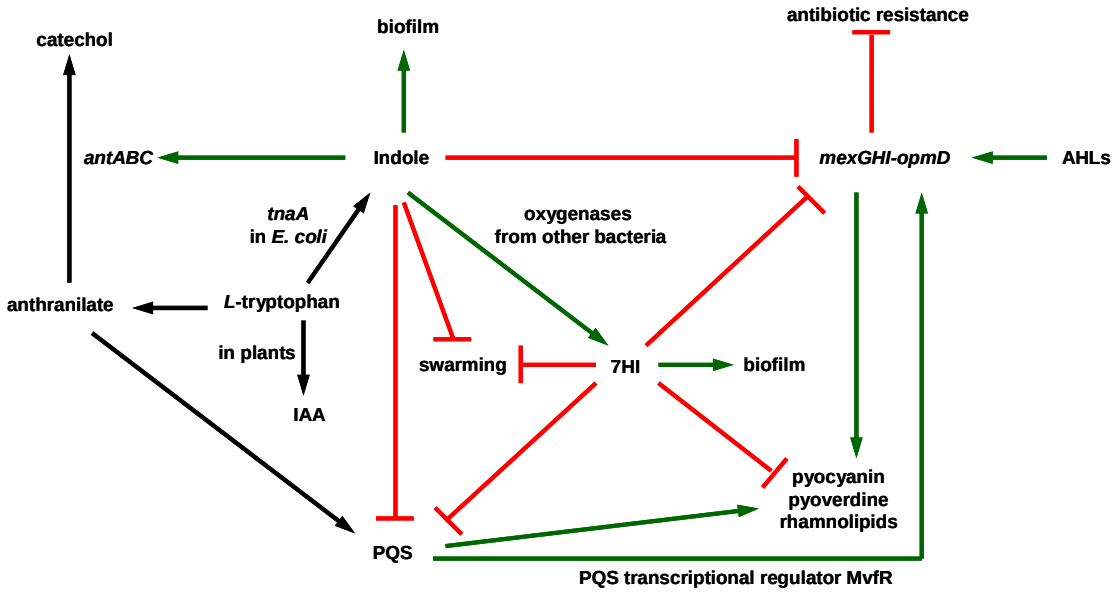


Fig. 5.9. Summary of indole-affected processes in *P. aeruginosa*. → indicates induction of gene expression or stimulation of a phenotype, ⊥ indicates repression of gene expression or repression of a phenotype, and black arrows indicate conversion.

of MexGHI-OmpD reduces production of rhamnolipids, pyocyanin, pyoverdine, elastase, and swarming motility (Aendekerk *et al.*, 2002), impairs cell growth (Aendekerk *et al.*, 2005), enhances antibiotic resistance (Aendekerk *et al.*, 2005), and attenuates virulence via PQS (Aendekerk *et al.*, 2005). As observed with *mexI* and *opmD* mutants (Aendekerk *et al.*, 2005), indole and 7HI decreased the production of virulence factors without affecting the transcriptional levels of *lasI* and *rhlI*; hence, indole and 7HI probably inhibit virulence factors due to a similar mechanism, through PQS regulation at the post-transcriptional level (Aendekerk *et al.*, 2005). Therefore, the reduction of virulence factors, swarming motility, and antibiotic sensitivity may be partially explained by the repression of this locus by indole and 7HI.

Also, the PQS transcriptional regulator MvfR induces the *mexGHI-opmD* genes, pyocyanin synthesis (*phz* operon), PA0284, PA1914, PA2329, and PA2331 (Déziel *et al.*, 2005), while both indole and 7HI repressed the *mexGHI-opmD* genes and repressed PA2328, PA2329, PA2330, and PA2331, and indole repressed PA0284 (14-fold) and 7HI repressed PA1914 (6-fold, Table 5.1). Interestingly, indole and 7HI were less effective for the production of virulence factors in the *mvfR* mutants (Fig. 5.2C), which suggested that MvfR was also required for the indole mechanism. Swarming motility is related to QS (Köhler *et al.*, 2000) and plays a role in the biofilm development of *P. aeruginosa* (Caiazza *et al.*, 2007; Overhage *et al.*, 2007). Overhage et al. (2007) showed a direct relationship between swarming motility and biofilm formation from the study of 5,000 *P. aeruginosa* insertion mutants. In contrast, Caiazza et al. (2007) showed an inverse regulation of biofilm formation and swarming motility. In the present study, both indole and 7HI decrease slightly swimming motility, 7HI abolishes swarming motility (Fig. 5.4), and both lead to more biofilm formation (Fig. 5.1). Hence, we also observed an inverse relationship between biofilm formation and swarming motility with 7HI. In addition, the reduction of rhamnolipids by indole and 7HI (Fig. 5.2A) may be a partial reason for the

reduced swarming motility (Fig. 5.4) since rhamnolipids modulate swarming motility patterns in *P. aeruginosa* (Caiazza *et al.*, 2005).

Decreasing rhamnolipid concentrations also promotes the clearance of *P. aeruginosa* biofilms *in vivo* (Jensen *et al.*, 2007), which suggests a role for rhamnolipids in the reduced pulmonary colonization and increased clearance of *P. aeruginosa* by 7HI in the guinea pigs (Fig. 5.8). Colonization of multiple tissues by *P. aeruginosa* after aerosol infection is consistent with previous observations in mice demonstrating the greatest degree of infection as shown by *in vivo* imaging (DiGiandomenico *et al.*, 2007). Both the number of bacteria present in the lungs and the bioluminescent signal produced after infecting guinea pigs with *P. aeruginosa* that have been pre-treated with 7HI support this conclusion. Our *in vivo* bioluminescence imaging also suggests that dissemination of *P. aeruginosa* to extrapulmonary sites may be affected by 7HI pre-treatment, but further studies are needed before this conclusion can be made. These observations suggest that hydroxylated indole represents a potential therapeutic approach or preventative measure for pulmonary infections caused by *P. aeruginosa*.

Previously, we reported that several hydroxyindoles affect biofilm formation of a pathogenic *E. coli* via different mechanisms; for example, doubly-hydroxylated indole, isatin, enhanced *E. coli* O157:H7 biofilm by inducing flagellar genes and repressing genes encoding AI-2 transporters *lsrABCDFGKR* (reminiscent of effects caused by autoinducer-2), and indole synthesis genes *tnaABC*, but indole, 5-hydroxyindole, and 7HI inhibited *E. coli* biofilm through cysteine (sulfur) metabolism (Lee *et al.*, 2007a). Here, both indole and 7HI stimulate biofilm formation in *P. aeruginosa*, inhibit production of QS-derived virulence factors, and enhance antibiotic resistance via similar mechanisms (Fig. 5.5) since both compounds differentially induced or repressed many common genes (Table 5.1). However, the impact of 7HI on the production of virulence factors was more significant than that with indole. Also, indole alone

specifically induced several oxygenases, and 7HI alone repressed the type IVb pilus *flp-tad-rcp* gene cluster and abolished swarming motility of *P. aeruginosa*, suggesting a different physiological role of indole and hydroxylated indole. This result is additional support of the hypothesis that hydroxylation of abundant indole by non-specific oxygenases (that are probably present in consortia) can lead to the formation of hydroxyindoles that alter cellular functions in *P. aeruginosa* as well as in *E. coli* in a manner different than indole (Lee *et al.*, 2007a). The pattern of the modification of indole is important as the control compound IAA did not influence the expression of quorum sensing-related genes (Table 5.1) or influence the production of virulence factors (Fig. 5.2).

In this study, we have demonstrated that the *E. coli* cell signal indole and hydroxylated indole (7HI), diminish several QS-controlled virulence factors in *P. aeruginosa*, reduce its virulence in guinea pigs, enhance its biofilm formation, enhance its antibiotic resistance, and are degraded readily (Fig. 5.6). Hence, we have shown that there is possibly interference of the *E. coli* signal indole by *P. aeruginosa* in that *P. aeruginosa* has a defense system to degrade indole. In a manner similar to the way *P. aeruginosa* may utilize AHLs as an interference strategy to preclude encroachment by competing bacteria (Kaufmann *et al.*, 2005), *E. coli* may utilize indole to compete against *P. aeruginosa* (a pathogen that threatens its host) by decreasing the production of its siderophores (pyoverdine and pyochelin) and virulence factors. Although highly speculative, another possibility is that *P. aeruginosa* may use the indole to increase its biofilm formation in the intestinal tract to initially form a colony to avoid washout from the host; at this point the opportunistic pathogen down-regulates virulence factors with the prevalent indole to avoid detection until a suitable cell density is reached (Williams, 2007). Since AHLs have been shown to have other functions other than QS (Kaufmann *et al.*, 2005), indole and 7HI may have functions other than inhibiting virulence factors in *P. aeruginosa*.

Given our results showing indole diminishes virulence factors and virulence, that an indole derivative (CBR-4830) has been shown to inhibit *P. aeruginosa* growth through a multi-drug efflux pump, *mexAB-oprM* (Robertson *et al.*, 2007), and that some natural indole derivatives, such as indole-3-carbinol and 3,3'-diindolylmethane derived from Cruciferous vegetables, show antimicrobial, antiviral, and anticancer activity (Higdon *et al.*, 2007) , indole and indole derivatives appear to have pharmaceutical potential as treatments against pathogenic *P. aeruginosa*. Most importantly, indole and 7HI present an opportunity for anti-virulence therapies (Cegelski *et al.*, 2008; Lesic *et al.*, 2007) which are also known as anti-pathogenic drugs (Rasmussen and Givskov, 2006). Anti-virulence compounds are an important way to fight infectious diseases because unlike antimicrobials, anti-virulence compounds like indole do not affect growth and so there is less chance of developing resistance (Hentzer *et al.*, 2002; Lesic *et al.*, 2007).

5.5 Experimental procedures

5.5.1 Bacterial strains, materials, and growth rate measurements

All experiments were conducted at 37^{o}C, and Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) was used except for the pyoverdine assay and swarming motility assay. *P. aeruginosa* PAO1 used in this study was the sequenced Holloway strain (Stover *et al.*, 2000), and *P. aeruginosa* PAO1 isogenic *antA*, *mexI*, and *mvfR* mutants (Jacobs *et al.*, 2003) were obtained from Prof. Colin Manoil. *P. aeruginosa* PA14 *phzS, rhlR, pqsA,*and *lasB* mutants (Liberati *et al.*, 2006) were used only as negative controls in virulence factor assays. The transposon mutations were confirmed (*antA* by us, *mexI* and *mvfR* by the source) by four PCR reactions using two primers in the IS*phoA*/hah or IS*lacZ*/hah transposon (5'-CGGGTGCAGTAATATCGCCCT-3'

and 5'-GGGTAACGCCAGGGTTTTCC-3') and two primers in the target gene (5'-GTGAGAACGCATGAACGCTA-3' and 5'-CTGACGATCTCGGTACGGTT-3' for *antA*, 5'-ATCCGCCGCAACAACTAC-3' and 5'-GTAGACCTGGTCGAGCTTGC-3' for *mexI*, and 5'-CTGCATGCTGGAATTGCTC-3' and 5'-ACTGAAGATCTCCCGCTTCA-3' for *mvfR*, respectively).

Indole, 7HI, and IAA were used at 1.0 mM, 0.5 mM, and 1.0 mM, respectively except for the biofilm dose response and biodegradation experiments. Indole and 7HI were purchased from Fisher Scientific (Pittsburg, PA). IAA was purchased from USB Biochemicals (Cleveland, OH). The toxicity of indole, 7HI, and IAA was evaluated using the specific growth rate with two independent cultures; these compounds were dissolved in DMF, and DMF was added as a control at 0.1 volume percent for all experiments. To test for utilization of indole and 7HI as a carbon source by *P. aeruginosa*, cell numbers were measured for four days in M9 minimal medium (Rodriguez and Tait, 1983) with 1.0 and 2.0 mM indole and 0.5 mM and 2.0 mM 7HI.

5.5.2 Total RNA isolation and microarray analysis

For the microarray experiments, 10 g of glass wool (Corning Glass Works, Corning, N.Y.) was used to form biofilms (Ren *et al.*, 2004b) for 7 h in 250 mL in 1 L Erlenmeyer shake flasks which were inoculated with overnight cultures that were diluted 1:100. Indole (1 mM), 7HI (0.5 mM), or IAA (1.0 mM) in 250 μL DMF, or 250 μL DMF alone was added. Glass wool was used to increase the surface area so that RNA could be readily obtained for the microarrays. RNA was isolated from the suspension and biofilm cells as described previously (Ren *et al.*, 2004b)

The Genechip *P. aeruginosa* Genome Array (Affymetrix, P/N 900339) contains 5,500 of the 5,570 ORFs of *P. aeruginosa* (Whiteley *et al.*, 2001). cDNA synthesis, fragmentation, and

hybridizations were as described previously (González-Barrios *et al.*, 2006b). Hybridization was performed for 16 h at 50°C, and the total cell intensity was scaled to an average value of 500. The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA control were used to monitor the labeling process. For each binary microarray comparison of differential genes expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 13 probe pairs (*p*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *p*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher (2-fold) than the standard deviation for the whole microarrays (Ren *et al.*, 2004a) (1.5-fold for indole, 1.6-fold for 7HI, and 1.1-fold for IAA). Gene functions were obtained from the Affymetrix–NetAffx Analysis Center (https://www.affymetrix.com/analysis/netaffx/index.affx).

5.5.3 RT-PCR

To corroborate the DNA microarray data, the transcription level of six prominent genes was quantified using RT-PCR: *mexG* (forward primer 5'-GGCGAAGCTGTTCGACTATC-3'; reverse primer 5'-AGAAGGTGTGGACGATGAGG-3'), *mexH* (forward primer 5'-GAAAAGCAATTTCTCCCTGGAC-3'; reverse primer 5'-GTTGATCTGTCCGGAAGTCACT A-3'), *mexI* (forward primer 5'-CTCTACCGGACCATGGAAGA-3'; reverse primer 5'-AGCGGTTGACGTAGTTCTCG-3'), *pvdS* (forward primer 5'-TAACCGTACGATCCTGGTG AAGA-3'; reverse primer 5'-ACGATCTGGAACAGGTAGCTGAG-3'), *ssuF* (forward primer 5'-CATCAACGTTCGTAACCAGTTCA-3'; reverse primer 5'-GATGGAGACCTCGGTGGAC

TT-3'), and PA0284 (forward primer 5'-ACCCTCAGAAGCCTGGATG-3'; reverse primer 5'-GTTGCTGCAGACGGAATTTT-3'). The expression level of the housekeeping gene *proC* (forward primer 5'-CAGGCCGGGCAGTTGCTGTC-3'; reverse primer 5'-GGTCAGGCGCGA GGCTGTCT-3') (Savli *et al.*, 2003) was used to normalize the expression data of interesting genes. An independent RNA sample using identical DNA microarray conditions were used for these studies (63 RT-PCR reactions based on three RT-PCR reactions for each of six genes including the *proC* housekeeping gene, with DMF, indole, and 7HI). RT-PCR was performed in triplicate using a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA). The relative fold changes for RT-PCR were calculated from threshold cycle numbers measured using StepOne software v2.0 (Applied Biosystems). Using another housekeeping gene *rpoN* (forward primer 5'-GAGCTCGACGAAGTGGAAGT-3'; reverse primer 5'- CGCATCAATTGGCTGTA GTC-3') and 9 RT-PCR reactions, similar results were obtained.

5.5.4 Crystal violet biofilm assay

This assay was adapted (Pratt and Kolter, 1998); overnight cultures diluted to an optical density of 600 nm of 0.05 and were incubated in polystyrene 96 well plates for 24 h without shaking. The dye staining the biofilms (air-liquid interface biofilm as well as bottom liquid-solid biofilm) was dissolved in 95% ethanol, and an absorbance at 540 nm (OD_{540}) was measured to quantify the total biofilm mass. Each data point was averaged from more than twelve replicate wells (six wells from two independent cultures).

5.5.5 Virulence factor assays

Except for the pyoverdine assay, overnight cultures were diluted 1:100 and contacted with indole (1 mM), 7HI (0.5 mM), IAA (1.0 mM), or diluent DMF (negative control). The

pyocyanin assay was adapted (Essar *et al.*, 1990); after growth for 7 h, supernatants were extracted with chloroform and 0.2 N HCL, and analyzed spectrophotometrically. The *phzS* mutant was used as a negative control. The rhamnolipid assay was adapted (Wilhelm *et al.*, 2007); after growth for 7 h, supernatants were assayed for rhamnolipids using the orcinol colorimetric assay (Wilhelm *et al.*, 2007). The *rhlR* mutant was used as a negative control. The PQS assay was adapted (Attila *et al.*, 2008b) after growth for 7 h, supernatants were extracted with acidified ethyl acetate and analyzed by thin layer chromatography. As the standard, a synthetic PQS obtained from Prof. Marvin Whiteley was used (Fig. 5.3), and the *pqsA* mutant was used as a negative control. The elastase assay was adapted (Ohman *et al.*, 1980); after growth to a turbidity of 2 at 600 nm, supernatants were incubated with elastin-Congo Red (MP Biomedicals, 101637), and the absorbance was measured at 495 nm to determine the elastase activity. The *lasB* mutant was used as a negative control. The pyoverdine assay was adapted (Ren *et al.*, 2005b); after growth in minimal succinate medium (Ren *et al.*, 2005b), cells were diluted to a turbidity of 0.05 at 600 nm in fresh minimal succinate medium and were grown for 8 h. The pyoverdine concentration was measured spectrophotometrically at 405 nm (Stintzi *et al.*, 1998). Each experiment was performed using at least two independent cultures.

5.5.6 Antibiotic resistance assays

Overnight cultures were diluted 1:100 and grown to a turbidity of 1.5 at 600 nm with indole, 7HI, or DMF. Antibiotics (0.06 mg/mL gentamicin, 10 mg/mL kanamycin, 0.4 mg/mL tetracycline, and 5 mM $VOSO_4 \bullet 2H_2O$) were mixed with cells and incubated for 60 min without shaking; cells were enumerated with LB agar plates. To test the antibiotic ampicillin, the MIC of ampicillin was determined using an LB agar dilution technique (Andrews, 2001). Cells were grown to mid-log phase (turbidity of 1.5 at 600 nm) and ten μL of diluted cells at 10^4 cells/ml

was added to the surface of LB agar plates with indole or 7HI and 100 to 800 μg/mL ampicillin. All plates were incubated for 18 h. Two independent cultures were used for each strain.

5.5.7 Swimming, swarming, and twitching motility

For swimming motility, 0.3% agar with 1% tryptone and 0.25% NaCl was used (Sperandio *et al.*, 2002), for swarming motility, BM2 swarming medium (62 mM potassium phosphate buffer at pH 7, 2 mM $MgSO_4$, 10 μM FeSO4, 0.4% glucose, 0.1% casamino acids, and 0.5% agar) was used (Overhage *et al.*, 2007), and for twitching motility, LB with 1.0% agar was used (Overhage *et al.*, 2007). Briefly, strains were grown from diluted overnight cultures to a turbidity of 1.0 at 600 nm. Indole and 7HI dissolved in DMF were added to the motility agar. DMF (0.1%) was added as the negative control. The halo diameter at 24 h was measured for swimming motility. Each experiment was performed using two independent cultures.

5.5.8 Degradation and detection of indole and 7HI

For the degradation of indole and 7HI, overnight cultures were diluted to a turbidity of 1.0 at 600 nm and were re-grown with indole (0.5 mM) and 7HI (0.5 mM) at 250 rpm. As a negative control, autoclaved cells (turbidity of 1.0 at 600 nm) were contacted with indole or 7HI at the same conditions to confirm that there is no evaporation or adsorption of indole or 7HI. Extracellular concentrations of indole or 7HI were measured directly from filtered supernatants with reverse-phase HPLC using a 100 x 4.6 mm Chromolith Performance RP-18e column (Merck KGaA, Darmstadt, Germany) and gradient elution with H_2O-0.1% formic acid and acetonitrile as the mobile phases at a flow rate of 1 mL/min (65:35 for 0-5 min, 35:65 for 5-12 min, and 65:35 at 12 min) (Lee *et al.*, 2007a). Under these conditions, the retention times and the absorbance maxima were 3.6 min/264 nm for 7HI and 5.9 min/271 nm for indole. Each

experiment was performed with two independent cultures. To determine the total protein, the Modified Lowry Protein Assay Kit from Pierce Biotechnology (Rockford, IL) was used. The protein content of *P. aeruginosa* was 0.255 mg protein/(mL OD).

5.5.9 Phenotype microarray

Phenotype PM1-8 microarray plates (12111, 12112, 12121, 12131, 12141, 12181, 12182, 12183) (Biolog, Hayward, CA) were used to investigate 756 different phenotypes. Briefly, overnight cells were removed from BUG+B agar plates with a sterile swab and placed into IF-O base buffer, and the cell turbidity at 600 nm was adjusted to 0.065. Bacterial inocula were prepared, and 100 μL of each cell suspension was inoculated into the plates.

5.5.10 Acute model of *Pseudomonas* infection in guinea pigs

Random bred Hartley strain guinea pigs weighing 200-300 g were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). The animals were housed individually in polycarbonate cages in a temperature- and humidity-controlled environment; ambient lighting was automatically controlled to provide 12 h light and 12 h dark cycles. Animals were given commercial chow and tap water *ad libitum*. All procedures were reviewed and approved by the Texas A&M University Laboratory Animal Care Committee. *P. aeruginosa* was cultured with 7HI or DMF alone as a control in LB for 7 h, and guinea pigs were infected by aerosol with equal amounts of washed cells using a Madison chamber aerosol generation device calibrated to deliver between 10^4-10^5 colony forming units (CFU) of *P. aeruginosa* into the lungs. At each time point (immediately after infection, 4 h, 24 h, and 48 h), five guinea pigs in each group were euthanized by overdose with sodium pentabarbitol and necropsy was performed to obtain total lung mass. Lungs were homogenized in saline and dilutions plated to determine the total cfu

present at each time point.

5.5.11 *In vivo* infection imaging

In order to confirm differences observed in colonization of guinea pig tissues, four guinea pigs were infected in the same manner as for the acute model of infection and two animals from each group were examined by quantitative *in vivo* imaging. Dorsal and lateral images were acquired for each animal immediately after infection, at 4 h and at 24 h. *P. aeruginosa* with the *Photorhabdus luminescens luxCDABE* operon stably inserted into the chromosome (single copy) was used (strain Xen41 from Xenogen, Alameda, CA); addition of 7HI did not change luminescence. Images were acquired using the luminescence settings on the Spectrum In Vivo Imaging System (IVIS) CCD camera and analyzed with Living Image 3.0 software (Xenogen). Colonization of all organs was quantified by measurement of total photons from each animal and from the pulmonary region by measurement of total photons from the pulmonary region of each animal.

5.5.12 Cytotoxicity

Assays to determine cytotoxicity to red blood cells with indole and 7HI vs. DMF alone were conducted as described previously (Attila *et al.*, 2008a) with *P. aeruginosa* cultured for 7 h.

5.5.13 Microarray accession numbers

The expression data for biofilm samples with and without indole or 7HI are summarized in Table 5.1 and have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through accession number GSE10065 (Edgar *et al.*, 2002).

CHAPTER VI

URACIL INFLUENCES QUORUM SENSING AND BIOFILM FORMATION IN *PSEUDOMONAS AERUGINOSA* AND FLUOROURACIL IS AN ANTAGONIST*

6.1 Overview

Pseudomonas aeruginosa is an ubiquitous, opportunistic pathogen whose biofilms are notoriously difficult to control. Here we discover uracil influences all three known quorum-sensing (QS) pathways of *P. aeruginosa*. By screening 5,850 transposon mutants for altered biofilm formation, we identified seven uracil-related mutations that abolished biofilm formation. Whole-transcriptome studies showed the uracil mutations (e.g., *pyrF* that catalyzes the last step in uridine monophosphate synthesis) alter the regulation of all three QS pathways (LasR-, RhlR-, and 2-heptyl-3-hydroxy-4-quinolone (PQS)-related regulons); addition of extracellular uracil restored global wild-type regulation. Phenotypic studies confirmed uracil influences the LasR (elastase), RhlR (pyocyanin, rhamnolipids), PQS, and swarming regulons. Our results also demonstrate uracil influences virulence (the *pyrF* mutant was less virulent to barley). Additionally, we found an anti-cancer uracil analog, 5-fluorouracil, that repressed biofilm formation, abolished QS phenotypes, and reduced virulence. Hence, we have identified a central regulator of an important pathogen and a potential novel class of efficacious drugs for

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* Reprinted with permission from "Uracil influences quorum sensing and biofilm formation in *Pseudomonas aeruginosa* and fluorouracil is an antagonist" by Akihiro Ueda, Can Attila, Marvin Whiteley, and Thomas K. Wood, 2008, *Microbial Biotechnology*, in press, Copyright Wiley-Blackwell. C. Attila was responsible for the PQS experiments and the growth rate experiments. A. Ueda was responsible for all the other experiments.

controlling cellular behavior (e.g., biofilm formation and virulence).

6.2 Introduction

The diverse small signal molecules used for quorum sensing (QS) (Camilli and Bassler, 2006) transform independent cells into specialized tissues (Battin *et al.*, 2007). These small molecules are used to regulate biofilm formation (Davies *et al.*, 1998; González-Barrios *et al.*, 2006b). The ubiquitous pathogen *P. aeruginosa,* which is one of the major causes of chronic lung infections of cystic fibrosis patients (Stover *et al.*, 2000) and a major cause of hospital-acquired infections, thrives in many environments due to its exquisite gene regulation which consists of myriad two-component systems (Stover *et al.*, 2000). In this organism, expression of many genes is regulated via three distinct QS systems.

The Las QS system is activated by *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL), and the LasR transcription factor controls expression of $3OC_{12}$-HSL synthesis (*lasI*) and virulence factors (e.g., *lasAB*, *toxA*, and *apr*) (Wagner *et al.*, 2004). The Rhl system is activated by *N*-butyryl-HSL (C_4-HSL), and the RhlR transcription factor controls expression of C_4-HSL synthesis (*rhlI*), *rhlAB*, *lasB*, and pyocyanin production (Wagner *et al.*, 2004). The Las and Rhl QS systems regulate more than 300 genes (Schuster *et al.*, 2003). The *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4-quinolone) also controls the production of pyocyanin, rhamnolipids, and elastase (Diggle *et al.*, 2006). The hierarchy of QS regulation is that the LasRI controls RhlRI regulation, and the PQS system is related to both the LasRI and RhlRI systems (Diggle *et al.*, 2006). Hence, these QS systems regulate virulence factors such as extracellular enzymes (LasA protease, LasB elastase, and alkaline proteases), metabolites (pyocyanin and hydrogen cyanide), and biofilm formation that cause persistent infections by *P. aeruginosa*.

Ribonucleic acid secondary messengers, including cyclic adenosine monophosphate (cAMP) (Baker and Kelly, 2004), cyclic guanosine monophosphate (cGMP) (Baker and Kelly, 2004), and cyclic-diguanosine monophosphate (c-diGMP) (Cotter and Stibitz, 2007), serve as signals for diverse biological functions. c-diGMP regulates virulence factors and biofilm formation in *P. aeruginosa* (Cotter and Stibitz, 2007), and cAMP regulates various *in vivo* bacterial functions by binding to intracellular receptors and protein kinases (Baker and Kelly, 2004). In humans, uridine-5'-triphosphate (UTP) is an extracellular signal that regulates a broad spectrum of cell functions via the $P2Y_2$ receptor, a G protein-coupled membrane receptor that regulates phospholipases and mitogen-activated protein kinases (Lazarowski and Boucher, 2001). Hence, ribonucleic acid signals are important biological regulators.

By screening thousands of transposon mutants to discern genes related to biofilm formation, we discovered that uracil influences virulence, biofilm formation, and other QS-controlled phenotypes that are regulated by well-studied QS pathways (e.g., LasR, RhlR, and PQS). Whole-transcriptome analysis indicated that strains with an altered uridine monophosphate (UMP) synthesis pathway had hundreds of QS genes repressed, and transcription of these genes was restored by exogenous uracil. We then hypothesized that uracil analogs may inhibit biofilm formation which led to the discovery that the well-studied 5-fluorouracil (5-FU) is a potent inhibitor of *P. aeruginosa* biofilm formation; hence, 5-FU shows promise for preventing biofilm formation.

6.3 Results

6.3.1 Biofilm screening identifies UMP-related genes

To understand how *P. aeruginosa* regulates biofilm formation, we screened 5,850 mutants

that cover 4,596 of the 5,962 predicted PA14 genes (Liberati *et al.*, 2006) to discern which genes impact biofilm formation using a crystal violet biofilm screen. We identified 137 mutants with over 3-fold enhanced biofilm formation and identified 88 mutants with over 10-fold reduced biofilm formation. The mutants with decreased biofilm formation include 20 flagella and related proteins (*flgABCEFHIJKL*, *flhAB*, *fliFHKLMQ, motAB*), and two type IV fimbriae/pili biogenesis proteins (*pilFX*) that are well-characterized for affecting biofilm formation (Klausen *et al.*, 2003). Among those with decreased biofilm formation, all seven mutants (*carA*, *carB*, *pyrB*, *pyrC*, *pyrD*, *pyrE*, and *pyrF*) of the UMP synthetic pathway (Fig. 6.1) formed dramatically reduced biofilms (Fig. 6.2A). These mutants grew more slowly in LB (Fig. 6.3A and Table 6.1); for example, the *pyrF* mutant had a specific growth rate of 0.90 ± 0.01/h compared to 1.59 ± 0.04/h for the wild-type strain. All of the six other mutants (*carA*, *carB*, *pyrB*, *pyrC*, *pyrD*, and *pyrE*) showed similar growth and biofilm formation to the *pyrF* mutant (Table 6.1).

The mutants for the biosynthesis of other pyrimidines and purines were not identified by screening for altered biofilm formation. Hence, the effects were not due to growth defects nor were they related to general metabolic defects in nucleic acid biosynthesis. This suggested that the products of this metabolic pathway regulate biofilm formation, so we investigated whether UMP, UTP, or uracil may serve as an internal signal for biofilm formation by using a *pyrF* mutant (PyrF catalyzes the last step of UMP synthesis and encodes orotidine-5'-phosphate decarboxylase). Addition of uracil (0.1 to 1 mM), but not UMP and UTP, increased biofilm formation in the *pyrF* mutant (Fig. 6.2B); note that LB medium contains approximately 0.2 mM uracil, and complementation by uracil was expected since *P. aeruginosa* has a pyrimidine salvage pathway to utilize uracil but not UMP or UTP. Neither UMP nor UTP affected growth of the wild-type strain and the *pyrF* mutant at 1 mM; hence, both nucleotides are probably not transported into cells. Since higher concentrations of uracil (10 mM) enhanced biofilm

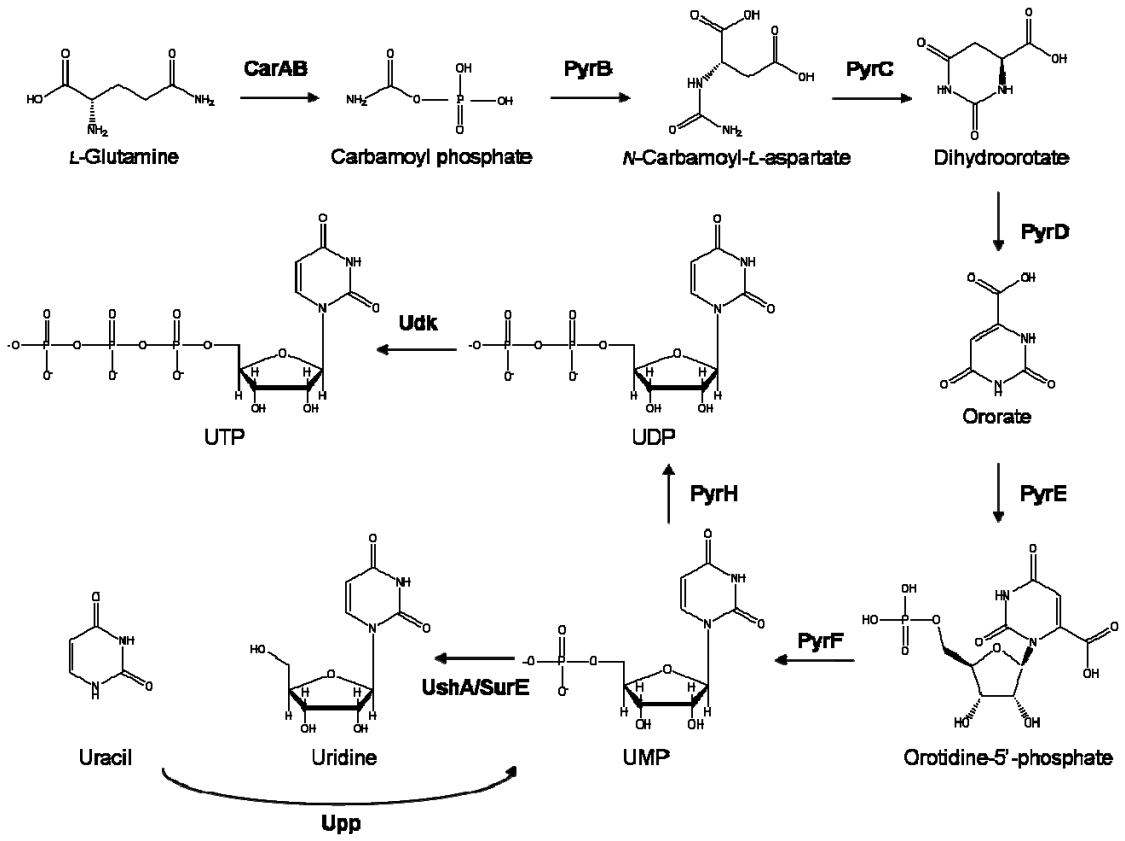

Fig. 6.1. UMP synthesis pathway in *P. aeruginosa* PA14 (Kanehisa and Goto, 2000). Abbreviations are CarA for the carbamoylphosphate synthetase large subunit, CarB for the carbamoylphosphate synthetase small subunit, PyrB for aspartate carbamoyltransferase, PyrC for dihydroorotase, PyrD for dihydroorotate dehydrogenase, PyrE for orotate phosphoribosyl-transferase, PyrF for orotidine-5'-phosphate decarboxylase, PyrH for uridylate kinase, Udk for uridylate diphosphate kinase, UshA for bifunctional UDP-sugar hydrolase and 5'-nucleotidase, SurE for stationary-phase survival protein, and Upp for uracil phosphoribosyltransferase.

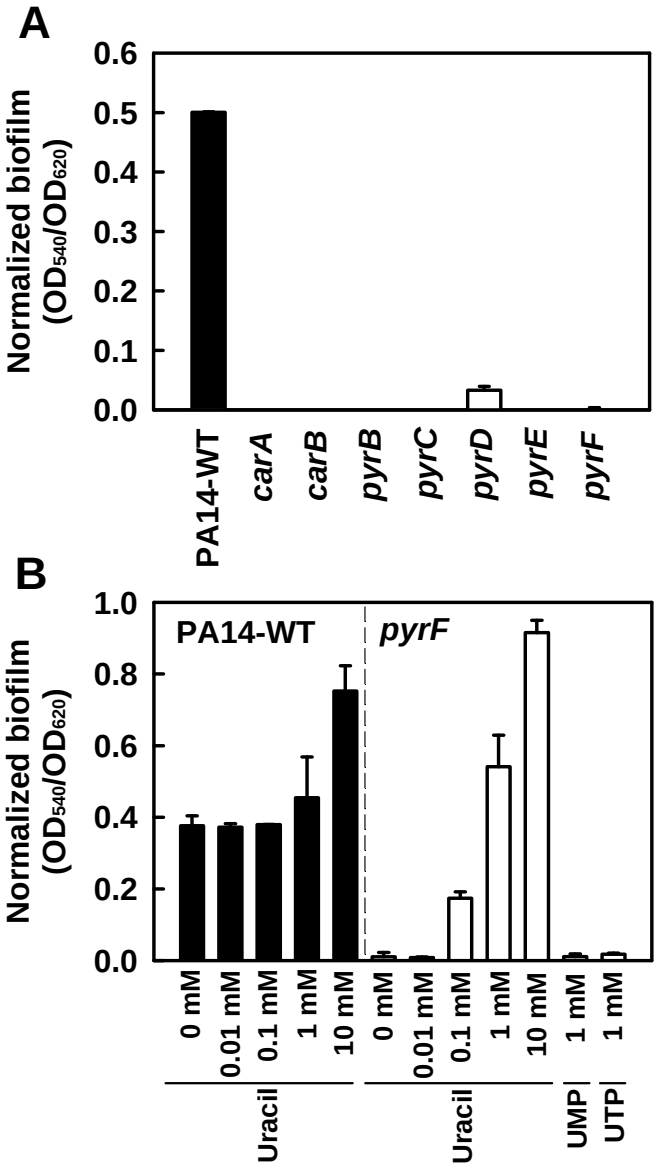

Fig. 6.2. Uracil controls biofilm formation. (A) Biofilm formation in the *P. aeruginosa* PA14 uracil-synthesis mutants *carA, carB, pyrB, pyrC, pyrD, pyrE,* and *pyrF*. (B) Biofilm formation of the *pyrF* mutant upon addition of uracil, UMP, and UTP. Biofilm formation was examined in LB medium after 24 h. Six to ten wells were used for each culture. Biofilm was normalized by cell growth, and data show the average of the two independent experiments ± s.d.

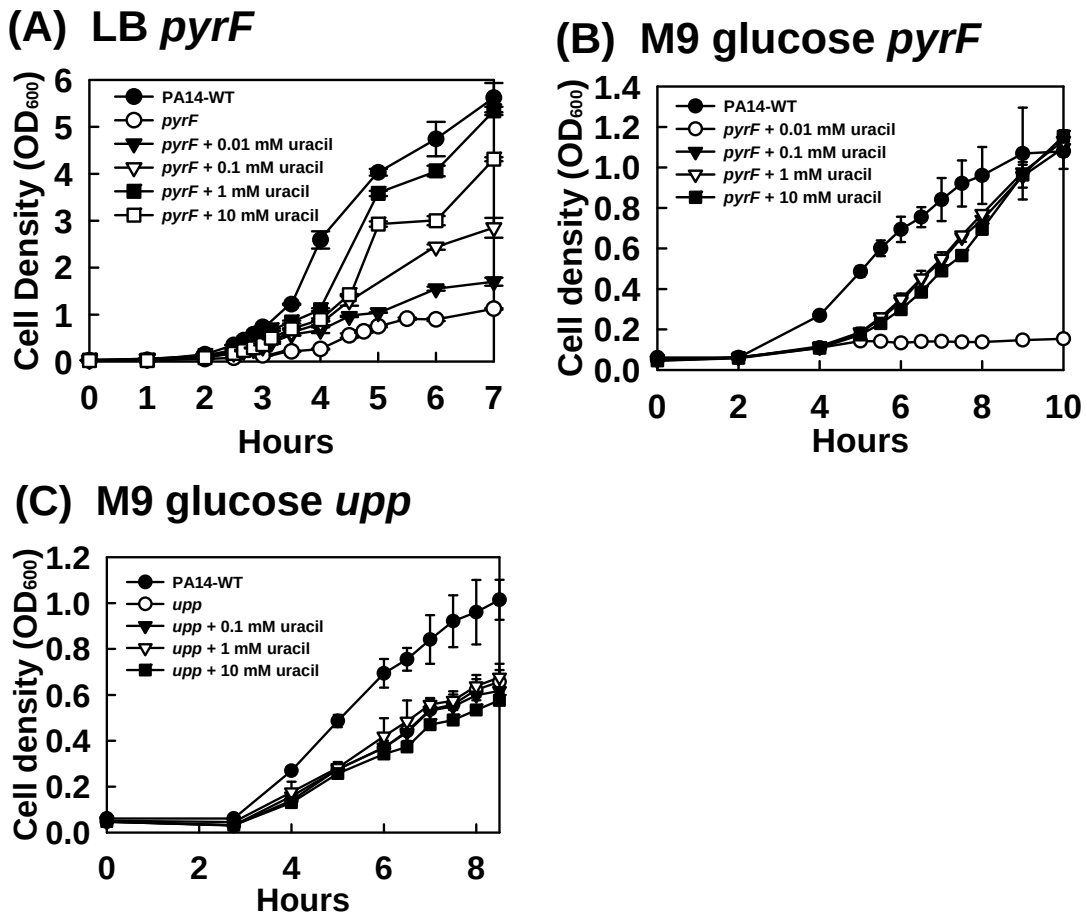

Fig. 6.3. Growth of the *pyrF* mutant in LB medium and in M9 glucose medium supplemented with uracil. Wild-type *P. aeruginosa* PA14 and the *pyrF* mutant were grown in (A) LB medium supplemented with 0.01 to 10 mM uracil and in (B) M9 glucose medium supplemented with 0.01 to 10 mM uracil. (C) Growth of wild-type *P. aeruginosa* PA14 and the *upp* mutant in M9 glucose medium supplemented with 0.1 to 10 mM uracil. Data show the average of two independent experiments ± s.d.

Table 6.1. Biofilm formation and growth of the PA14 uracil-synthesis mutants, *carA, carB, pyrB, pyrC, pyrD, pyrE,* and *pyrF*. Biofilm formation was examined in LB after 24 h.

Strain	Growth (O_{D620})	Biofilm (OD_{540})	Normalized biofilm (OD_{540}/OD_{620})
PA14-WT	0.91 ± 0.04	0.45 ± 0.01	0.50 ± 0.05
carA	0.35 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
carB	0.35 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
pyrB	0.36 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
pyrC	0.37 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
pyrD	0.40 ± 0.01	0.01 ± 0.00	0.03 ± 0.04
pyrE	0.39 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
pyrF	0.39 ± 0.01	0.00 ± 0.00	0.00 ± 0.00

formation of wild-type PA14 (Fig. 6.2B), uracil, but not UMP and UTP, serves as a consistent positive regulator of biofilm formation in *P. aeruginosa*; note these experiments do not rule out the possibility that UMP or UTP may mediate the biofilm effect intracellularly.

6.3.2 Purines do not affect biofilm formation

To examine whether purine nucleotide synthesis also regulates biofilm formation like the pyrimidine uracil, we tested biofilm formation with mutants lacking the purine nucleotide synthetic pathway. The *purH* mutant, deficient in inosine monophosphate synthesis (used for adenosine and guanosine), formed biofilms as well as wild-type PA14 (Fig. 6.4). Additionally, the *purE* mutant (lacks phosphoribosylaminoimidazole carboxylase for 5'-phosphoribosyl-4-carboxy-5-aminoimidazole synthesis) and the PA14_01760 mutant (putative non-specific ribonucleoside hydrolase that may catalyze adenosine to adenine and guanosine to guanine) showed normal biofilm formation. Thus, the purine nucleotides, adenine and guanine, do not affect biofilm formation whereas all of the *car* and *pyr* mutants related to uracil synthesis showed decreased biofilm formation.

6.3.3 UMP synthesis induces QS genes

To determine which genes are regulated by uracil in biofilms, we analyzed the whole transcriptome for the *pyrF* mutant vs. wild-type *P. aeruginosa* for cells grown on glass wool (Table 6.1). Remarkably, all three known QS systems were repressed upon inhibition of UMP synthesis (overall 298 genes were repressed and 147 were induced). The *pyrF* mutation repressed the transcriptional factor for the Rhl QS system, *rhlR*, -4.9-fold as well as repressed *rhlA* (-21-fold) and *rhlB* (-20-fold). The deficiency in UMP synthesis also repressed *lasAB* of the Las QS system -16- to -39-fold and repressed *pqsH* (encodes a FAD-dependent

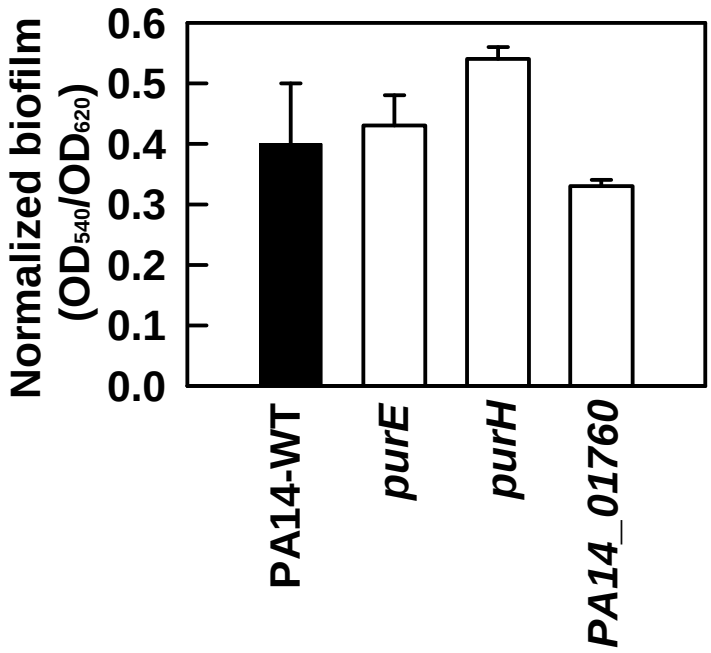

Fig. 6.4. **Biofilm formation of mutants deficient in adenine and guanine synthesis.** Biofilm formation was examined in LB medium after 24 h, and 10 wells were used for each culture. Biofilm formation was normalized by cell growth, and data show the average of the two independent experiments ± s.d.

Table 6.2. Uracil regulates QS and virulence factors. Partial list of differentially-expressed genes in biofilm cells in LB medium after 7 h for the *pyrF* mutant vs. wild-type PA14 (WT), for the *pyrF* mutant with 1 mM uracil vs. WT, and for the *pyrF* mutant with 1 mM uracil vs. the *pyrF* mutant without uracil.

			Fold changes			
PA14 #	**PAO1 #**	**Gene**	***pyrF* vs. WT**	***pyrF* + uracil vs. WT**	***pyrF* + uracil vs. *pyrF***	**Description**
Repressed genes upon deleting *pyrF*						
Quorum sensing						
PA14_19120	PA3477	*rhlR*	-4.9	1.1	4.9	Transcriptional regulator RhlR
PA14_19110	PA3478	*rhlB*	-21.1	-1.2	17.1	Rhamnosyltransferase chain B
PA14_19100	PA3479	*rhlA*	-19.7	-1.4	16	Rhamnosyltransferase chain A
PA14_45950	PA1431	*rsaL*	-2.1	1.4	2.6	Regulatory protein RsaL
PA14_40290	PA1871	*lasA*	-39.4	1	32	LasA protease precursor
PA14_16250	PA3724	*lasB*	-16	1.1	19.7	Elastase LasB
PA14_30630	PA2587	*pqsH*	-2.6	-1.1	2.5	FAD-dependent monooxygenase
Phenazine synthesis						
PA14_09460	PA1901	*phzC2*	-17.1	-1.1	13.9	Phenazine biosynthesis protein PhzC
PA14_09450	PA1902	*phzD2*	-18.4	1.3	19.7	Phenazine biosynthesis protein PhzD
PA14_09440	PA1903	*phzE2*	-14.9	-1.1	14.9	Phenazine biosynthesis protein PhzE
PA14_09420	PA1904	*phzF2*	-17.1	1	17.1	Phenazine biosynthesis protein
PA14_09410	PA1905	*phzG2*	-12.1	1.2	13.9	Pyridoxamine 5'-phosphate oxidase
PA14_09490	PA4209	*phzM*	-3.5	1.1	3.5	Phenazine-specific methyltransferase
PA14_09480	PA4210	*phzA1*	-10.6	-1.1	8.6	Phenazine biosynthesis protein
PA14_09470	PA4211	*phzB1*	-18.4	1	18.4	Phenazine biosynthesis protein
PA14_09400	PA4217	*phzS*	-7.5	1.2	9.8	Flavin-containing monooxygenase
Pyochelin synthesis						
PA14_09290	PA4224	*pchG*	-5.7	-1.9	2.8	Pyochelin biosynthesis protein PchG
PA14_09280	PA4225	*pchF*	-7	-2.5	3.5	Pyochelin synthetase
PA14_09270	PA4226	*pchE*	-5.7	-1.7	3	Dihydroaeruginoic acid synthetase
PA14_09260	PA4227	*pchR*	-2.1	1.1	2.5	Transcriptional regulator PchR
PA14_09240	PA4228	*pchD*	-5.3	-1.3	4	Pyochelin biosynthesis protein PchD
PA14_09230	PA4229	*pchC*	-5.7	-1.7	3.5	Pyochelin biosynthesis protein PchC
PA14_09220	PA4230	*pchB*	-5.7	-1.3	4	Salicylate biosynthesis protein PchB
Pyoverdine synthesis						
PA14_33260	PA2426	*pvdS*	-11.3	-4.6	1.9	Sigma factor PvdS
Chemotaxis						
PA14_02200	PA0175		-2.8	1.2	3.5	Probable chemotaxis methyltransferase
PA14_02220	PA0176	*aer2*	-3.5	1.3	4.9	Aerotaxis transducer Aer2
PA14_02230	PA0177		-2.8	1.1	3.2	Probable purine-binding chemotaxis

Table 6.2. (continued)

			Fold changes			
PA14 #	**PAO1 #**	**Gene**	***pyrF* vs. WT**	***pyrF* + uracil vs. WT**	***pyrF* + uracil vs. *pyrF***	**Description**
Repressed genes upon deleting *pyrF*						
PA14_02250	PA0178		-3.7	-1.1	3.5	Probable 2-component sensor
PA14_02260	PA0179		-3.7	1.1	4.3	Probable 2-component response regulator
PA14_02270	PA0180		-3.7	-1.1	3.7	Probable chemotaxis transducer
PA14_28050	PA2788		-14.9	-1.1	12.1	Probable chemotaxis transducer
Multidrug efflux transporter						
PA14_09540	PA4205	*mexG*	-32	-1.2	26	Hypothetical protein
PA14_09530	PA4206	*mexH*	-24.3	1.1	22.6	Efflux membrane fusion protein precursor
PA14_09520	PA4207	*mexI*	-14.9	-1.1	13	Efflux transporter
PA14_09500	PA4208	*opmD*	-16	-1.1	13.9	Probable outer membrane efflux protein
Virulence factors						
PA14_48115	PA1246	*aprD*	-6.1	-0.2	4.9	Alkaline protease secretion protein AprD
PA14_48100	PA1247	*aprE*	-4.9	0.2	5.7	Alkaline protease secretion protein AprE
PA14_48090	PA1248	*aprF*	-4	0.3	4.3	Alkaline protease secretion protein AprF
PA14_48060	PA1249	*aprA*	-10.6	0.6	36.8	Alkaline metalloproteinase precursor
PA14_48040	PA1250	*aprI*	-2.6	0.5	3.5	Alkaline proteinase inhibitor AprI
PA14_36330	PA2193	*hcnA*	-5.7	-1.1	4.9	Hydrogen cyanide synthase HcnA
PA14_36320	PA2194	*hcnB*	-5.7	-1.1	5.3	Hydrogen cyanide synthase HcnB
PA14_36310	PA2195	*hcnC*	-5.7	1.2	7	Hydrogen cyanide synthase HcnC
PA14_34870	PA2300	*chiC*	-32	-1.2	34.3	Chitinase
Type II secretion system/TypeIV pilus						
PA14_55850	PA4299	*tadD*	-2.6	1.4	4.3	Flp pilus assembly protein TadD
PA14_55860	PA4300	*tadC*	-2.8	1.1	3	Flp pilus assembly protein TadC
PA14_55880	PA4301	*tadB*	-2	-1.1	2	Flp pilus assembly protein TadB
PA14_55890	PA4302	*tadA*	-4.6	1.3	6.5	Flp pilus assembly protein, ATPase CpaF
PA14_55900	PA4303	*tadZ*	-6.5	1	4.3	Flp pilus assembly protein, ATPase CpaE
PA14_55920	PA4304	*rcpA*	-7	-1.2	7	Flp pilus assembly protein, secretin CpaC
PA14_55930	PA4305	*rcpC*	-5.7	1.5	9.8	Flp pilus assembly protein CpaB
PA14_55940	PA4306	*flp*	-29.9	1.6	59.7	Flp pilus assembly protein, pilin Flp

Table 6.2. (continued)

			Fold changes			
PA14 #	**PAO1 #**	**Gene**	***pyrF* vs. WT**	***pyrF* + uracil vs. WT**	***pyrF* + uracil vs. *pyrF***	**Description**
Repressed genes upon deleting *pyrF*						
Others						
PA14_53250	PA0852	*cpbD*	-14.9	1.1	16	Chitin-binding protein CbpD precursor
PA14_39780	PA1914		-16	1.9	36.8	Conserved hypothetical protein
PA14_37770	PA2067		-16	-1.1	12.1	Probable hydrolase
PA14_37745	PA2069		-42.2	-1.1	32	Probable carbamoyl transferase
PA14_35160	PA2274		-13.9	-1.2	13	Hypothetical protein
PA14_33870	PA2381		-22.6	1.1	24.3	Hypothetical protein
PA14_31350	PA2566		-17.1	1.5	21.1	Conserved hypothetical protein
PA14_31290	PA2570	*lecA*	-3	1.7	4.9	PA-I galactophilic lectin
PA14_26020	PA2939		-22.6	-1.1	22.6	Probable aminopeptidase
PA14_24650	PA3049	*rmf*	-22.6	1.6	39.4	Ribosome modulation factor
PA14_20610	PA3361	*lecB*	-2.1	1.2	3	Fucose-binding lectin PA-IIL
PA14_18120	PA3570	*mmsA*	-16	-1.4	11.3	Methylmalonate-semialdehyde dehydrogenase
PA14_11140	PA4078		-17.1	1.1	16	Probable nonribosomal peptide synthetase
PA14_10560	PA4129		-29.9	-1.1	13.9	Hypothetical protein
PA14_10550	PA4130		-26	1	26	Probable sulfite or nitrite reductase
PA14_10540	PA4131		-18.4	1	17.1	Probable iron-sulfur protein
PA14_10500	PA4133		-29.9	1.1	29.9	Cytochrome c oxidase subunit (cbb3-type)
PA14_10490	PA4134		-16	-1.3	13	Hypothetical protein
Induced genes upon deleting *pyrF*						
-	AF241171		4.9	1.1	-6.1	No significant similarity
PA14_00570	PA0045		4.6	1.1	-3.7	Hypothetical protein
PA14_00580	PA0046		4.6	1.1	-4.3	Hypothetical protein
PA14_03830	PA0293	*aguB*	10.6	-1.1	-9.8	N-carbamoylputrescine amidohydrolase
PA14_06420	PA0492		9.8	-2.1	-24.3	Conserved hypothetical protein
PA14_06430	PA0493		7	-1.6	-10.6	Probable biotin-requiring enzyme
PA14_06480	PA0496		6.5	-1.6	-8.6	Conserved hypothetical protein
PA14_54520	PA0755	*opdH*	12.1	1.4	-8.6	Cis-aconitate porin OpdH
PA14_54170	PA0782	*putA*	6.5	-1.1	-8.6	Proline dehydrogenase PutA
PA14_50770	PA1051		4.9	-1.2	-6.5	Probable transporter
PA14_46080	PA1420		5.3	1.7	-4	Hypothetical protein
PA14_46070	PA1421	*speB2*	9.8	-1.1	-9.8	Agmatinase

Table 6.2. (continued)

			Fold changes			
PA14 #	**PAO1 #**	**Gene**	***pyrF* vs. WT**	***pyrF* + uracil vs. WT**	***pyrF* + uracil vs. *pyrF***	**Description**
Induced genes upon deleting *pyrF*						
PA14_46070	PA1421	*speB2*	9.8	-1.1	-9.8	Agmatinase
PA14_38170	PA2038		4.9	-1.3	-5.7	Hypothetical protein
PA14_35460	PA2252		7	1.2	-6.1	Probable Na/alanine/glycine symporter
PA14_27370	PA2840		4.9	-1.1	-3.7	Probable ATP-dependent RNA helicase
PA14_26910	PA2875		5.7	7	1.3	Conserved hypothetical protein
PA14_19470	PA3452	*mqoA*	5.3	1	-4.6	Malate:quinone oxidoreductase
PA14_17960	PA3582	*glpK*	6.1	-1.1	-5.7	Glycerol kinase
PA14_17930	PA3584	*glpD*	5.7	-1.1	-8	Glycerol-3-phosphate dehydrogenase
PA14_16010	PA3741		6.1	-1.1	-7.5	Hypothetical protein
PA14_13660	PA3885		5.7	1.5	-1.9	Hypothetical protein
PA14_11150	PA4077		7.5	4.9	-1.3	Probable transcriptional regulator
PA14_09660	PA4198		16	1.1	-13.9	Probable AMP-binding enzyme
PA14_67190	PA5088		7.5	2	-3	Hypothetical protein
PA14_67860	PA5139		4.9	-2.3	-10.6	Hypothetical protein
PA14_71890	PA5445		4.9	-1.1	-7.5	Probable coenzyme A transferase
PA14_72180	PA5469		7	-1.6	-10.6	Conserved hypothetical protein
PA14_72650	PA5506		6.5	1.9	-4	Hypothetical protein
PA14_72700	PA5509		6.1	1.1	-5.3	Hypothetical protein
PA14_72960	PA5530		4.9	1.1	-4.6	Probable MFS dicarboxylate transporter

monooxygenase for the last step of PQS synthesis) -2.6-fold. In addition, many virulence factors were repressed upon inhibition of UMP synthesis as seen by repression of the genes for phenazine synthesis, chemotaxis, alkaline proteases, type II secretion, and type IV pilus formation (Table 6.2).

To corroborate these microarray results and to show they are directly related to uracil, we analyzed the changes in differential gene expression of the whole transcriptome upon addition of uracil to the *pyrF* cells in biofilms. As expected, uracil addition induced expression of nearly all of the genes repressed by the *pyrF* mutation (Table 6.2); therefore, the changes in the QS pathways are caused by uracil, since extracellular uracil restored transcription of 252 out of 298 genes repressed by *pyrF* mutation.

To explore the effect of uracil further, we also added 10 mM uracil to the wild-type strain in LB medium and studied the whole transcriptome response of biofilm cells. As expected, uracil treatment induced the genes for uracil catabolism (8- to 74-fold increases for PA0439-PA0444), and *lasI* was induced by 2-fold (the *pyrF* mutation repressed *lasAB*). Interestingly, 10 mM uracil repressed genes related to iron acquisition (-2.1 to -2.3-fold for the two component system *pfeRS*, -13-fold for PA2426 *pvdS* which encodes sigma factor PvdS, -2.3-fold for the siderophore receptor PA0931, and -1.3 to -4.3-fold for pyochelin synthesis genes PA4224-PA4231) (Table 6.3). It is not clear in detail how uracil regulates expression of these genes, but uracil may enhance iron uptake, and then the increased iron concentrations repress these iron-related genes including virulence factors which are regulated by iron (Ochsner *et al.*, 2002b).

Table 6.3. List of the repressed genes related to iron acquisition in biofilm cells of PA14 wild-type in LB medium after 7 h in the presence of 10 mM uracil.

PA14 #	PAO1 #	Gene	Fold change	Description
Two-component system				
PA14_29375	PA2686	*pfeR*	-2.1	Two-component response regulator PfeR
PA14_29360	PA2687	*pfeS*	-2.3	Two-component sensor PfeS
Pyoverdine synthesis				
PA14_33260	PA2426	*pvdS*	-13.0	Sigma factor PvdS
Pyochelin synthesis and transport				
PA14_09380	PA4218	*fptX*	-2.8	Probable transporter
PA14_09370	PA4219	*fptC*	-3.7	Hypothetical protein
PA14_09350	PA4220	*fptB*	-3.5	Hypothetical protein
PA14_09340	PA4221	*fptA*	-2.1	Fe(III)-pyochelin receptor precursor
PA14_09320	PA4222	*pchI*	-2.5	Probable ATP-binding component of ABC transporter
PA14_09300	PA4223	*pchH*	-2.5	Probable ATP-binding component of ABC transporter
PA14_09290	PA4224	*pchG*	-3.0	Pyochelin biosynthetic protein
PA14_09280	PA4225	*pchF*	-4.3	Pyochelin synthetase
PA14_09270	PA4226	*pchE*	-2.8	Dihydroaeruginoic acid synthetase
PA14_09240	PA4228	*pchD*	-2.3	Pyochelin biosynthesis protein PchD
PA14_09230	PA4229	*pchC*	-2.6	Pyochelin biosynthetic protein PchC
PA14_09210	PA4231	*pchA*	-2.1	Salicylate biosynthesis isochorismate synthase
Heme uptake and utilization				
PA14_55580	PA0672	*hemO*	-3.0	Heme oxygenase
PA14_62300	PA4708	*phuT*	-3.0	Hypothetical protein
PA14_62330	PA4709	*phuS*	-2.5	Probable hemin degrading factor
PA14_62350	PA4710	*phuR*	-4.0	Probable outer membrane hemin receptor
Other iron-regulated genes				
PA14_55300	PA0697		-2.0	Hypothetical protein
PA14_47400	PA1300		-3.5	Probable sigma-70 factor
PA14_38220	PA2033		-2.3	Hypothetical protein
PA14_18680	PA3530	*bfd*	-4.0	Hypothetical protein
PA14_58000	PA4468	*sodM*	-3.7	Superoxide dismutase
PA14_58010	PA4469		-6.1	Hypothetical protein
PA14_58030	PA4470	*fumC1*	-6.1	Fumarate hydratase
PA14_58040	PA4471	*fagA*	-5.7	Hypothetical protein

Table 6.3. (continued)

PA14 #	PAO1 #	Gene	Fold change	Description
PA14_60480	PA4570		-3.0	Hypothetical protein
PA14_72970	PA5531	*tonB*	-2.3	TonB protein
PA14_52230	PA0931	*pirA*	-2.3	Ferric enterobactin receptor PirA
PA14_58570	PA4514		-9.8	Probable outer membrane receptor for iron transport
PA14_56680	PA4358		-2.3	probable ferrous iron transport protein
PA14_56690	PA4359		-2.1	Fe(II) transport system protein A

6.3.4 UMP synthesis increases QS phenotypes

The DNA microarray results suggested uracil influences genes related to QS for all three known QS systems in *P. aeruginosa*. Since QS systems are regulated by cell growth, we investigated the growth of the *pyrF* mutant in defined M9 glucose medium with exogenous uracil. The *pyrF* mutant grew well in minimal medium with 0.1, 1, or 10 mM uracil supplement, but not with 0.01 mM uracil (Fig. 6.3). The specific growth rate was 0.51 ± 0.05 h^{-1} for wild-type PA14, and 0.49 ± 0.01 h^{-1}, 0.47 ± 0.02 h^{-1}, and 0.45 ± 0.04 h^{-1} for the *pyrF* mutant with 0.1, 1, and 10 mM uracil, respectively. Therefore, 0.1 mM uracil in M9 glucose medium restores normal growth, and higher concentrations of uracil are not utilized as a carbon, nitrogen, or energy source. Hence, we tested the QS assays with uracil concentrations greater than 0.1 mM.

Since LasB (elastase) is regulated by LasR (Wagner *et al.*, 2004), we assayed elastase activity as an indicator of the Las QS system and found elastase activity increased 1.9- to 3.9-fold in the *pyrF* mutant compared to the wild-type strain upon adding uracil (Fig. 6.5A). We also examined three RhlR-regulated phenotypes, pyocyanin production (Wagner *et al.*, 2004), rhamnolipid production (Wagner *et al.*, 2004), and swarming motility (Déziel *et al.*, 2003). As expected, poor pyocyanin production was found in the *phzM* mutant (PhzM is one of the key enzymes for pyocyanin production), and addition of uracil to the *pyrF* mutant (1 mM and 10 mM) enhanced pyocyanin production by 2.2-fold and 3.8-fold, respectively. Rhamnolipid production also increased by 1.3-fold with additional uracil (Fig. 6.5A). Swarming motility was abolished in the *pyrF* mutant with 0.1 mM uracil but 1 mM uracil addition restored it (Fig. 6.5A). We also quantified PQS production because *pqsH* was repressed in the *pyrF* mutant (Table 6.2) and found PQS production by the *pyrF* mutant doubled as uracil concentrations increased from 0.1 to 1.0 mM uracil (Fig. 6.5A). Therefore, additional uracil in minimal medium increased consistently elastase activity, pyocyanin production, rhamnolipid production

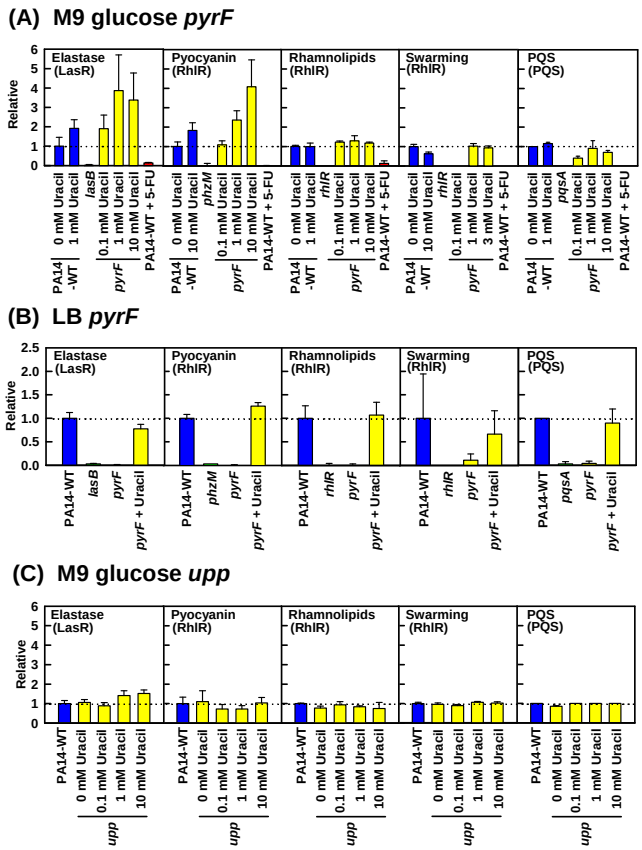

Fig. 6.5. Uracil and 5-fluoruracil control QS phenotypes. (A) Effect of uracil addition (0.1 to 10 mM) on wild-type *P. aeruginosa* PA14 and the *pyrF* mutant and of addition of 10 µM 5-fluorouracil to the wild-type strain in M9 glucose medium for LasB elastase activity, pyocyanin production, rhamnolipid production, swarming motility, and PQS production. Negative controls were *lasB* (for elastase), *phzM* (for pyocyanin), *rhlR* (rhamnolipid and swarming), and *pqsA* (PQS). Data show the average of two independent experiments ± s.d. Wild-type values were 0.03±0.01, elastase absorbance at 495 nm/cell growth, 0.011±0.002 pyocyanin absorbance at 520 nm/cell growth, 53±2 µg rhamnolipid/mL, and 4.6±0.4 cm for swarming. PQS production was quantified and compared to that of the wild-type strain and standard. (B) Effect of the *pyrF* mutation in LB medium and of adding 1 mM uracil to the *pyrF* mutant on LasB elastase activity, pyocyanin production, rhamnolipid production, swarming motility, and PQS production. Data show the average of two independent experiments ± s.d. Wild-type values were 0.12±0.01 elastase absorbance at 495 nm/cell growth, 0.017±0.001 pyocyanin absorbance at 520 nm/cell growth, 25±5 µg rhamnolipid/mL, and 0.9±0.4 cm for swarming. PQS production was quantified and compared to that of the wild-type strain and standard. (C) Effect of uracil addition (0.1 to 10 mM) on elastase activity, pyocyanin production, rhamnolipid production, swarming motility, and PQS production with the *P. aeruginosa upp* mutant in M9 glucose medium. Data show the average of two independent experiments ± s.d. Wild-type values were 0.069±0.008 elastase absorbance at 495 nm/cell growth, 0.022±0.005 pyocyanin absorbance at 520 nm/cell growth, 56±1 µg rhamnolipid/mL, and 4.4±1.1 cm for swarming. PQS production was quantified and compared to that of the wild-type strain and a purified standard.

(slightly), swarming, and PQS production in the *pyrF* mutant.

We also examined the effect of uracil addition to wild-type PA14 in M9 glucose medium (Fig. 6.5A). Elastase activity increased 1.9-fold with 1 mM uracil, and pyocyanin increased by 1.8-fold with 10 mM uracil. However, rhamnolipid and PQS synthesis were not significantly changed with uracil addition, and swarming decreased with 10 mM uracil.

In LB medium, elastase activity, pyocyanin production, rhamnolipid production, swarming, and PQS production were all abolished in the *pyrF* mutant and were all restored by uracil addition to the *pyrF* mutant (Fig. 6.5B). Therefore, results in both media show conclusively that UMP synthesis and extracellular uracil influence all five QS phenotypes and that the changes in QS phenotypes are not related to growth effects.

6.3.5 Upp is required to affect QS

P. aeruginosa PA14 possesses uracil phosphoribosyltansferase (Upp) that participates in the uracil salvage pathway (Andersen *et al.*, 1992). Upp catalyzes uracil to UMP, hence the *upp* mutant is not able to utilize uracil for salvage and UMP synthesis. We hypothesized that if exogenous uracil influences QS phenotypes in the *upp* mutant, uracil itself impacts the regulation of QS or uracil may be utilized as a carbon/nitrogen source. If exogenous uracil does not influence QS phenotypes in the *upp* mutant, then an unidentified nucleotide derived from uracil may influence QS. We tested our hypothesis by performing QS assays with the *upp* mutant in presence of uracil (Fig. 6.5C). Although exogenous uracil increased elastase activity by 1.5-fold, this increase is not significant in comparison with the results of the *pyrF* mutant (Fig. 6.5A). Exogenous uracil (0.1 to 10 mM) also did not increase the other QS phenotypes (Fig. 6.5C). Note that exogenous uracil did not affect the growth of the *upp* mutant in M9 glucose medium (Fig. 6.3C); hence, again, uracil did not serve as a carbon, nitrogen, or energy source. These

results suggest uracil utilization by Upp is necessary to influence the QS phenotypes.

6.3.6 UMP synthesis increases virulence

Since genes related to seven virulence factors (*lasA, lasB, rhlRAB, phzABCDEFGMS, chiC, aprADEFI,* and *tadZABCDG-rcpAC-flp*) were repressed in the *pyrF* mutant, we compared the pathogenicity of wild-type PA14 and the *pyrF* mutant using our barley germination assay (Attila *et al.*, 2008a); previously, we had used this assay to corroborate virulence factors we identified in the poplar tree rhizosphere. The *pyrF* mutation increased barley germination by 1.8 ± 0.7-fold compared to wild-type PA14; hence, it reduced virulence. Furthermore, addition of 1 mM uracil to the *pyrF* mutant restored pathogenicity to wild-type levels, and addition of 1 mM uracil made the wild-type strain 2.0 ± 1.6-fold more virulent. These results show uracil synthesis and extracellular uracil influence virulence.

Since extracellular uracil was clearly transported into cells as evidenced by the change in QS phenotypes, virulence, and whole-genome transcription upon uracil addition, we checked to see if uracil was exported by *P. aeruginosa* PA14 cells and thereby was perhaps functioning as an extracellular signal. Using minimal medium to reduce the complexity of extracellular components and to avoid uracil in LB medium, we found that over a 24 h period, extracellular uracil increased 13-fold to 3.8 μM (similar results were obtained with *P. aeruginosa* PAO1); however, the small concentration implies uracil works intracellularly (not as a QS signal), and these small amounts may be due to cell lysis.

6.3.7 5-FU inhibits biofilm formation and QS phenotypes

Inhibition of biofilm formation is important since biofilms cause persistent infections that are responsible for many human diseases related to bacteria; therefore, discovering novel

biofilm inhibitors is valuable. As shown in Fig. 6.2B, uracil regulates biofilm formation for both the *pyrF* mutant and wild-type PA14; hence, we screened uracil analogs for biofilm inhibition. Among the six uracil structural analogs tested, we identified 5-FU as an effective biofilm inhibitor (Fig. 6.6A). In LB medium, 5-FU (25 μM) decreased PA14 biofilm formation 3-fold with a 20% reduction in the specific growth rate, and 200 μM 5-FU inhibited biofilm formation 33-fold with a 50% reduction in the specific growth rate (Fig. 6.6B). In M9 glucose medium, we found that 10 μM 5-FU inhibited biofilm formation by 56% (Fig. 6.6C) without affecting growth (in planktonic cultures, final turbidity at 620 nm was 0.132 ± 0.005 without 5-FU and 0.133 ± 0.001 with 5-FU). At higher concentrations, 25 μM 5-FU, biofilm formation was inhibited by 61%, but growth was also inhibited (final turbidity at 620 nm was 0.107 ± 0.001).

Additionally, we found 5-FU inhibits QS-regulated virulence factors for wild-type *P. aeruginosa* PA14 (Fig. 6.5A). In M9 glucose medium, all five QS phenotypes were nearly abolished: 5-FU (10 μM) repressed significantly elastase activity (86%) and the RhlR-regulated phenotypes of pyocyanin production (100%), rhamnolipid production (87%), and swarming (100%) as well as abolished PQS production. In addition, 5-FU decreased wild-type PA14 pathogenicity for barley (1.8 ± 1.2-fold more germination at 25 μM 5-FU). Hence, 5-FU is an effective biofilm inhibitor that works by inhibiting QS phenotypes.

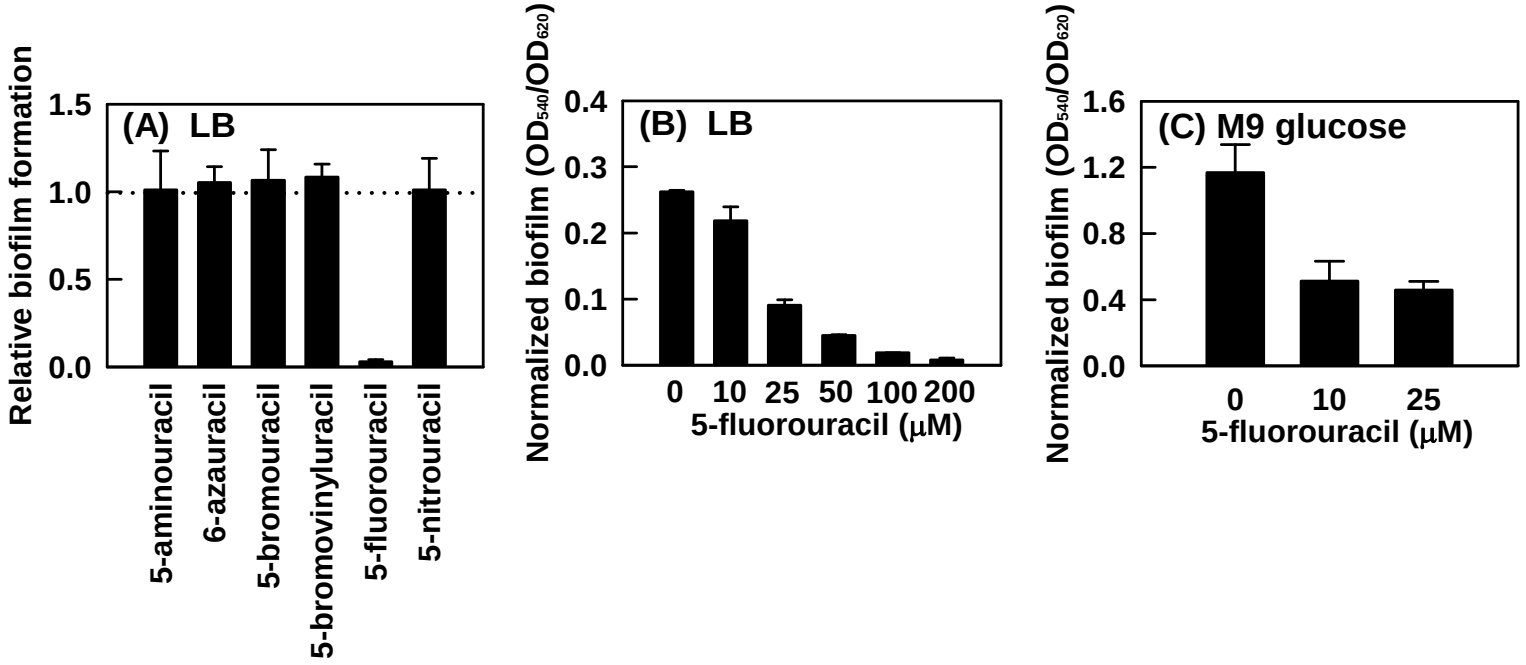

Fig. 6.6. 5-fluorouracil inhibits biofilm formation. Biofilm formation (OD_{540}) was normalized by cell growth (OD_{620}). (A) Biofilm formation of wild-type *P. aeruginosa* PA14 upon addition of the uracil analogs 5-aminouracil, 6-azauracil, 5-bromouracil, 5-bromovinyluracil, 5-fluorouracil, and 5-nitrouracil in LB medium. Each analog was tested at 200 μM. Relative biofilm formation was calculated as the ratio of uracil analog treatment to no treatment. (B) Biofilm formation of wild-type *P. aeruginosa* upon addition of 5-fluorouracil in LB medium. Biofilm formation was examined after 24 h, and 10 wells were used for each culture. Data show the average of the two independent experiments ± s.d. (C) Biofilm formation of wild-type *P. aeruginosa* upon addition of 5-fluorouracil in M9 glucose medium. Biofilm formation was examined after 24 h, and 10 wells were used for each culture. Data show the average of the two independent experiments ± s.d.

6.4 Discussion

By carefully cataloging the genes related to biofilm formation, we discovered here that uracil influences all three known QS pathways of the pathogen *P. aeruginosa*; hence, uracil is important for QS since in order to influence all three QS pathways it must be upstream of all three regulatory circuits. The lines of evidence showing the influence of uracil on QS are (i) disruption of UMP synthesis via the *pyrF* mutation represses transcription of hundreds of QS genes (Table 6.2), (ii) addition of extracellular uracil to the *pyrF* mutant restores transcription of these QS genes to wild-type levels (Table 6.2), (iii) addition of uracil to the wild-type strain increases some QS phenotypes (Fig. 6.5A), (iv) the *pyrF* mutation abolishes at least five QS phenotypes including those regulated by LasR (elastase), RhlR (pyocyanin, rhamnolipid, and swarming), and PQS (PQS production), and the addition of uracil restores these phenotypes (Fig. 6.5), (v) the disruption of UMP synthesis by seven independent mutations abolishes biofilm formation (Fig. 6.2A), and the addition of extracellular uracil restores biofilm formation (Fig.6. 2B), (vi) mutations in purine synthesis do not affect biofilm formation, and (vii) the *pyrF* mutation reduces virulence as shown by barley germination, and addition of uracil accentuates virulence.

Due to its importance, it is expected that others should have seen uracil-related genes in whole-transcriptome QS and biofilm studies. As expected, altered *carAB* and *pyrBCDEF* expression was observed in *P. aeruginosa* under various conditions; for example, *pyrQ* (a homolog of *pyrC*) was one of the most induced genes (45-fold) in cystic fibrosis sputum medium vs. synthetic medium (Palmer *et al.*, 2007), and this gene was induced 40-fold in sputum compared to minimal glucose medium (Palmer *et al.*, 2005). Similarly, *carAB* and *pyrF* are induced 4-fold in more pathogenic *P. aeruginosa* strains (Chugani and Greenberg, 2007). These results corroborate our barley germination results with the *pyrF* mutant by confirming that

synthesis of uracil is important for pathogenesis and show that uracil-related genes are in the literature.

Metabolites are often identified as signaling molecules; for example, polyamines regulate swarming and biofilm formation, and rhamnolipids regulate swarming and biofilm structure (Monds and O'Toole, 2007). Indole from tryptophan is a cell signal for *E. coli,* and it regulates biofilm formation in a different manner for *E. coli* and pseudomonads (Lee *et al.*, 2007b). In this research, we show uracil enhances biofilm formation and QS-regulated virulence factors (elastase activity, pyocyanin production, and rhamnolipids production) (Figs. 6.2 and 6.5) without growth inhibition (Fig. 6.3). These findings support that uracil may serve as a regulator for these phenotypes.

Iron availability is mediated by pyoverdine which regulates swarming for *P. putida* (Matilla *et al.*, 2007). In the *pyrF* mutant, *pvdS,* which encodes a sigma factor for iron acquisition, was highly repressed; hence, one possible mechanism is that swarming of the *pyrF* mutant is regulated by iron availability via uracil control of *pvdS* expression. Further evidence of this link between iron and uracil was seen upon adding uracil to wild-type biofilm cells since many iron-related genes were differentially expressed (Table 6.3).

The QS-regulated transcriptome was examined previously with the mutant *P. aeruginosa* PAO-JP2, which lacks both *lasI* and *rhlI* (Wagner *et al.*, 2003). Addition of autoinducers induced a variety of the virulence factors, such as alkaline proteases (*aprADI*), phenazine biosynthesis (*phzACDEFGMS*), chitinase (*cpbD*), LasAB, RhlRAB, MexGHI-OpmD, and type II secretion (*tadACD-rcpAC-flp*). We found most of these genes are repressed in the *pyrF* mutant, and that uracil restores gene expression of these loci. This supports that uracil regulates many of the virulence factors via QS.

Since uracil is a component for mRNA, one possible mechanism for global gene

expression regulation by uracil is its influence on the transcription of AU-rich genes. Therefore, we examined the GC content of the top 10 induced and repressed genes in the *pyrF* vs. wild-type microarray data (Table 6.2). The average GC content is 64.9% and 66.9% for the top 10 induced and repressed genes, respectively. These values are not significantly different than the GC content of whole PAO1 genome, 66.6% (Stover *et al.*, 2000). Therefore, uracil regulates gene expression independently of their GC content.

The impact of uracil on QS may be general in that all cells have uracil, and we have found that the main set of genes (e.g., *carAB, pyrBCDFIL,* and *uraA*) induced in *E. coli* by the species-independent signal autoinducer-2 (Camilli and Bassler, 2006) are related to UMP biosynthesis and uracil transport (Lee *et al.*, 2008). In addition, we have also found the cell signal indole (Lee *et al.*, 2007a) represses this same set of genes in *E. coli* more than any others (e.g., *carAB, pyrBCDIL,* and *uraA*) (Lee *et al.*, 2008). Hence, both known *E. coli* signals function through uracil synthesis and uracil-, AI-2-, and indole-based signaling are intertwined.

It is logical that procaryotic cells should use uracil in that they make use of three other RNA-based intracellular signals, cAMP, cGMP, and c-diGMP. Our results show uracil addition to the *upp* mutant is not able to enhance QS phenotypes (Fig. 6.5C), although uracil addition to the *pyrF* mutant enhanced all five QS phenotypes (Fig. 6.5A and 6.5B). This suggests that the QS phenotypes are regulated in *P. aeruginosa* when uracil is utilized for pyrimidine nucleotide metabolism. In eucaryotes, UTP is an extracellular signal for exciting sympathetic neurons, for muscle cell proliferation, and for endothelial cell adhesion (Lazarowski and Boucher, 2001)UTP release is stimulated in both neural and non-neural cells by mechanical stress (Lazarowski and Boucher, 2001).

Since we discovered that uracil influences QS in *P. aeruginosa*, we hypothesized that well-known human anti-cancer drugs may be used to control *P. aeruginosa* pathogenicity. This

realization is important in that this class of drugs is already screened for human toxicity and may be used rapidly in trials for diseases such as cystic fibrosis. Specifically, we showed 5-FU inhibits biofilm formation of *P. aeruginosa* (Fig. 6.6), and is non-toxic to *P. aeruginosa*, and this compound is already approved for treatment of human colon cancer (Wiebke *et al.*, 2003); so it is relatively non-toxic to humans. Our results corroborate a previous report (Hussain *et al.*, 1992) that showed 5-FU inhibits the biofilm formation of *Staphylococcus epidermidis* and suggest those original surprising results may be related to uracil.

Therefore, for *P. aeruginosa,* 5-FU is one of the few known biofilm-inhibiting compounds that is non-toxic such as brominated furanones (Ren *et al.*, 2001), ursolic acid (Ren *et al.*, 2005a), and indole derivatives (Lee *et al.*, 2007b). Furthermore, 5-FU is one of the few known anti-virulence compounds (Cegelski *et al.*, 2008); anti-virulence compounds are an important way to fight infectious diseases because unlike antimicrobials, anti-virulence compounds like 5-FU do not affect growth and so there is less chance of developing resistance (Hentzer *et al.*, 2002).

6.5 Experimental procedures

6.5.1 Bacterial strains

P. aeruginosa PA14 and the isogenic transposon-insertion mutants were obtained from Dr. Frederick Ausubel (Liberati *et al.*, 2006) and are listed in Table 6.4. Strains were routinely pre-cultured in Luria Bertani (LB) medium (Sambrook *et al.*, 1989) or M9 minimal medium (Ausubel *et al.*, 1998) with 0.2% glucose as a carbon source for PA14 or LB with 15 μg/mL gentamicin for the isogenic mutants. All experiments were conducted at 37°C. Cell growth was

Table 6.4. Strains used in this study. PA14 wild-type and all of the isogenic mutants were described by Liberati *et al.* (2006)

Strain	Genotype
P. aeruginosa PA14	Wild type strain
P. aeruginosa PA14_62930 (*carA*)	PA14_62930 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_62910 (*carB*)	PA14_62910 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_05260 (*pyrB*)	PA14_05260 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_05250 (*pyrC*)	PA14_05250 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_24640 (*pyrD*)	PA14_24640 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_70370 (*pyrE*)	PA14_70370 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_26890 (*pyrF*)	PA14_26890 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_16250 (*lasI*)	PA14_45940 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_16250 (*lasB*)	PA14_16250 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_09490 (*phzM*)	PA14_09490 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_19120 (*rhlR*)	PA14_19120 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_51430 *(pqsA)*	PA14_51430 Ω Mar2xT7 GmR
P. aeruginosa PA14_51420 *(pqsB)*	PA14_51420 Ω Mar2xT7 GmR
P. aeruginosa PA14_23990 (*xcpR*)	PA14_23990 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_12330 (*phoH*)	PA14_12330 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_09300 (*PA4223*)	PA14_09300 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_60860 (*nfxB*)	PA14_60860 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_65320 (*miaA*)	PA14_65320 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_68370 (*cysQ*)	PA14_68370 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_68680 (*envZ*)	PA14_68680 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_51240 (*purC*)	PA14_51240 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_71620 (*purE*)	PA14_71620 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_64200 (*purH*)	PA14_64200 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_01760 (*PA0143*)	PA14_01760 Ω Mar2xT7 Gm^R
P. aeruginosa PA14_61470 (*upp*)	PA14_61470 Ω Mar2xT7 Gm^R

measured using turbidity at 620 nm for the biofilm assay with 96-well plates or 600 nm for all other experiments. Conformation of the transposon insertion for the *pyrF* mutant was performed as described previously (Ueda and Wood, 2008). Gene specific primers were designed as PA14_26890-VF (5'-GGGTGAAGGTCGGCAAGGAACTCTT-3') and PA14_26890-VR (5'-GGAGAATCTCATCGACCGCCTTCAG-3') to amplify wild-type *pyrF* gene. Using chromosomal DNA from PA14 wild-type, 899 bp of the *pyrF* gene was amplified; this band was not amplified for the *pyrF* mutant. A DNA fragment corresponding to the end of the MAR2xT7 transposon and upstream flanking *pyrF* was amplified using the transposon specific primer GB3a (5'-TACAGTTTACGAACCGAACAGGC-3') and chromosomal primer PA14_26890-VF and using transposon specific primer R1 (5'-ATCGACCCAAGTACCGCCAC-3') and downstream chromosomal primer PA14_26890-VR with chromosomal DNA from the *pyrF* mutant; these two bands were not amplified for the PA14 wild-type.

6.5.2 Biofilm assay

Comprehensive screening for altered biofilm mutants was carried out with the 5,850 clones of the PA14 non-redundant mutant library (Liberati *et al.*, 2006). The initial screen of biofilm formation was examined in LB medium using 96-well polystyrene plates and crystal violet staining (Lee *et al.*, 2007a) using one well for each strain. Mutants with biofilm formation altered over 3-fold (inhibited or stimulated) were re-screened with ten replicate wells and two independent cultures. Biofilm formation was normalized by planktonic cell growth to take into account changes in growth and is shown as normalized biofilm (OD_{540}/OD_{620}).

6.5.3 Biofilm inhibition with uracil analogs

Uracil analogs 5-aminouracil, 6-azauracil, 5-bromouracil, 5-fluorouracil (Fisher

Scientific, Hanover Park, IL), 5-(*trans*-2-bromovinyl)-uracil (Sigma-Aldrich, St. Louis, MO), and 5-nitrouracil (MP Biomedical, Solon, OH) were tested as biofilm inhibitors in LB medium at 200 μM (10 to 200 μM for 5-FU). 5-FU was also tested for biofilm inhibition with M9 glucose medium at 10 μM. *P. aeruginosa* PA14 wild-type was grown in LB medium or M9 glucose medium overnight, overnight cultures were diluted to a turbidity at 600 nm of 0.05 with LB medium or M9 glucose medium containing an uracil analog, and biofilm formation in 96-well polystyrene plates was examined after 24 h. Ten wells were used for each condition, and two independent cultures were tested.

6.5.4 QS assays

LasB elastase activity was measured with cells grown to a turbidity at 600 nm of 2 as described previously based on spectrophotometric determination of the amount of elastin-congo red reaction (Ohman *et al.*, 1980). Elastin-congo red was purchased from Sigma-Aldrich, and the *lasB* mutant was used as a negative control. Pyocyanin production was measured as described previously based on spectrophotometric determination after extraction with chloroform and 0.2 N HCl (Essar *et al.*, 1990); the *phzM* mutant was used as a negative control. Rhamnolipids were quantified spectrophotometrically after diethylether extraction and sulfur acid/orcinol addition (Wilhelm *et al.*, 2007); rhamnose was used as a standard (Fisher Scientific), and the *rhlR* mutant was used as a negative control. Swarming motility (translocation on top of agar plates) was examined with BM-2 plates (62 mM potassium phosphate, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.1% casamino acid, 0.4% glucose, 0.5% Bacto agar) (Overhage *et al.*, 2008) with cells grown to a turbidity of 1 at 600 nm (Morohoshi *et al.*, 2007) after 24 h; five plates were tested for each experiment and two independent cultures were used, and the *rhlR* mutant was used as a negative control. PQS was extracted and quantified by thin

layer chromatography (Gallagher *et al.*, 2002) with the modifications of glass silica gel F_{254} pre-coated plates (10 x 20 cm, VWR, West Chester, PA) and a 95:5 mixture of dichloromethane:methanol. The plate image was quantified by a Bio-Rad VersaDoc 3000 imaging system (Bio-RAD, Hercules, CA), and PQS (500 ng) was used as a positive control (Syntech Solution, San Diego, CA).

6.5.5 Barley virulence assay

Pathogenicity of *P. aeruginosa* was tested by a barley germination assay (Attila *et al.*, 2008a). Barley seeds (cultivar Belford, Stover Seed Company, Los Angeles, CA) were surface-sterilized in 1% sodium hypochlorite for 30 min. After washing the seeds with sterile water ten times, seeds were germinated in absence or presence of bacteria. Germinated seeds were counted after 3 days.

6.5.6 Whole-transcriptome analysis

The *P. aeruginosa* genome array (Affymetrix, P/N 510596) was used to investigate differential gene expression in biofilms for PA14 vs. the *pyrF* mutant, and in biofilms for the *pyrF* mutant vs. the *pyrF* mutant with 1 mM uracil as described previously (Attila *et al.*, 2008a). Biofilm cells were harvested from 10 g of glass wool after incubation for 7 h in LB with shaking at 250 rpm, and RNA was extracted with a RNeasy Mini Kit (Qiagen, Valencia, CA). Global scaling was applied so the average signal intensity was 500. The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA controls were used to monitor the labeling process. For each binary microarray comparison of differential genes expression, if the gene with the larger transcription rate did not have a

consistent transcription rate based on the 13 probe pairs (*p*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *p*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher than the standard deviation for the whole microarrays, (2.74 for the *pyrF* mutant vs. wild-type PA14, 1.73 for the *pyrF* mutant with uracil vs. wild-type PA14, and 3.15 for the *pyrF* mutant vs. the *pyrF* mutant with uracil) (Ren *et al.*, 2004a). The microarray raw data are deposited at the Gene Expression Omnibus (GSE9592) of the National Center for Biotechnology Information.

6.5.7 Extracellular uracil

Uracil was quantified as a function of cell density in M9 minimal medium supplemented with 0.2% glucose and 0.2% sodium succinate using a reverse-phase high-pressure liquid chromatograph (Waters 515 with photodiode array detector, Milford, MA) with a Nova-Pak® C18 column (Waters, 150 x 3.9 mm, 4 μm) and gradient elution with 100 mM ammonium acetate (pH 5.0) and acetonitrile as the mobile phases at a flow rate of 1 ml min^{-1} (100:0 at 0 min, 94:6 at 8 min, and 100:0 at 10 min). Under these conditions, the retention time for uracil was 1.75 min, and the absorbance maximum was 259 nm (uracil standard was used to verify peaks by co-elution).

CHAPTER VII

5-FLUOROURACIL REPRESSES BIOFILM FORMATION IN *ESCHERICHIA COLI* K-12 THROUGH AriR

7.1 Overview

The uracil analog, 5-fluorouracil (5-FU) represses virulence factors and biofilm formation of *Pseudomonas aeruginosa* PA14 without affecting its growth. As 5-FU is an approved anticancer drug, its anti-virulence attributes in *P. aeruginosa* prompted us to examine the effect of this compound on three different *Escherichia coli* K-12 strains and its effect on virulence genes in *E. coli* O157:H7 (EHEC); the mechanism by which it functions was also examined. 5-FU decreased biofilm formation in a dose-dependent manner in *E. coli* K-12 and repressed the expression of virulence genes in EHEC. Five other uracil analogs were also tested for their effects on biofilm formation, and none of these compounds affected the biofilm formation in *E. coli* K-12. Whole-transcriptome analysis revealed that 5-FU induced the expression of 157 genes and repressed the expression of 19 genes. Biofilm formation with the addition of 5-FU was checked in 21 isogenic knock-out mutants whose gene expression was induced in the microarray data; we found 5-FU does not decrease biofilm formation of the cells that lack AriR, a global DNA regulator that controls acid resistance in *E. coli*. Hence, 5-FU represses biofilm formation of *E. coli* K-12 through AriR and is a novel anti-virulence compound for this strain.

7.2 Introduction

Bacteria prefer to live in the biofilm state (Sauer et al. 2004). Biofilms acquire higher resistance to surfactants, phagocytes, and antibiotics (Costerton et al. 1995). The slow penetration of antibiotics into the biofilms, altered chemical microenvironment within the biofilm, and the different phenotypic state of bacteria in the biofilm state render biofilms antibiotic resistant (Stewart and Costerton 2001). Furthermore, the biofilm matrix protects cells from penetration (Costerton et al. 1999) and diffusion (Donlan and Costerton 2002) of antimicrobial molecules. Biofilms cause serious problems in medicine and in industry (Donlan and Costerton 2002). In the US, the annual cost of biofilms in cardiovascular and orthopedic implants is more than $3 billion (Klemm et al. 2007). Therefore, biofilm prevention and control is gaining importance (Labbate et al. 2004).

Thus far, only a few compounds have been discovered to combat *E. coli* biofilms without affecting growth. Furanone, from the marine alga *Delisea pulchra* (Ren et al. 2001), indole (Lee et al. 2007b), and indole derivatives (5-hydroxyindole and 7-hydroxyindole) (Lee et al. 2007a) repress biofilm formation of *E. coli*. By screening 13,000 plant extracts, ursolic acid was also discovered to inhibit *E. coli* biofilm formation (Ren et al. 2005a). Compounds that attenuate virulence factors without affecting growth are better than the antimicrobials since cells are less likely to develop resistance against them (Hentzer et al. 2002).

In *E. coli*, uracil-related genes, the genes involved in uridine monophosphate (UMP) biosynthesis and uracil transport (*carAB*, *pyrLBI*, *pyrC*, *pyrD*, *pyrF*, and *uraA*), are repressed by the addition of indole and the presence of SdiA at 30°C and are induced by the addition of AI-2 at 37°C (Lee et al. 2008). Also, in *E. coli*, SdiA- and AI-2-based signaling are intertwined (Lee et al. 2008). Furthermore, by screening 5,850 transposon mutants for altered biofilm formation of *P. aeruginosa* PA14, the same uracil-related mutations (*carA*, *carB*, *pyrB*, *pyrC*, *pyrD, pyrE*,

and *pyrF*) abolish biofilm formation and quorum sensing phenotypes (elastase activity, pyocyanin production, PQS production, rhamnolipid production, and swarming motility), whereas the mutations for other pyrimidines and purine biosynthesis did not alter biofilm formation (Ueda et al. 2008). Hence, uracil or a uracil derivative appears to affect quorum sensing in both *E. coli* and *P. aeruginosa*.

These results prompted us to examine uracil analogs as biofilm effectors. By screening uracil analogs for biofilm formation of *P. aeruginosa*, 5-FU was discovered as a biofilm inhibitor of *P. aeruginosa* (Ueda et al. 2008). 5-FU is also a biofilm inhibitor of *Staphylococcus epidermis* (Hussain et al. 1992). Effective biofilm inhibitors could benefit many populations by impacting treatments for various diseases (Cegelski et al. 2008).

In this study, we screened uracil analogs to observe their effects on the biofilm formation of *E. coli* K-12 and found that 5-FU also inhibits the biofilm formation of *E. coli* K-12. We also investigated the genetic basis for the effects of 5-FU by looking at the whole transcriptome. Our biofilm and microarray results suggest that 5-FU works through AriR. AriR, previously known as YmgB (Lee et al. 2007c), is a regulator that inhibits biofilm formation and motility and protects cells from acid (Lee et al. 2007c).

7.3 Results

7.3.1 5-fluorouracil inhibits *E. coli* biofilm formation

As uracil influences virulence and biofilm formation in *P. aeruginosa* (Ueda et al. 2008), we investigated the effects of uracil analogs on the biofilm formation of *E. coli* K-12 ATCC 25404. This strain was chosen because it is a wild-type strain. Of the examined uracil analogs, only 5-FU reduced the biofilm formation of *E. coli* K-12 ATCC 25404 (Fig. 7.1). We also

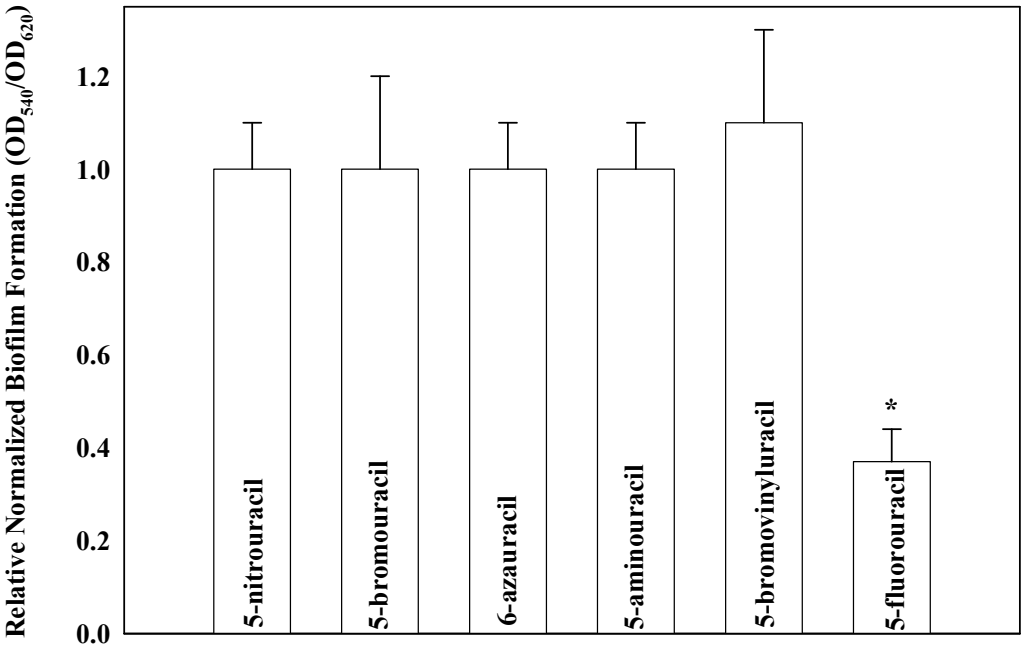

Fig. 7.1. **Effect of uracil derivatives at 10 µM (5-nitrouracil, 5-bromouracil, 6-azauracil, 5-aminouracil, 5-bromovinyluracil, and 5-fluorouracil) on the biofilm formation of *E. coli* K-12 ATCC 25404 WT strain at 37°C after 7 h in 96-well plates in LB medium.** At least two independent experiments were conducted (total of 12 wells), and error bars indicate one standard deviation. Asterisks indicate significant differences as estimated by Student's t-test ($p < 0.05$).

examined the effects of 5-FU (0 to 50 μM) on the biofilm formation of three different *E. coli* K-12 strains to ensure the effect of 5-FU was general (ATCC 25404, BW25113, and MG1655). In all three strains, 5-FU reduced the biofilm formation in a dose-dependent manner in LB medium (Fig. 7.2). In order to circumvent any growth effect, all the biofilm formation results were normalized by the growth rate. In all three strains, biofilm formation was decreased by around 5-fold with the addition of 25 μM of 5-FU.

To determine the toxicity of 5-FU, *E. coli* K-12 ATCC 25404 was used. 25 μM of 5-FU repressed the specific growth rate by 42 ± 3 %, and 10 μM of 5-FU repressed the specific growth rate by 25 ± 5 %. Hence, 5-FU reduces biofilm formation of *E. coli* K-12 without significant toxicity at 10 μM so this concentration was utilized for subsequent experiments.

7.3.2 Differential gene expression in biofilms upon 5-FU contact

To explore the mechanism by which 5-FU represses the biofilm formation of *E. coli*, a whole transcriptome analysis in biofilms was performed at 7 h after the addition of 5-FU (10 μM) in LB medium. This concentration of 5-FU was utilized so that changes were not due to differences in growth. 5-FU induced the expression of 19 genes and repressed the expression of 157 genes with more than 1.5-fold change. A partial list of altered genes are shown in Table 7.1.

Unlike ursolic acid and 7-hydroxyindole, which repressed genes with functions for cysteine metabolism (Lee et al. 2007a; Ren et al. 2005a), and furanone, which repressed the expression of AI-2 induced genes (Ren et al. 2004b), 5-FU did not alter the expression of these genes. 5-FU induced the expression of seven histidine genes (*hisABCDGHL*) as well as the stress-related genes *yciE*, *yciF*, and *yciG*. YciG is also necessary for swarming motility in *E. coli*

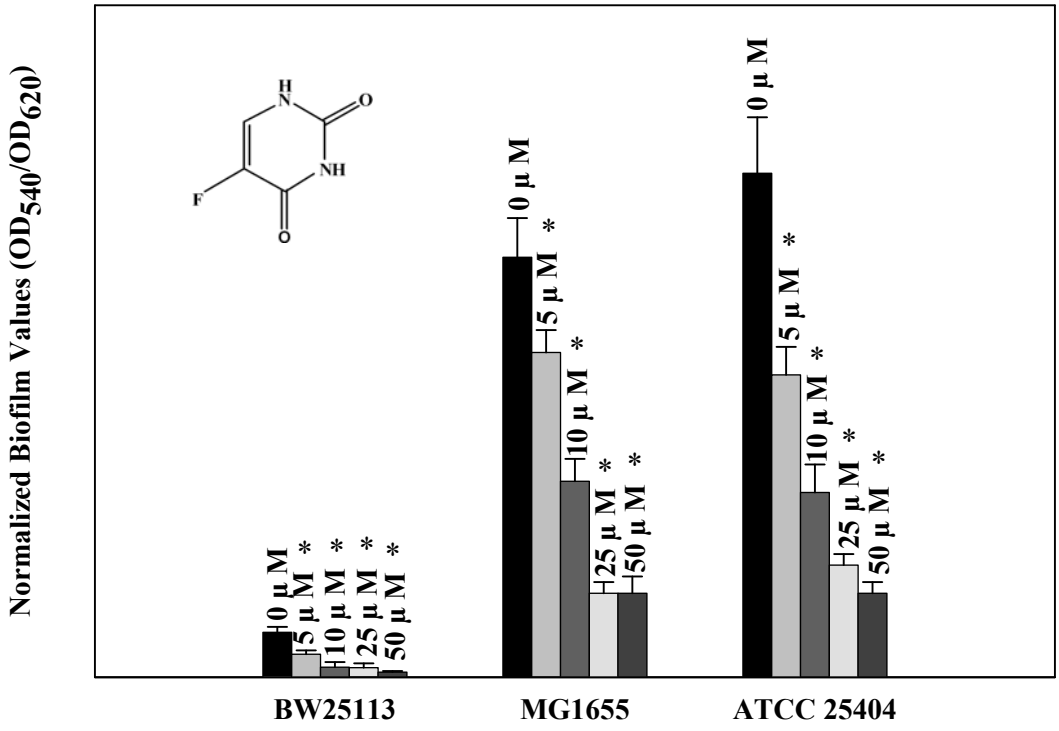

Fig. 7.2. Effect of 5-fluorouracil (0, 5, 10, 25, and 50 µM) on the biofilm formation of *E. coli* K-12 BW25113, *E. coli* K-12 MG1655, and *E. coli* K-12 ATCC 25404 WT at 37°C after 7 h in 96-well plates in LB medium. At least two independent experiments were conducted (total of 12 wells), and error bars indicate one standard deviation. Asterisk indicates significant difference as estimated by Student's t-test ($p < 0.05$).

Table 7.1. Partial list of altered genes for *E. coli* K-12 ATCC 25404 wild-type contacted with 10 µM 5-fluorouracil versus *E. coli* K-12 ATCC 25404 wild-type in biofilms formed on glass wool in LB at 37°C after 7 h.

B #	Gene name	Fold change	Descriptions
Acid resistance and cold shock protein			
b1164	*ycgZ*	1.6	Cold shock protein, hypothetical function
b1165	*ymgA*	1.6	Hypothetical function
b1166	*ariR*	1.6	Regulator of acid resistance
b1823	*cspC*	-1.7	Cold shock protein
Acyl carrier protein			
b1094	*acpP*	-1.5	Acyl carrier protein
DNA binding			
b0440	*hupB*	1.6	Histone-like protein HU-beta, HU-1
b1158	*pinE*	2.3	DNA invertase, site-specific recombination, e14 prophage
Histidine related			
b2018	*hisL*	1.5	his operon leader peptide
b2019	*hisG*	1.6	ATP-phosphoribosyltransferase
b2020	*hisD*	1.6	Histidinol dehydrogenase
b2021	*hisC*	1.7	Histidinol-phosphate aminotransferase
b2022	*hisB*	1.5	Imidazoleglycerolphosphate dehydratase/histidinol phosphatase; bifunctional enzyme; HAD21
b2023	*hisH*	1.5	Amidotransferase component of imidazole glycerol phosphate (IGP) synthase
b2024	*hisA*	1.5	*N*-(5'-phospho-L-ribosylformimino)-5-amino-1-(5'-phosphoribosyl)-4-imidazolecarboxamide 636 isomerase
Initiation factor			
b1718	*infC*	-1.5	Protein initiation factor
Lysozyme inhibitor			
b0220	*ivy*	1.5	Inhibitor of vertebrate lysozyme
Maltose transport			

Table 7.1. (continued)

B #	Gene name	Fold change	Descriptions
b4034	*malE*	1.5	Maltose-binding protein, periplasmic; MBP; substrate recognition for active transport of and chemotaxis toward maltose and maltodextrin
Membrane protein			
b1782	*mipA*	1.5	MltA-interacting protein; outer membrane; binds MrcB and MltA in a heterotrimer
Nucleotide binding			
b1713	*pheS*	1.6	Phenylalanine--tRNA ligase, beta-subunit
b1714	*pheT*	1.6	Phenylalanine--tRNA ligase, alpha-subunit
Outer membrane protein			
b0565	*ompT*	1.5	Outer membrane protease VII, DLP12 prophage; OM protein 2b; omptin
b2215	*ompC*	-1.6	Outer membrane protein C
Periplasmic protein			
b4376	*osmY*	-1.6	Osmotically inducible periplasmic protein
Ribosomal protein			
b0953	*rmf*	-1.7	Ribosome modulation factor
b3341	*rpsG*	-2.6	30S ribosomal subunit protein S7
RNA polymerase			
b2741	*rpoS*	-1.6	RNA polymerase subunit
Stress related			
b1257	*yciE*	1.5	Hypothetical protein
b1258	*yciF*	1.5	H-NS repressed; dimeric; rubrerythrin/ferritin-like putative metal-binding protein
b1259	*yciG*	1.5	Required for swarming phenotype, function unknown
Trigger factor			
b0436	*tig*	1.5	Trigger factor, protein folding chaperone; also peptidyl-prolyl cis-trans isomerase
Tryptophan synthesis			

Table 7.1. (continued)

B #	Gene name	Fold change	Descriptions
b1261	*trpB*	1.5	Tryptophan synthase, beta subunit
Unknown functions			
b1088	*yceD*	1.5	Hypothetical protein
b1550	*gnsB*	2.0	Overexpression increases unsaturated fatty acid content of phospholipids
b2089	*ygdI*	1.6	Hypothetical protein

Biofilm-related genes *ymgA* and *ariR* were also up-regulated in the microarray; deletion of these genes increases biofilm formation (Lee et al. 2007c). qRT-PCR showed *ymgA* and *ariR* were induced by 2.6- and 3.7-fold, respectively; hence, qRT-PCR corroborated that 5-FU induces the expression of *ymgA* and *ariR*. Therefore, YmgA and AriR reduce biofilm formation and their induction by 5-FU may explain the reduction in biofilm formation see upon addition of 5-FU.

7.3.3 5-FU controls biofilm formation through AriR

To determine which of the genes identified by the whole-transcriptome analysis were related to the reduction of biofilm by 5-FU, we examined the biofilm formation of 21 knock-out mutants of *E. coli* K-12 with 5-FU (Table 7.1). Three mutations (*hisA*, *ivy*, and *yceD*) decreased biofilm formation, and the other 18 mutations enhanced biofilm formation in LB medium (Fig. 7.3). These results indicate that 5-FU induces, in general, the expression of biofilm-repressing genes.

Of the 21 mutants, upon addition of 10 μM 5-FU, only the biofilm formation of the *ariR* mutant was not affected by the addition of 5-FU. Hence, biofilm reduction by 5-FU requires YmgB. Since YmgB represses biofilm as reported previously (Domka et al. 2007; Lee et al. 2007c) and as shown here (Fig. 7.3), this result is consistent.

To corroborate these results, we also examined the biofilm formation of *ariR* mutant in LB glucose (0.2%) medium. In LB glu medium also, the biofilm formation of *ariR* mutant was increased (3-fold) relative to the wild-type strain and was not affected by the addition of 5-FU (Fig. 7.4). Hence, 5-FU consistently requires YmgB to reduce biofilms.

To learn more about the interaction of 5-FU and *ariR*, we examined the biofilm formation of over-expressed *ariR* with 5-FU. However, over-expressed *ariR* abolished the biofilm formation

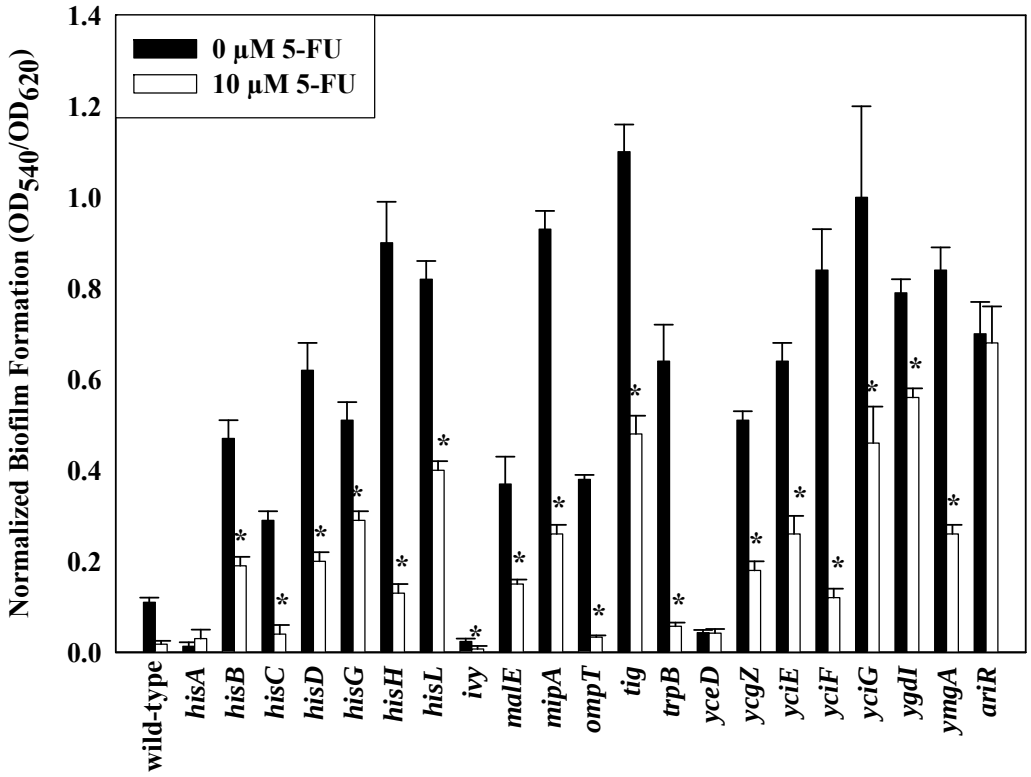

Fig. 7.3. Effect of 5-fluorouracil (0 and 10 µM) on biofilm formation of *E. coli* K-12 BW25113 WT and 21 mutants at 37°C after 7 h in 96-well plates in LB medium. Asterisks indicate significant differences as indicated by Student's t-test ($p < 0.05$).

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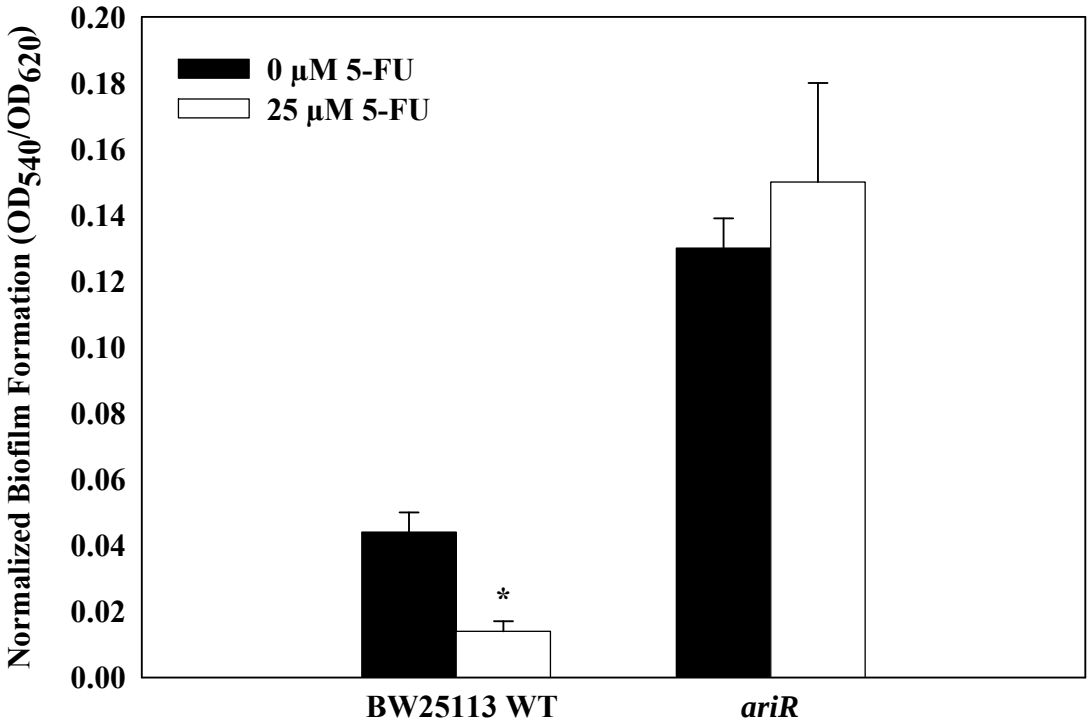

Fig. 7.4. Effect of 5-fluorouracil (0 and 25 µM) on biofilm formation of *E. coli* K-12 BW25113 WT and *ariR* mutant at 37°C after 7 h in 96-well plates in LB glu medium. Asterisk indicates significant difference as indicated by Student's t-test ($p < 0.05$).

(data not shown) as expected since AriR is reduces biofilm formation (Fig. 7.3 and Fig. 7.4). Therefore, the effect of 5-FU on over-expressed *ariR* could not be measured.

7.3.4 5-FU does not affect the swimming motility of *E. coli* K-12 ATCC 25404

As 5-FU decreases the biofilm formation of *E. coli* K-12 ATCC 25404, and *E. coli* biofilm formation and motility are often related (Wood *et al.*, 2006), we examined the swimming motility of ATCC 25404 by the addition of 5-FU. After 5 h, neither 5 nor 10 μM of 5-FU altered the swimming motility of this strain. After 8 h, 10 μM of 5-FU increased the swimming motility of ATCC 25404 by only 1.4-fold. Hence, the mechanism of biofilm inhibition by 5-FU in ATCC 25404 is not related to cell motility.

7.3.5 5-FU represses the expression of virulence genes of EHEC and reduces attachmnent

As 5-FU inhibits virulence phenotypes in *P. aeruginosa* PA14 (Ueda et al. 2008), we examined if 5-FU affects the virulence genes of EHEC. In DMEM medium, 10 μM 5-FU inhibits completely the growth of EHEC and 1 μM inhibits the growth by 2.0 ± 0.2-fold); hence, we tried lower concentrations. The growth rate of EHEC in DMEM medium with 0.5 μM 5-FU repressed the growth by 28 ± 5 % therefore, we used 0.5 μM of 5-FU for the RT-PCR experiments in DMEM medium. EHEC possesses the pathogenicity island named the locus of enterocyte effacement (LEE), which is necessary for the attachment and effacing lesions caused by EHEC (Walters and Sperandio 2006a). LEE is composed of five operons (*LEE1* to *LEE5*) and contains 41 genes (Walters and Sperandio 2006a). In DMEM medium, 0.5 μM of 5-FU repressed the expression of *eae*, *escC*, and *espA* genes by 2.8-, 3.4-, and 2.2-fold versus EHEC without 5-FU. *eae* gene belongs to *LEE5* operon (Sharma et al. 2005), *escC* gene belongs to

LEE2 operon (Roe et al. 2003), and *espA* gene belongs to *LEE4* operon (Sharma et al. 2005). Our results indicate that 5-FU represses the expression of the examined three *LEE* operons.

As 5-FU repressed the expression of virulence genes required for attachment of EHEC in DMEM medium, we measured the *in vitro* adhesion of EHEC to the human epithelial intestinal cells, HCT-8, in RPMI medium. *in vitro* adhesion of EHEC to HCT-8 cells, normalized by colony forming units in RPMI medium, was not altered in RPMI medium with the addition of 0.5 μM of 5-FU (results not shown).

7.4 Discussion

As *E. coli* is the best-studied microorganism (Lugtenberg et al. 2002) and its knock-out mutants are readily available (Baba et al. 2006), using *E. coli* to unravel the mechanism of biofilm inhibition is advantageous (Wood 2008). By analyzing the impact of 5-FU on the whole transcriptome, we found that 5-FU induces the expression of a known biofilm repressor AriR (Lee et al. 2007c). The effect of 5-FU was examined with 21 microarray-related mutants. Except for the *ariR* mutant, all the biofilm-induced mutants repressed the biofilm formation with 5-FU addition. We also checked the biofilm formation in LB glu medium. In this medium also, 5-FU did not affect the biofilm formation of the *ariR* mutant, while 5-FU repressed the biofilm formation of the wild-type. Hence, 5-FU represses biofilm formation by inducing the expression of *ariR* and becomes ineffective in the *ariR* mutant.

5-FU induced the expression of seven histidine genes. We examined the biofilm formation of these seven histidine mutants with 5-FU addition. Except the *hisA* mutant, the six histidine mutants induced biofilm formation vs. the wild-type (Fig. 7.3); hence, histidine biosynthesis also might be important for biofilm formation. Also, further experiments with phenotype microarrays would be important to learn how 5-FU affects the histidine metabolism

phenotypically. 5-FU induced the expression of stress genes *yciEFG*; *yciE* and *yciF* are both induced in *E. coli* contacted with two antibiotics: ampicillin and orfloxacin (Kaldalu et al. 2004). The mutation in these three genes induced the biofilm formation; in this study, an indirect relation between the biofilm formation and stress-related genes was observed. Previously deletion of *ycfR* increased both biofilm formation and the expression of stress-related genes (Zhang et al. 2007).

When bacteria encounter the growth inhibitors, they develop resistance, which makes treatment of bacterial infections more difficult (Rasmussen and Givskov 2006). Compounds controlling the expression of virulence without affecting growth are thereby superior (Lesic et al. 2007). In this study, based on our knowledge of uracil influencing biofilm formation and virulence factors (Lee et al. 2008; Ueda et al. 2008); we revealed that the uracil analog, 5-FU, is a biofilm inhibitor of *E. coli* and works through AriR.

7.5 Experimental procedures

7.5.1 Bacterial strains, media, growth conditions, and chemicals

The strains used in this study are listed in Table 7.2; they were grown at 37°C. LB medium (Sambrook et al. 1989) and LB medium supplemented with 0.2% glucose were used for the crystal violet biofilm experiments and the glass wool biofilm DNA microarray experiments. LB and DMEM (Dulbecco's modified Eagle's medium) with glucose (4.5 g/L) and 10 mM HEPES (Hyclone, Logan, UT) were used for the quantitative real-time, reverse transcription polymerase chain reaction (qRT-PCR) experiments. RPMI (Roswell Park Memorial Institute) medium with 10% horse serum, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL penicillin,

Table 7.2. *E. coli* K-12 and *E. coli* O157:H7 strains were used in this study. Km^R is kanamycin resistance.

Strains	Genotype	Source
E. coli K-12	Wild type	ATCC 25404
E. coli K-12 MG1655	$F^- \lambda^-$ *ilvG rfb-50 rph-1*	(Blattner *et al.*, 1997)
E. coli K-12 BW25113	$lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$	(Datsenko and Wanner, 2000)
E. coli K-12 BW25113 Δ*hisA*	K-12 BW25113 Δ*hisA* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*hisB*	K-12 BW25113 Δ*hisB* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*hisC*	K-12 BW25113 Δ*hisC* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*hisD*	K-12 BW25113 Δ*hisD* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*hisG*	K-12 BW25113 Δ*hisG* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*hisH*	K-12 BW25113 Δ*hisH* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*hisL*	K-12 BW25113 Δ*hisL* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*ivy*	K-12 BW25113 Δ*ivy* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*malE*	K-12 BW25113 Δ*malE* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*mipA*	K-12 BW25113 Δ*mipA* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*ompT*	K-12 BW25113 Δ*ompT* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*tig*	K-12 BW25113 Δ*tig* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*trpB*	K-12 BW25113 Δ*trpB* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*yceD*	K-12 BW25113 Δ*yceD* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*ycgZ*	K-12 BW25113 Δ*ycgZ* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*yciE*	K-12 BW25113 Δ*yciE* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*yciF*	K-12 BW25113 Δ*yciF* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*yciG*	K-12 BW25113 Δ*yciG* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*ygdI*	K-12 BW25113 Δ*ygdI* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*ymgA*	K-12 BW25113 Δ*ymgA* Ω Km^r	(Baba *et al.*, 2006)
E. coli K-12 BW25113 Δ*ariR*	K-12 BW25113 ΔariR Ω Km^r	(Baba *et al.*, 2006)
E. coli O157:H7	EHEC $Stx1^+$ and $Stx2^+$	(Strockbine *et al.*, 1986)

and 100 µg/mL streptomycin was used for *in vitro* adhesion to the human colonic epithelial cells HCT-8 (ATCC). Kanamycin was used to select the *E. coli* K-12 BW25113 knock-out mutants.

7.5.2 Biofilm formation

Biofilm formation of the wild-type strain and the mutants was performed in 96-well polystyrene plates as indicated previously (Pratt and Kolter 1998) with 300 µL of crystal violet per well, and the absorbance was measured at 540 nm. In brief, overnight cultures were inoculated with an initial turbidity of 0.05 at 600 nm for 7 h without shaking. Twelve replicate wells were averaged to obtain each data point and two independent cultures were used. Uracil analogs 5-aminouracil, 6-azauracil, 5-bromouracil, 5-fluorouracil (Fisher Scientific, Hanover Park, IL), and 5-nitrouracil (MP Biomedical, Solon, OH) were diluted in dimethylformamide. 5-(*trans*-2-bromovinyl)-uracil (Sigma-Aldrich, St. Louis, MO) was diluted in ethanol. Stock solutions (0, 5, 10, 25, and 50 mM) were added at 0.1 vol% to *E. coli* K-12 ATCC 25404 and dimethylformamide and ethanol were added at 0.1 vol% as solvent negative controls.

7.5.3 Swimming motility

Swimming motility assays were performed as explained previously (Sperandio *et al.*, 2002) by measuring halos on agar plates after 5 h and 8 h. In brief, overnight cultures were re-grown to a turbidity of 1 at 600 nm. The cultures were inoculated into the plates containing 5-FU with a toothpick, and halos were measured after 5 h and 8 h. Ten identical plates (five plates from each of two independent cultures) were used to evaluate the swimming motility for each condition.

7.5.4 RNA isolation in biofilms

To identify the genes controlled by 5-FU, *E. coli* K-12 ATCC 25404 was grown in LB medium, one mL of the overnight cultures (turbidity 7.5 at 600 nm) was inoculated into 250 mL of fresh LB medium supplemented with 250 μL of 10 mM 5-FU or DMF and containing 10 g of glass wool (Corning Glass Works, Corning, NY) (Ren et al. 2004a), and the cultures were incubated for 7 h at 37°C with shaking. Total RNA was obtained from the biofilm cells by using the RNeasy Mini kit (Qiagen Sciences, Germantown, MD) as described previously (Ren et al. 2005a).

7.5.5 DNA microarrays

The *E. coli* GeneChip Genome 2.0 arrays containing 10,208 probe sets for open reading frames, rRNA, tRNA, and intergenic regions for four *E. coli* strains (MG1655, CFT073, O157:H7-Sakai, and O157:H7-EDL933) were used for the DNA microarray experiments. cDNA synthesis, fragmentation, and hybridization were performed as described previously (González-Barrios et al. 2006a). Hybridization was performed for 16 h, and the total cell intensity was scaled automatically in the software to an average value of 500. The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA controls were used to monitor the labeling process. For each binary microarray comparison of differential genes expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 11-15 probe pairs (p-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the p-value for comparing two chips was lower than 0.05 (to assure that the changes in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher

(1.5-fold) than the standard deviation (1.3-fold) for all the genes of the microarray (Ren et al. 2004b).

7.5.6 qRT-PCR

To determine the effects of 5-FU on the expression of virulence genes (*eae*, *escC*, and *espA*) (Table 7.3), *E. coli* O157:H7 (EHEC) was grown in LB medium overnight. The overnight culture was diluted to a turbidity of 0.05 at 600 nm in 10 mL of DMEM medium, and 10 μL of 0.5 mM 5-FU or 10 μL of DMF were added to 10 mL of cultures. Cells were incubated to a turbidity of 1.0 at 600 nm at 37°C with shaking and were centrifuged at 10,000 *g* for 2 min. RNA was obtained from the cell pellets using the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD) as described previously (Ren et al. 2005a). The expression of *eae* (contained within the operon of virulence genes known as the locus of enterocyte effacement 5 (*LEE5*) (Sharma et al. 2005)), *escC* (*LEE2*), and *espA* (*LEE4*) was determined by using qRT-PCR. The housekeeping gene *rpoA* was used to normalize the gene expression data. qRT-PCR was performed in triplicate for each sample.

To corroborate the DNA microarray data, the transcription level of two induced genes, *ariR* and *ymgA* was quantified using by the qRT-PCR by using the same RNA used for the DNA microarrays. The housekeeping gene *rrsG* was used to normalize the expression data of the interesting genes.

7.5.7 *In vitro* adhesion assays

Human colonic epithelial cells HCT-8 cells were cultured and propagated in RPMI medium with 10% horse serum, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin. HCT-8 cells were cultured in 24-well tissue culture plates at 37°C

Table 7.3. Primers used for RT-PCR experiments. *eae*, *escC*, *espA*,and *rpoA* primers were obtained from Kendall *et al.* (2007).

Primer Name	Nucleotide sequence (5' to 3')	Primer Name	Nucleotide sequence (5' to 3')
eae-F	GCTGGCCCTTGGTTTGATCA	*eae*-R	GCGGAGATGACTTCAGCACTT
espA-F	TCAGAATCGCAGCCTGAAAA	*espA*-R	CGAAGGATGAGGTGGTTAAGCT
escC-F	GCGTAAACTGGTCCGGTACGT	*escC*-R	TGCGGGTAGAGCTTTAAAGGCAAT
rpoA-F	GCGCTCATCTTCTTCCGAAT	*rpoA*-R	CGCGGTCGTGGTTATGTG
ymgA-F	TGCTCCAGATACTGCGCATGAAGA	*ymgA*-R	TTCTTCGGTTTGGCCCTCCTGAAT
ariR-F	GTTAGGGCAGGCTGTCACCAATTT	*ariR*-R	GCTGTGTATCGCAACACGATTTCC
rrsG-F	ACTTAACAAACCGCCTGCGT	*rrsG*-R	TATTGCACAATGGGCGCAAG

in 5% CO_2 until 80% confluence was reached. The *in vitro* adhesion of EHEC cells to HCT-8 cells was performed as described previously (Bansal et al. 2007).

7.5.8 Microarray accession numbers

The expression data for biofilm samples with and without 5-FU are summarized in Table 7.3 and have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through accession number GSE12750 (Edgar et al. 2002).

CHAPTER VIII

QUORUM SIGNAL AI-2 UPTAKE REGULATORS IN *ESCHERICHIA COLI* AFFECTS SRNA REGULATIONS AND BIOFILM ARCHITECTURE*

8.1 Overview

The regulatory network for the uptake of *Escherichia coli* autoinducer 2 (AI-2) is comprised of a transporter complex, LsrABCD; its repressor, LsrR; and a cognate signal kinase, LsrK. This network is an integral part of the AI-2 quorum sensing (QS) system. Because LsrR and LsrK directly regulate AI-2 uptake, we hypothesized that they might play a wider role in regulating other QS-related cellular functions. In this study, we characterized physiological changes due to the genomic deletion of *lsrR* and *lsrK*. We discovered that many genes were co-regulated by *lsrK* and *lsrR* but in a distinctly different manner than that for the *lsr* operon (where LsrR serves as a repressor that is de-repressed by the binding of phospho-AI-2 to the LsrR protein). An extended model for AI-2 signaling that is consistent with all current data on AI-2, LuxS, and the LuxS regulon is proposed. Additionally, we found that both the quantity and architecture of biofilms were regulated by this distinct mechanism, as *lsrK* and *lsrR* knockouts behaved identically. Similar biofilm architectures probably resulted from the concerted response

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of a set of genes including *flu* and *wza*, the expression of which is influenced by *lsrRK*. We also found for the first time that the generation of several small RNAs (including DsrA, which was previously linked to QS systems in *Vibrio harveyi*) was affected by LsrR. Our results suggest that AI-2 is indeed a QS signal in *E. coli*, especially when it acts through the transcriptional regulator LsrR.

8.2 Introduction

Bacteria communicate with each other through small "hormone-like" organic molecules referred to as autoinducers. Autoinducer-based bacterial cell-to-cell communication, enabling population-based multicellularity, has been termed quorum sensing (QS) (Fuqua *et al.*, 1994). Cellular functions controlled by QS are varied and reflect the needs of a particular bacterial species for inhabiting a given niche (Camilli and Bassler, 2006; Keller and Surette, 2006; Vendeville *et al.*, 2005).

QS among *Escherichia coli* and *Salmonella* strains has been a topic of great interest, and different intercellular signaling systems have been identified: that mediated by the LuxR homolog SdiA: the LuxS/autoinducer 2 (AI-2) system, an AI-3 system, and a signaling system mediated indole (Ahmer, 2004; De Keersmaecker *et al.*, 2006; Sitnikov *et al.*, 1996; Sperandio *et al.*, 2003; van Houdt *et al.*, 2006; Walters and Sperandio, 2006b). Among these systems, the LuxS/AI-2 system possesses the unique feature of endowing cell population-dependent behavior while interacting with central metabolism through the intracellular activated methyl cycle (DeLisa *et al.*, 2001; DeLisa and Bentley, 2002; March and Bentley, 2004; Vendeville *et al.*, 2005; Winzer *et al.*, 2003). Therefore, it has the potential to influence both gene regulation and bacterial fitness.

AI-2's function has been studied using *luxS* mutants and by adding either conditioned medium or *in vitro*-synthesized AI-2 to bacterial cultures. It is noteworthy that the *luxS* transcription profile is not synchronous with the accumulation profile of extracellular AI-2 in bacterial supernatants (Beeston and Surette, 2002; Hardie *et al.*, 2003; Xavier and Bassler, 2005b). In *E. coli*, extracellular AI-2 activity peaks during the mid- to late-exponential phase and rapidly decreases during entry into the stationary phase. A corresponding decrease in LuxS protein levels is not observed (Hardie *et al.*, 2003; Xavier and Bassler, 2005b). The disappearance of extracellular AI-2 activity in *E. coli* and *Salmonella enterica* serovar Typhimurium is due to its uptake, carried out by its import through an ATP-binding cassette (ABC) transporter named the *luxS*-regulated (Lsr) transporter (Taga *et al.*, 2003; Wang *et al.*, 2005b; Xavier and Bassler, 2005b). The transporter proteins are part of the *lsr* operon, which is regulated by cyclic AMP/cyclic AMP receptor protein and proteins transcribed two genes, *lsrK* and *lsrR*, located immediately upstream of *lsr* and divergently transcribed in its own *lsrRK* operon (Wang *et al.*, 2005c). The cytoplasmic kinase, LsrK, phosphorylates AI-2 into an activated molecule that is suggested to bind and de-repress the *lsr* repressor, LsrR. A conceptual model of the LsrR/phospho-AI-2 circuit is provided in Fig. 8.1.

LsrR and LsrK were among the first positively identified QS regulators in *E. coli* (De Keersmaecker *et al.*, 2006; Wang *et al.*, 2005c; Xavier and Bassler, 2005b). Since QS regulators are responsible for mediating many cellular phenotypes and morphologies (Davies *et al.*, 1998; Hammer and Bassler, 2003; Henke and Bassler, 2004), the functions of LsrR and LsrK are of great interest. Furthermore, it is well known that AI-2 uptake is an integral part of the *E. coli* QS network; thus, it remains intriguing that these bacteria actively transport its QS autoinducer. In many other systems, the signal molecule is freely diffused or binds a cognate receptor, triggering a sensor-kinase couple. It is possible that AI-2 signaling bacteria import and internalize AI-2 to

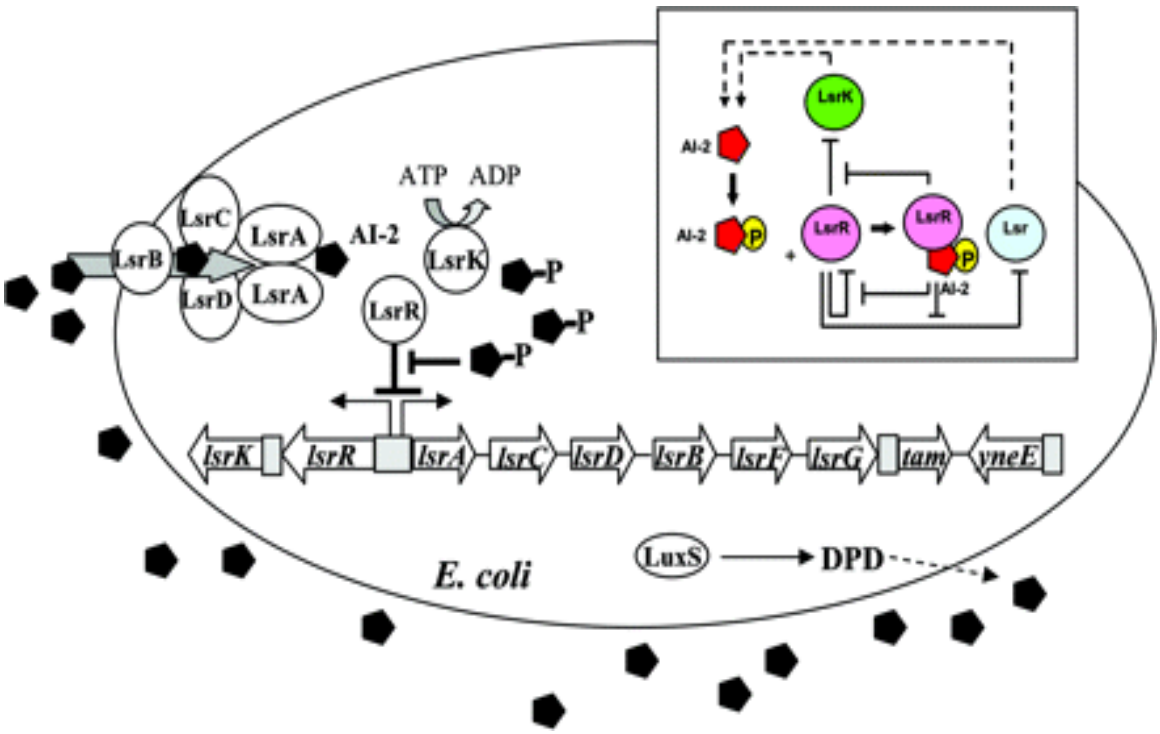


Fig. 8.1. Regulatory mechanisms of the LsrR/phospho-AI-2 circuit in *E. coli* AI-2 uptake (modified from (Wang *et al.*, 2005c)). The AI-2 uptake repressor LsrR represses many genes including the *lsr* operon (comprised of *lsrACDBFG*) and the *lsrRK* operon. AI-2 can be imported back inside the cell via LsrACDB. Imported AI-2 is processed as phospho-AI-2 via the kinase LsrK. Phospho-AI-2 has been reported to bind LsrR and relieve its repression of the lsr transporter genes, triggering their expression. This in turn stimulates additional AI-2 uptake. DPD, 4,5-dihydroxy-2,3-pentanedione.

terminate extracellular AI-2-dependent cellular responses and alternatively trigger cytoplasmic AI-2-dependent gene expression, akin to a genetic switching mechanism.

The physiological functions associated with either extracellular or cytoplasmic AI-2 can be partially revealed using *lsrK* and *lsrR* mutants. In *lsrK* mutants, Lsr transporter expression is repressed, and AI-2 remains in the supernatant (extracellular AI-2) (Wang *et al.*, 2005b). In *lsrR* mutants, the Lsr transporter is expressed, and extracellular AI-2 is continuously imported into the cell (cytoplasmic AI-2), irrespective of its accumulation (Taga *et al.*, 2003; Wang *et al.*, 2005b). We carried out genome-wide transcriptome analyses of *lsrR* and *lsrK* mutants relative to the isogenic parent strain W3110. We further evaluated physiological changes (biofilm formation, motility, etc.) resulting from the mutations. We found that *lsrR* and *lsrK* serve as global regulators of gene expression and affect biofilm architecture through the coordinate regulation of biofilm-related genes such as *wza* (responsible for colanic acid) and the autoaggregation gene *flu*. While the expression of many important genes was found to be altered by the *lsrR* and *lsrK* deletions (and are putatively regulated by LsrRK), those associated with host invasion, stress responses, and foreign DNA were most prevalent. For the first time, small riboregulators were shown to respond to the QS regulators *lsrR* and *lsrK*. Finally, and perhaps most importantly, our results suggest that *lsrR* and *lsrK* (or, more specifically, LsrR and AI-2) operate in tandem and in the inverse of their role in regulating AI-2 uptake. Positive identification of LsrR/AI-2 signaling sheds new light on the widely discussed differences between AI-2, the metabolic by-product, and AI-2, the QS signaling molecule (Wang *et al.*, 2005c; Winzer *et al.*, 2003).

8.3 Results

8.3.1 Deletion of *lsrR* and *lsrK* does not affect growth or motility

We observed no changes in growth rate and cell motility due to a deletion of either *lsrR* or *lsrK* when the cells were grown in LB (data not shown). No significant differences in biofilm formation were found using a crystal violet assay for cells cultivated up to 24 h in various media (data not shown). We then used SEM to visualize biofilm morphology. W3110 Δ*lsrR* and Δ*lsrK* strains appeared with significantly different structures than that of the wild-type: an extracellular matrix not present on the wild-type was observed on the surface of both mutants (Fig. 8.2). To investigate associated gene regulation, we carried out a complete transcriptome analysis of the *lsrR* and *lsrK* mutants and later reexamined biofilm formation using a more comprehensive flow cell chamber and confocal microscopy.

8.3.2 *lsrR* and *lsrK* mutations reveal targets of AI-2 signaling

For transcriptome analysis, cells were grown in LB medium (without glucose) to an OD_{600} of 2.4 ± 0.1 (early stationary phase). At this point, extracellular AI-2 in cultures of wild-type cells being transported back into the cells is nearly completely depleted from Δ*lsrR* mutants and is retained at near-peak levels in Δ*lsrK* mutants (Wang *et al.*, 2005b). To report the number of genes differentially expressed, we took the commonly used twofold ratio as a cutoff limit (Canales *et al.*, 2006; Ichikawa *et al.*, 2000), together with a *P*-value of <0.05 to ensure statistical significance. Furthermore, a set of selected genes whose expression was changed by the *lsrR* and/or *lsrK* gene was verified with real-time RT-PCR measurements. Figure 8.3 shows that there was a strong positive correlation ($r^2 = 0.98$ for *lsrR* mutants and $r^2 = 0.96$ for *lsrK* mutants) between the two techniques. Results indicate that there were 119 and 27 genes induced and

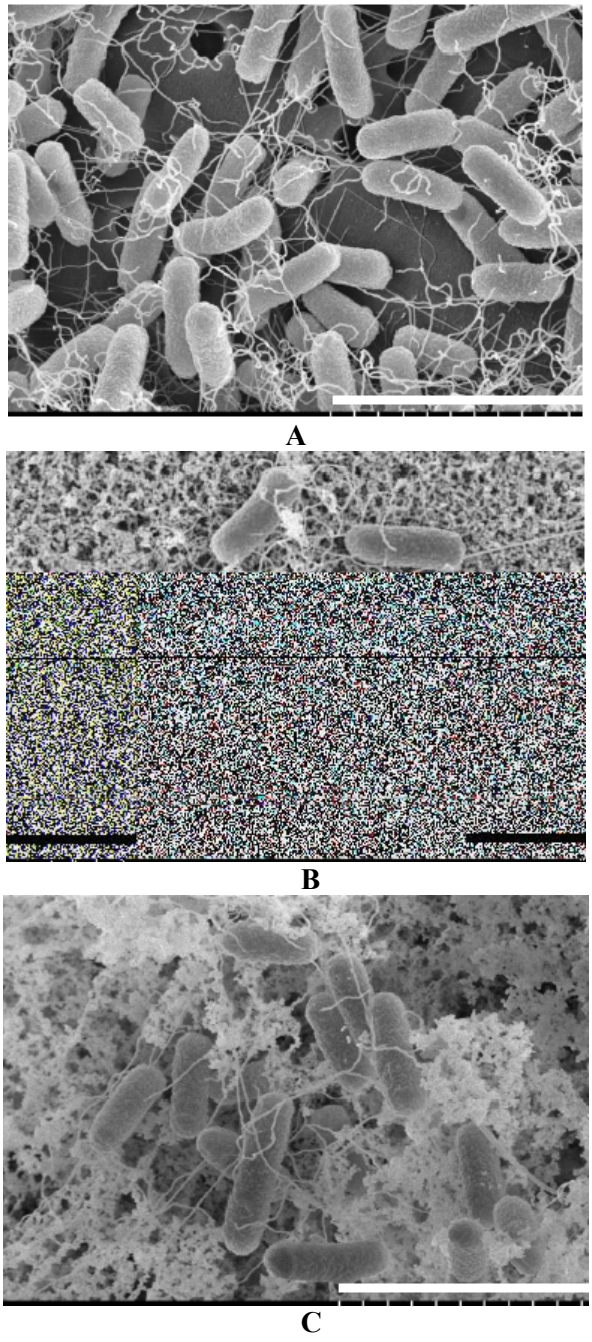
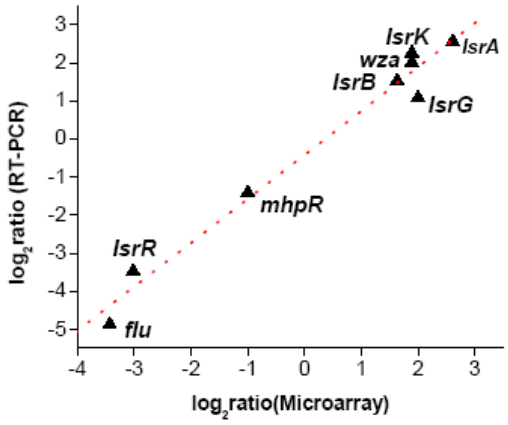
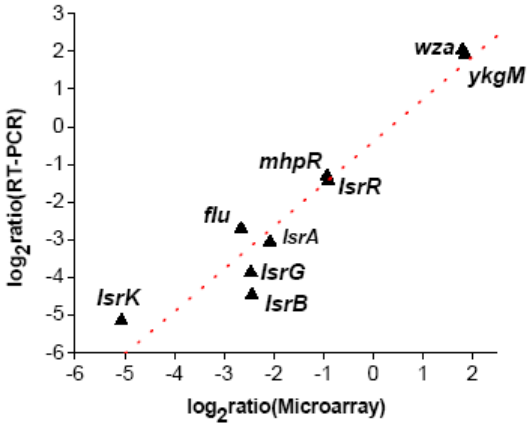


Fig. 8.2. Scanning electron micrographs of wild-type W3110 and isogenic *lsrR* and *lsrK* mutants. (A) Wild-type strain W3110. (B) Isogenic lsrR mutants. (C) Isogenic lsrK mutants. Length scale is indicated by bars. Scale bar 4 μm.



A



B

Fig. 8.3. Correlation between microarray and real-time RT-PCR results. The differences in expression of eight *lsrR*-controlled genes and nine *lsrK*-controlled were $\log_2$ transformed and plotted (microarray versus real-time RT-PCR). (A) Symbols: ▲, W3110 Δ*lsrR*; dashed line, corresponding linear correlation between microarray data and real-time RT-PCR results. (B) Symbols: ▲, W3110 Δ*lsrK*; dotted line, corresponding linear correlation between microarray data and real-time RT-PCR results.

Table 8.1. The list of genes co-regulated by *lsrR* and *lsrK*. "co-regulated" refers to the expression of genes being affected by mutation of both *lsrR* and *lsrK*. Genes consistently induced or repressed ($P < 0.05$) more than two-fold are shown.

B #	Gene	Gene product[b]	Fold change	
			Δ*lsrR*/WT	Δ*lsrK*/WT
Divergently regulated genes				
b0564	*appY*	regulatory protein affecting appA and other genes	-2.2	2.6
b1511	*lsrK*	putative kinase	3.7	-33.5
b1513	*lsrA*	putative ATP-binding component of a transport system	6.1	-4.2
b1514	*lsrC*	putative transport system permease protein	2.5	-2.0
b1515	*lsrD*	putative transport system permease protein	3.0	-3.2
b1516	*lsrB*	putative LACI-type transcriptional regulator	3.1	-5.4
b1517	*lsrF*	orf, hypothetical protein	3.1	-2.9
b1518	*lsrG*	orf, hypothetical protein	4.0	-5.5
b1519	*tam*	putative enzyme	2.1	-2.0
Co-regulated genes				
b1512	*lsrR*	putative transcriptional regulator, sorC family	-8.1	-2.0
b0218	*yafU*	orf, hypothetical protein	3.0	4.2
b0296	*ykgM*	putative ribosomal protein	3.2	3.6
b0326	*yahL*	orf, hypothetical protein	2.9	2.5
b0327	*yahM*	orf, hypothetical protein	3.2	2.2
b0334	*prpD*	orf, hypothetical protein	-2.0	-2.2
b0346	*mhpR*	transcriptional regulator for mhp operon	-2.0	-2.1
b0450	*glnK*	nitrogen regulatory protein P-II 2	4.4	3.6
b0451	*amtB*	probable ammonium transporter	2.3	2.2
b0521	*arcC*	putative carbamate kinase	-2.5	-2.6
b0527	*ybcI*	orf, hypothetical protein	2.0	2.6
b0685	*ybfE*	orf, hypothetical protein	2.4	2.4
b0718	*ybgQ*	putative outer membrane protein	2.0	2.4
b0787	*ybhM*	orf, hypothetical protein	4.2	2.9
b0939	*ycbR*	putative chaperone	2.5	2.1
b1031	*ycdV*	putative ribosomal protein	-2.0	-3.4
b1137	*ymfD*	orf, hypothetical protein	2.6	2.7
b1140	*intE*	prophage e14 integrase	2.3	2.2
b1160	*ycgW*	orf, hypothetical protein	2.0	3.2
b1161	*ycgX*	orf, hypothetical protein	4.6	4.1
b1196	*ycgY*	orf, hypothetical protein	2.0	2.7
b1450		orf, hypothetical protein	6.6	2.4
b1454		putative transferase	2.4	2.5
b1555		orf, hypothetical protein	2.0	2.0
b1558	*cspF*	cold shock protein	3.0	3.9
b1571	*ydfA*	orf, hypothetical protein	4.1	3.5
b1575	*dicB*	inhibition of cell division	2.2	2.3
b1625		orf, hypothetical protein	3.1	3.7

Table 8.1. (continued)

B #	Gene	Gene product[b]	Fold change	
			Δ*lsrR*/WT	Δ*lsrK*/WT
b1697	*ydiQ*	putative transport protein	2.0	2.1
b1796	*yoaG*	orf, hypothetical protein	4.8	2.2
b1798	*yeaS*	orf, hypothetical protein	2.2	2.2
b1954	*dsrA*	regulatory RNA; positive regulation of promoters	.6	4.4
b2000	*flu*	sensitive to HNS negative regulation	-10.8	-6.3
b2001		outer membrane fluffing protein, similar to adhesin orf, hypothetical protein	-5.2	-3.5
b2014	*yeeF*	putative amino acid/amine transport protein	-3.1	-2.2
b2020	*hisD*	L-histidinal:NAD+ oxidoreductase; L-histidinol:NAD+ oxidoreductase	-2.1	-2.0
b2062	*wza*	putative polysaccharide export protein	3.7	3.5
b2110	*yehC*	putative chaperone	2.5	2.3
b2274		orf, hypothetical protein	3.7	3.5
b2312	*purF*	amidophosphoribosyltransferase = PRPP	-2.0	-2.0
b2483	*hyfC*	hydrogenase 4 membrane subunit	2.3	2.5
b2629	*yfjM*	orf, hypothetical protein	4.7	4.0
b2734	*pphB*	protein phosphatase 2	2.8	2.6
b2832		putative transport protein	3.9	3.4
b2848	*yqeJ*	orf, hypothetical protein	3.8	3.7
b2850	*ygeF*	orf, hypothetical protein	3.6	2.0
b2854		orf, hypothetical protein	2.3	2.0
b3109	*yhaN*	orf, hypothetical protein	2.0	2.0
b3118	*tdcA*	transcriptional activator of tdc operon	2.2	2.5
b3120	*yhaB*	orf, hypothetical protein	4.0	3.6
b3136	*agaS*	putative tagatose-6-phosphate aldose isomerase	2.8	3.5
b3264	*envR*	putative transcriptional regulator	2.8	2.5
b3324	*yheE*	putative general secretion pathway for protein export	2.4	2.1
b3484	*yhhI*	putative receptor	3.2	2.5
b3490	*yhiL*	orf, hypothetical protein	2.3	3.8
b3507	*yhiF*	orf, hypothetical protein	3.3	5.2
b3512	*yhiE*	orf, hypothetical protein	3.3	5.1
b3578	*yiaN*	putative membrane protein	2.4	2.0
b3587	*yiaW*	orf, hypothetical protein	2.5	2.6
b3595	*yibJ*	orf, hypothetical protein	2.2	2.5
b3596	*yibG*	orf, hypothetical protein	2.1	2.6
b3817	*yigF*	orf, hypothetical protein	2.2	3.5
b3903	*rhaA*	L-rhamnose isomerase	3.6	2.5
b3989	*htrC*	heat shock protein htrC	2.5	2.0
b4072	*nrfC*	formate-dependent nitrite reductase; Fe-S centers	2.2	2.0
b4075	*nrfF*	part of formate-dependent nitrite reductase complex	2.3	2.8
b4116	*adiY*	putative ARAC-type regulatory protein	2.6	3.3
b4239	*treC*	trehalase 6-P hydrolase	2.3	2.2
b4247	*yjgG*	orf, hypothetical protein	3.1	3.5

Table 8.2. The list of genes whose expression is affected by *lsrR*. Genes consistently induced or repressed ($P < 0.05$) more than two-fold are shown

B #	Gene	Gene product	Fold change Δ*lsrR*/WT
b0139	*htrE*	probable outer membrane porin protein involved in fimbrial assembly	-3.2
b1039	*csgE*	curli production assembly/transport component, 2nd curli operon	-3.0
b0586	*entF*	ATP-dependent serine activating enzyme (may be part of enterobactin synthase as component F)	-2.3
b1849	*purT*	phosphoribosylglycinamide formyltransferase 2	-2.3
b1393	*ydbS*	putative enzyme	-2.2
b4131	*cadA*	lysine decarboxylase 1	-2.2
b0270	*yagG*	putative permease	-2.1
b0484	*ybaR*	putative ATPase	-2.0
b0596	*entA*	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, enterochelin biosynthesis	-2.0
b1963		orf, hypothetical protein	-2.0
b2230	*yfaA*	orf, hypothetical protein	-2.0
b2725	*hycA*	transcriptional repression of hyc and hyp operons	-2.0
b0615	*citF*	citrate lyase alpha chain	-2.0
b0999	*yccD*	orf, hypothetical protein	-2.0
b1293	*sapB*	homolog of Salmonella peptide transport permease protein	-2.0
b1776		putative oxidoreductase	-2.0
b1141		orf, hypothetical protein	2.0
b0709	*ybgH*	putative transport protein	2.0
b1456	*rhsE*	rhsE protein in rhs element	2.0
b1550		orf, hypothetical protein	2.0
b1588		putative oxidoreductase, major subunit	2.0
b2497	*uraA*	uracil transport	2.0
b2775	*yqcE*	putative transport protein	2.0
b3060	*ygiP*	putative transcriptional regulator LYSR-type	2.0
b3214	*gltF*	regulator of gltBDF operon, induction of Ntr enzymes	2.0
b3215	*yhcA*	putative chaperone	2.0
b2648		orf, hypothetical protein	2.1
b1229	*tpr*	a protaminelike protein	2.1
b0236	*prfH*	probable peptide chain release factor	2.1
b0363	*yaiP*	polysaccharide metabolism	2.1
b0984	*ymcA*	orf, hypothetical protein	2.1
b1122	*ymfA*	orf, hypothetical protein	2.1
b1155		orf, hypothetical protein	2.1
b3159	*yhbV*	orf, hypothetical protein	2.1
b4063	*soxR*	redox-sensing activator of soxS	2.1
b4246	*pyrL*	pyrBI operon leader peptide	2.1
b4300	*sgcR*	putative DEOR-type transcriptional regulator	2.1

Table 8.2. (continued)

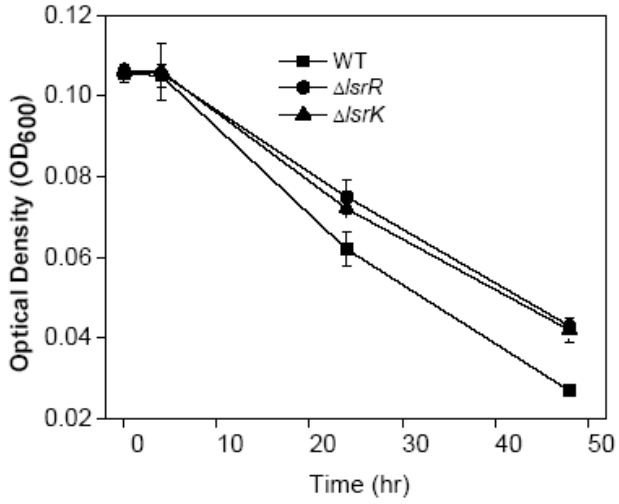
B #	Gene	Gene product	Fold change Δ*lsrR*/WT
b0942		putative fimbrial-like protein	2.2
b1146		orf, hypothetical protein	2.2
b1720		orf, hypothetical protein	2.2
b2120	*yehM*	orf, hypothetical protein	2.2
b2123	*yehR*	orf, hypothetical protein	2.2
b2646	*ypjF*	orf, hypothetical protein	2.2
b3325	*yheF*	putative general protein secretion protein	2.2
b3944	*yijF*	orf, hypothetical protein	2.2
b4212	*ytfH*	orf, hypothetical protein	2.2
b1265	*trpL*	trp operon leader peptide	2.3
b1675		orf, hypothetical protein	2.3
b2387		putative PTS system enzyme IIB component	2.3
b2657		putative enzyme	2.3
b0806	*ybiM*	orf, hypothetical protein	2.4
b1028		orf, hypothetical protein	2.4
b3437	*gntK*	gluconokinase 2, thermoresistant	2.4
b4204	*yjfZ*	orf, hypothetical protein	2.4
b1121		homolog of virulence factor	2.5
b2105	*yohL*	orf, hypothetical protein	2.5
b2852	*ygeH*	putative invasion protein	2.5
b3517	*gadA*	glutamate decarboxylase isozyme	2.5
b3564	*xylB*	xylulokinase	2.6
b0717	*ybgP*	putative chaperone	2.8
b1350	*recE*	exonuclease VIII, ds DNA exonuclease, 5 --> 3 specific	2.9
b4071	*nrfB*	formate-dependent nitrite reductase; a penta-haeme cytochrome c	2.9
b2348	*argW*	Arginine tRNA5	3.4
b0246	*yafW*	orf, hypothetical protein	3.4
b2059	*wcaA*	putative regulator	3.5
b3504	*yhiS*	orf, hypothetical protein	3.9
b3875	*yshA*	orf, hypothetical protein	4.3
b3119	*tdcR*	threonine dehydratase operon activator protein	5.1

Table 8.3. The list of genes whose expression is affected by *lsrK*. Genes consistently induced or repressed ($P < 0.05$) more than two-fold are shown

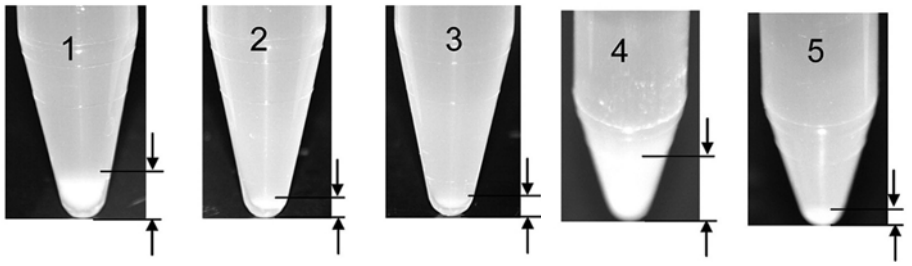
B #	Gene	Gene product	Fold change Δ*lsrK*/WT
b0396	*araJ*	involved in either transport or processing of arabinose polymers	-2.7
b0136	*yadK*	putative fimbrial protein	-2.4
b1980		orf, hypothetical protein	-2.4
b2651		orf, hypothetical protein	-2.1
b1318	*ycjV*	putative ATP-binding component of a transport system	-2.1
b2634	*yfjR*	orf, hypothetical protein	-2.1
b2932	*yggP*	orf, hypothetical protein	-2.1
b0044	*fixX*	putative ferredoxin	-2.0
b0108	*ppdD*	prelipin peptidase dependent protein	-2.0
b0141	*yadN*	putative fimbrial-like protein	-2.0
b0263		putative transport system permease protein	-2.0
b1256	*yciD*	putative outer membrane protein	-2.0
b1896	*otsA*	trehalose-6-phosphate synthase	-2.0
b2384		orf, hypothetical protein	-2.0
b1357	*ydaS*	orf, hypothetical protein	2.0
b1576	*ydfD*	orf, hypothetical protein	2.0
b0528	*ybcJ*	orf, hypothetical protein	2.0
b0901	*ycaK*	orf, hypothetical protein	2.0
b1012		orf, hypothetical protein	2.0
b1029	*ycdU*	orf, hypothetical protein	2.0
b1358	*ydaT*	orf, hypothetical protein	2.0
b1956		orf, hypothetical protein	2.0
b2253		putative enzyme	2.0
b2371	*yfdE*	putative enzyme	2.0
b2399	*yfeD*	orf, hypothetical protein	2.0
b2902	*ygfF*	putative oxidoreductase	2.0
b2921	*ygfI*	putative transcriptional regulator LYSR-type	2.0
b2972		putative peptidase	2.0
b3117	*tdcB*	threonine dehydratase, catabolic	2.0
b3489	*yhiK*	orf, hypothetical protein	2.0
b3556	*cspA*	cold shock protein 7.4, transcriptional activator of hns	2.0
b4338	*yjiP*	orf, hypothetical protein	2.0
b0532	*sfmD*	putative outer membrane protein, export function	2.1
b1409		putative phosphatidate cytidiltransferase	2.1
b1455		orf, hypothetical protein	2.1
b2374		putative enzyme	2.1
b3078	*ygjI*	putative oxidoreductase	2.1
b3906	*rhaR*	positive regulator for rhaRS operon	2.1
b3937	*yiiX*	orf, hypothetical protein	2.1
b4038	*yjbI*	orf, hypothetical protein	2.1

Table 8.3. (continued)

B #	Gene	Gene product	Fold change Δ*lsrK*/WT
b1147	*ymfL*	orf, hypothetical protein	2.2
b2969	*yghE*	putative general secretion pathway for protein export (GSP)	2.2
b3547	*yhjX*	putative resistance protein	2.2
b3808		orf, hypothetical protein	2.2
b4076	*nrfG*	part of formate-dependent nitrite reductase complex	2.2
b0702	*ybfB*	orf, hypothetical protein	2.3
b0691	*ybfH*	orf, hypothetical protein	2.3
b2886	*ygfS*	putative oxidoreductase, Fe-S subunit	2.3
b3659	*yicK*	two-module transport protein	2.3
b2853		orf, hypothetical protein	2.4
b0157	*yadS*	orf, hypothetical protein	2.4
b1159	*mcrA*	restriction of DNA at 5-methylcytosine residues; at locus of e14 element	2.4
b1557	*cspB*	cold shock protein; may affect transcription	2.4
b2357	*yfdN*	orf, hypothetical protein	2.4
b2846	*yqeH*	orf, hypothetical protein	2.4
b2849	*yqeK*	orf, hypothetical protein	2.4
b1353	*sieB*	phage superinfection exclusion protein	2.5
b1375	*ynaE*	orf, hypothetical protein	2.5
b2626	*yfjJ*	orf, hypothetical protein	2.5
b4066	*yjcF*	orf, hypothetical protein	2.5
b0364	*yaiS*	orf, hypothetical protein	2.6
b1722		orf, hypothetical protein	2.6
b3063	*ygjE*	orf, hypothetical protein	2.6
b3552	*yiaD*	putative outer membrane protein	2.6
b4205	*ytfA*	orf, hypothetical protein	2.8
b4385	*yjjJ*	orf, hypothetical protein	2.8
b0544	*ybcK*	orf, hypothetical protein	2.9
b1476	*fdnI*	formate dehydrogenase-N, nitrate-inducible, cytochrome B556(Fdn) gamma subunit	2.9
b1877	*yecT*	orf, hypothetical protein	2.9
b0558	*ybcV*	putative an envelope protein	3.0
b3946	*talC*	putative transaldolase	3.0



A



B

Fig. 8.4. **Autoaggregation assay of W3110 and isogenic mutants Δ*lsrR* and Δ*lsrK*.** (A) Time-resolved sedimentation results (autoaggregation assay). Symbols: ■, W3110; ●, W3110 Δ*lsrR*; ▲, W3110 Δ*lsrK*; □, ZK2686; ○, Δ*agn43* (isogenic mutant from parent strain ZK2686, called ZK2692). (B) Pictures of final pellets from the autoaggregation assay (A). 1, W3110; 2, W3110 Δ*lsrR*; 3, W3110 Δ*lsrK*; 4, ZK2686; 5, ZK2692.

repressed, respectively, at least twofold by *lsrR*, and there were 117 and 32 genes induced and repressed, respectively, by *lsrK*. Among these genes were 78 genes whose expression levels were changed by both *lsrR* and *lsrK* mutants (see Tables 8.1, 8.2, and 8.3).

The co-regulated genes comprise a number of genes that one might expect to be regulated by the QS mechanisms. For example, *flu,* which encodes phase-variable protein antigen 43 (Ag43), was dramatically derepressed, 10.8- and 6.3-fold in Δ*lsrR* and Δ*lsrK* mutants, respectively. Ag43 belongs to an autotransporter protein family, which regulates its own transport to the bacterial cell surface. Ag43 mediates cell-to-cell aggregation and thus enhances biofilm formation (Danese *et al.*, 2000a; Kjaergaard *et al.*, 2000; Reisner *et al.*, 2003). As Ag43 plays an important role in the initial recognition and attachment to host tissue surfaces, it also plays a role in the pathogenesis of disease-causing *E. coli* (Henderson *et al.*, 2004). We carried out an autoaggregation assay to see if *lsrRK* in fact played a role in aggregation, presumably through Ag43. An additional test between ZK2686 and its isogenic (Δ*flu*) mutant, ZK2692, was run to validate our assay as an indicator of Ag43 function. In Fig. 8.4, more wild-type cells settled to the bottom of the tubes, and faster, than both mutant strains (Fig. 8.4A), even though complete resolution of the assay took 2 days (Fig. 8.4B). We suspect that fimbrial blockage of autoaggregation may have contributed to this delay (Hasman *et al.*, 1999). The complete deficiency in autoaggregation of both *lsrR* and *lsrK* mutants is consistent with our microarray results and a regulatory model involving LsrR and LsrK.

There were 68 genes whose expression changed in an *lsrR* mutant alone (not changed in the *lsrK* mutant), and among these, 25 are hypothetical proteins, with unknown function (Table 8.2). We report a preponderance of genes associated with attachment, defense, and pathogenicity affected by the *lsrR* mutation. For example, a curli production assembly/transport component, *csgE*, from the second curli operon was repressed in the *lsrR* mutant. Curli is

associated with biofilm formation, host cell adhesion and invasion, and immune system activation, where CsgA is the major fiber subunit and CsgE, CsgF, and CsgG are nonstructural proteins involved in curli biogenesis (Barnhart and Chapman, 2006; Robinson *et al.*, 2006). *htrE*, a homolog of *papD* involved in type II pilus assembly (Raina *et al.*, 1993), was negatively regulated. Another putative fimbria-like protein, from b0942, was likely up-regulated (twofold increase in expression in the *lsrR* mutant). A transmembrane domain, *sapB* of the SapABCD system (homologs of the *S. enterica* serovar Typhimurium SapABCD proteins) (Parra-Lopez *et al.*, 1993), which is required for virulence and resistance to the antimicrobial peptides melittin and protamine, was repressed in the *lsrR* mutant. Meanwhile, an increase in the transcription of a protamine-like protein, *tpr*, was observed. *yheF* (also called GspD), which belongs to a secretin protein family and is involved in virulence and filamentous phage extrusion (Genin and Boucher, 1994), was up-regulated by the *lsrR* deletion. YheF proteins are not normally expressed and are silenced by the nucleoid-structuring protein H-NS (Francetic *et al.*, 2000). The upregulation of *yheF* due to the *lsrR* deletion is likely an example of bacterial self-protection.

There were 71 genes whose expression changed in an *lsrK* mutant only, and among these genes, 38 are annotated as being hypothetical proteins with unclear functions (Table 8.3). Like *lsrR*, a number of genes associated with attachment, defense, and pathogenicity were found to be regulated by *lsrK*, *ppdD*, which encodes a putative major type IV pilin, was repressed twofold in an *lsrK* mutant. PpdD was able to form type IV pili when expressed in *Pseudomonas aeruginosa*, as determined by immunogold labeling (Sauvonnet *et al.*, 2000a). PpdD also formed pili when pullulanase secretion proteins from *Klebsiella oxytoca* and *E. coli* K-12 *ppdD* were co-expressed in *E. coli* (Sauvonnet *et al.*, 2000b). Genes for two putative fimbrial proteins, *yadK* and *yadN*, were repressed 2.4- and 2-fold, respectively, in the *lsrK* mutant. *mcrA*, a type

IV site-specific DNase defending cells against foreign DNA such as bacteriophages (Anton and Raleigh, 2004), was up-regulated 2.4-fold. *sieB*, similar to a λ gene responsible for preventing phage superinfection (Faubladier and Bouche, 1994), was also upregulated 2.5-fold.

8.3.3 *lsrR* and *lsrK* regulate biofilm architecture and formation

It is known that flagella, fimbriae, type I pili, curli fibers, Ag43, exopolysaccharides (EPS), and other outer membrane adhesins are critical for biofilm development in *E. coli* (Danese *et al.*, 2000a; Danese *et al.*, 2000b; Prigent-Combaret *et al.*, 2000; Reisner *et al.*, 2003). However, several flagellum-related and motility-associated genes, such as the motility master regulon *flhDC* and type I adhesin, were unchanged in the *lsrR* and *lsrK* mutants compared to the parental strain. The complex nature by which EPS and capsular polysaccharides (CPS) exert influence on biofilm formation cannot be overstated. However, for example, in *Pseudomonas aeruginosa* and *V. cholerae*, QS is shown to control biofilm formation, in part, through the regulation of EPS synthesis (Davies *et al.*, 1998; Hammer and Bassler, 2003). Colanic acid synthesis is necessary for forming EPS and CPS during biofilm development (Danese *et al.*, 2000b; O'Toole *et al.*, 2000; Prigent-Combaret *et al.*, 2000). The product of the *wza* gene is associated with colanic acid synthesis for EPS and CPS surface expression and assembly (Beis *et al.*, 2004; Drummelsmith and Whitfield, 1999, 2000; Nesper *et al.*, 2003; Reid and Whitfield, 2005; Whitfield and Paiment, 2003). Many reports have described the importance of *wza* in biofilm formation, and changes in *wza* expression affect biofilm formation (Danese *et al.*, 2000b; O'Toole *et al.*, 2000; Prigent-Combaret *et al.*, 2000). Remarkably, this gene was induced 3.7- and 3.5-fold in *lsrR* and *lsrK* mutants, respectively. Also, a putative regulator for colanic acid synthesis, *wcaA*, was induced 3.5-fold in *lsrR* mutants.

Another biofilm-related gene, *flu*, an autotransporter, was significantly repressed in both

Table 8.4. Biofilm related genes from genomic profiling.

B #	Gene	Gene product	Fold Change	
			Δ*lsrR*/WT	Δ*lsrK*/WT
b2000	flu	outer membrane fluffing protein, similar to adhesin	-10.8	-6.3
b2062	wza	putative polysaccharide export protein	3.7	3.5
b0139	htrE	probable outer membrane porin protein involved in fimbrial assembly	-3.2	--
b1039	csgE	curli production assembly/transport component, 2nd curli operon	-3.0	--
b0942	b0942	putative fimbrial-like protein	2.2	--
b2059	wcaA	putative regulator	3.5	--
b0141	yadN	putative fimbrial-like protein	--	-2.0
b0136	yadK	putative fimbrial protein	--	-2.4

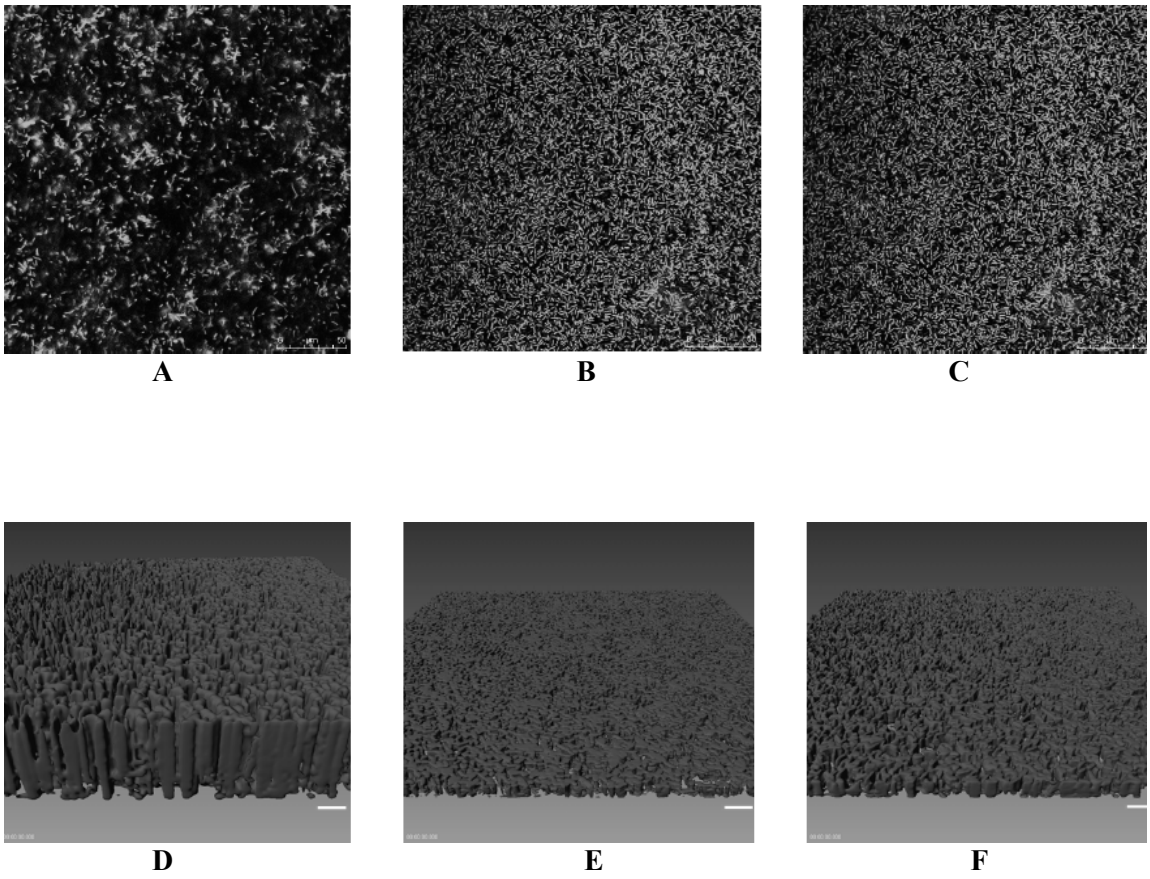


Fig. 8.5. Scanning confocal laser microscopic images of flow-cell-generated biofilms. (A and D) Wild-type W3110. (B and E) Isogenic *lsrR* mutant. (C and F) Isogenic *lsrK* mutant. We note that results for the *lsrR* mutant had a larger standard deviation than those for the *lsrK* mutant; the biofilm height was observed to fluctuate in the flow chamber (not shown here). A, B, and C are scanning confocal microscopic images; D, E, and F are reconstructed three-dimensional biofilm structures. The length scale is indicated by bars and the scale bar is 10 μM.

Table 8.5. Confocal analysis report of biofilm flow cell assay.

Strain	Biomass (μm^2/ μm^3)	Substratum Coverage (%)	Mean thickness (µm)	Roughness coefficient
W3110 type/pCM18	32.02± 3.2	20.4± 6.4	25.45 ±2.8	0.196± 0.08
Δ*lsrK*:kan^r/pCM18	3.64± 1.05	50.8 ± 4.7	2.95± 1.24	0.235± 0.05
Δ*lsrR*:kan^r/pCM18	13.43 ±14.43	44.6± 21.4	10.86± 11.85	0.229± 0.11

mutants (Table 8.4). The biofilm-related curli gene *csgE* and a putative fimbrial assembly gene, *htrE*, were both down-regulated upon *lsrR* deletion. In *lsrK* mutants, two putative fimbria-related genes, *yadK* and *yadN*, were down-regulated more than two-fold. Our SEM analysis revealed significant differences in cell-based fimbriae and matrices of both mutants compared to the wild-type (Fig. 8.2).

In order to further elucidate the variation in biofilm formation between the wild-type and mutants, we utilized confocal scanning microscopy on flow-cell-derived biofilms. Confocal images (Fig 8.5) show for the first time that more biofilm was formed at the substrate surface in both mutant strains than in the wild-type. Also, Imaris imaging demonstrated that the biofilm thicknesses of *lsrR* and *lsrK* mutants were similar and much less than that of the wild-type. Hence, the mean thickness and biomass of the wild-type were higher, while the substratum coverage was lower than that of the mutants (Table 8.5) (because more of the mutant biofilmsadhered to the bottom); that is, the bottom layer adjacent to the substrate appeared to be more substantive in the case of the mutants, and they tended to collapse and pack tightly onto the surface of the substrate.

In summary, two key functions known to affect biofilm formation and architecture colanic acid synthesis and fimbria formation, were shown to be regulated by genes of the QS regulators LsrR and LsrK. Also, biofilm structures were altered significantly in the *lsrR* and *lsrK* mutants.

8.3.4 Deletion of *lsrR* and *lsrK* affected sRNA expression

As shown recently by Lenz *et al.* (2004), small RNA (sRNA) species are involved in QS. We searched the intergenic regions for known and putative sRNAs (Table 8.6) and found that the global sRNA regulator DsrA was induced 3.6- and 4.4-fold in Δ*lsrR* and Δ*lsrK* strains,

Table 8.6. sRNAs affected by *lsrR* and *lsrK*.

Name	Start	End	Flanking gene	Fold Change		condition
				Δ*lsrR*/WT	Δ*lsrK*/WT	
DsrA	2023233	2023532	dsrB/yedP	3.6	4.4	confirmed
DicF	1647459	1647632	rzpQ/dicB	2.0	2.5	confirmed
RydB/tpe7	1762411	1762957	sufA(ydiC)/ydiH	4.9	--	confirmed
IS102	2069234	2069404	yeeP/flu	-2.3	-1.5	confirmed
Tpke70	2494586	2496690	ddg/yfdZ	2.0	--	confirmed
MicC (ISO63)	1434918	1435283	ompN/ydbK	3.2	--	confirmed
ayjiW	4577468	4577637	opposite yjiW	2.5	2.1	confirmed
SokX	2885243	2885600		--	3.5	confirmed
Unknown	2468480	2468778	yfdI/tfaS	2.3	3.2	predicted

respectively. DsrA is a riboregulator for RpoS and H-NS production, wherein DsrA enhances the translation of *rpoS* RNA by stabilizing *rpoS* mRNA. It also inhibits H-NS translation by sharply increasing *hns* mRNA turnover (Lease and Belfort, 2000; Majdalani *et al.*, 1998) and thereby curtails H-NS mediated transcriptional silencing. Correspondingly, DsrA RNA also affects CPS biosynthesis via increased production of the activator RcsA due its inhibitory effects on H-NS-mediated transcriptional silencing (Sledjeski *et al.*, 2001). DsrA also plays a regulatory role in acid resistance (Lease *et al.*, 2004). The regulatory effects of DsrA are mediated by specific RNA-to-RNA pairing interactions, while its stability and activity require the recruitment of Hfq (Brescia *et al.*, 2003; Moll *et al.*, 2003; Sledjeski and Gottesman, 1995; Sonnleitner *et al.*, 2004).

Since RpoS and H-NS play an important role in globally regulating genes in response to changing environments, it is not surprising that AI-2-related QS networks utilize their uptake regulators (e.g., LsrR and LuxP), together with DsrA, in a hierarchial modality for mediating prompt responses to environmental stimuli and extracellular stresses. Induction of components (*yheE* and *yheF*) in the type II secretion complex GspC to GspO is a good example of DsrA regulation: type II secretion was silenced by H-NS in wild-type *E. coli* (Francetic *et al.*, 2000), while DsrA anatagonizes the H-NS-mediated silencing of numerous promoters (Sledjeski and Gottesman, 1995). Therefore, the QS-mediated induction of DsrA resulting from *lsrR* and *lsrK* deletions leads to amplified expression of *yheE* and *yheF* (seemingly in the *lsrR* mutant only).

The sRNA cell division inhibitor DicF was induced by at least two-fold in both *lsrR* and *lsrK* mutants. A two-fold increase in expression of the cell division gene *dicB* was also observed, which is not surprising since both genes belong to the same cell division operon (Table 8.1) (Bouche and Bouche, 1989). *dicF* inhibits cell division in *E. coli* by decreasing the abundance and activity of FtsZ; therefore, DicF affects the septum formation and separation of

the replicated chromosomes into daughter nucleoids (Tetart *et al.*, 1992; Tetart and Bouche, 1992). However, this inhibition effect can be suppressed by an *rpoB* mutation, and the inhibition effect is partially counteracted by an *rpoS* mutation (Cam *et al.*, 1995). We note, however, that we did not see elongated cells in *lsrR* and *lsrK* mutants in our SEM studies. This is probably because cell division is a complex process controlled by many modes of regulation (Aarsman *et al.*, 2005; Chen and Beckwith, 2001; Hale and de Boer, 2002; Vicente and Rico, 2006).

Another sRNA immediately upstream of the *flu* gene was repressed in both mutants. This might account for the dramatic decrease in *flu* expression, 10.8- and 6.3-fold, respectively, in the *lsrR* and *lsrK* mutants described above, although a monocistronic RNA has not been identified. Two other sRNAs were found to be co-regulated by the *lsrR* and *lsrK*: one is *ayiW*, and the other, which is unnamed, is located between *yfdI* and *tfaS*. The function of these riboregulators remains unclear and awaits further research.

The remaining sRNAs revealed in our study (Table 8.6) include three from the *lsrR* deletion (RydB, MicC, and Tpke70) and one from the *lsrK* deletion (SokX). High-copy expression of RydB decreases *rpoS* expression during the stationary phase in LB medium (Wassarman *et al.*, 2001). It is intriguing to speculate that LsrR associates with RydB to assist in fine-tuning QS circuitry through the regulation of the global regulator RpoS. MicC works similarly as an antisense mechanism and is induced when cells are grown at low temperatures or in minimal medium (Chen *et al.*, 2002). MicC negatively regulates the translation of an outer membrane protein, OmpC (Chen *et al.*, 2004). Consistent with this posttranscriptional regulation, we did not see transcriptional changes in *ompC* expression or changes in the expression of its regulator, *ompF*. Finally, Tpke70 is an antisense RNA with an unknown function. In *lsrK* mutants, SokX, of unknown function was induced 3.5-fold.

8.4 Discussion

In contrast to our previous microarray studies of W3110 and a *luxS* mutant strain, LW7, where fewer than 50 genes were significantly affected by *luxS* mutation (Wang *et al.*, 2005c), our current study found many genes that were significantly affected by the *lsrR* and *lsrK* deletion (146 and 149 genes, respectively). Of these genes, only nine were regulated in exactly the same manner as that described previously for LuxS-regulated genes (Fig. 8.1) (Wang *et al.*, 2005c; Xavier and Bassler, 2005b). Deletion of *lsrR* results in the induction of the *lsr* operon (including the *lsrACDBFG* and *tam* genes), while deletion of *lsrK* results in the depression of those genes; that is, upon entry into the cell via the Lsr transporter, AI-2 is phosphorylated by LsrK. Phospho-AI-2 can bind the cognate transcriptional regulator LsrR and de-repress gene expression. The immediate targets of this de-repression are the very same AI-2 uptake genes (Fig. 8.1). Observations that AI-2 regulates its own uptake and transcriptome results indicating that few genes are impacted by the *luxS* mutation (Wang *et al.*, 2005c) have fueled speculation that AI-2 is limited in its role as a signal molecule in *E. coli*. Indeed, in our previous report, we suggested that the signaling role of AI-2 might require additional cellular factors (Wang *et al.*, 2005c).

A significant difference between the present *lsrR* and *lsrK* mutants and the *luxS* mutant is in the roles of the expressed proteins: signal perception versus signal generation. The present study enables a linkage between *lsrR* and *lsrK* to AI-2 as a signaling molecule. A key to this understanding was revealed previously but not reported for its importance (Wang *et al.*, 2005b; Wang *et al.*, 2005c): the *lsr-lacZ* and *lsrR-lacZ* reporters in *lsrR* and *lsr* operon mutants were both up-regulated manifold in both strains. Of note, the *lsr* transcription rate in an *lsr* operon mutant was up-regulated to an almost equivalent extent to that in an *lsrR* mutant strain, suggesting that the cells still possessed phospho-AI-2 even though they did not possess the

uptake complex. These cells did import AI-2 but a much slower rate than the wild-type and *lsrR* mutants. The same transcriptional reporter plasmid was nearly completely inactive in the *lsrK* mutant, as expected (consistent with the absence of de-repression afforded by phospho-AI-2). Interestingly, the extracellular AI-2 level in LsrK mutants never dropped, suggesting that AI-2 was not taken up by the cells in the absence of LsrK. These results suggest that AI-2, taken in by an alternative transporter (alluded to in (Wang *et al.*, 2005c)) or otherwise unsecreted AI-2, may still be phosphorylated by LsrK. These findings also suggest that (i) the Lsr transporter does not function without LsrK and (ii) LsrK can work with another transporter. These findings also give rise to the possibility that LsrK (and LsrR) may work on genes other than the *lsr* operon.

Indeed, the present analysis reveals a host of genes regulated by LsrR and LsrK, most of which did not appear in *luxS* mutants. Perhaps the most striking results of our current study are that (i) the majority of genes affected by the *lsrR* mutation are also affected by the *lsrK* mutation, (ii) the expression of the vast majority of these genes are identically affected (up or down) by both mutations, and (iii) these "co-regulated" genes are not those of the *lsr* regulon (*tam*, *metE*, *yneE*, and *lsr* operons) (Wang *et al.*, 2005c). These findings suggest that AI-2, in addition to phospho-AI-2, is an LsrR regulator; that is, for the apparently co-regulated genes, we suspect that a totally different regulatory mechanism than that is shown in Fig. 8.1 exists (in which LsrR is a repressor (and at times an activator) and its repression is released by phospho-AI-2). We propose an extended hypothesis that LsrR is a QS regulator that acts in tandem with unphosphorylated AI-2 or its anomer (Fig. 8.6); namely, AI-2 binds to LsrR and de-represses the transcription of a variety of genes, those identified in Table 8.1., which one might expect should be under QS regulation (as opposed to metabolic genes associated with the activated methyl cycle) (Wang *et al.*, 2005c).

Our extended model is either supported by or consistent with all studies reported to date

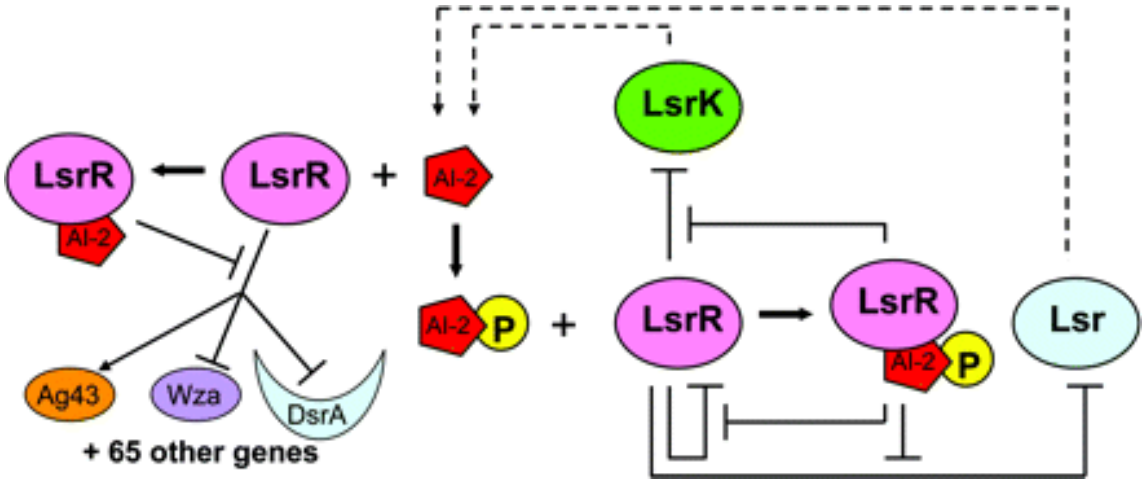


Fig. 8.6. Proposed AI-2 signaling with QS regulators LsrR and LsrK. During early and mid-exponential phases, AI-2 levels are low, insufficient for triggering rapid uptake. lsrR binds and represses many genes including *lsr* genes. As AI-2 accumulates extracellularly, it begins to be transported into the cells via a non-Lsr pathway or otherwise accumulates within the cells (or other pre-AI-2 anomers), which then bind to LsrR and derepress many QS genes including *lsrR*, *flu*, *wza*, and *dsrA* (crescents represents sRNA). Lsr-mediated AI-2 uptake remains repressed, as its derepression is due to phospho-AI-2. This temporal model is consistent with the activation of QS phenotypes that are switched on in late exponential phase. Finally, when the AI-2 concentration reaches the uptake "threshold" and cells sense nutrient depletion, *lsr* rapidly imports AI-2. Thereafter, cells phosphorylate the imported AI-2 signal, which results in the cessation of the LsrR/AI-2 regulation and amplification of Lsr/phosphor-AI-2 regulation. Hence, a rapid QS switch is manifested by a change in the phosphorylation state of AI-2 and its binding to LsrR.

concerning AI-2, *luxS*, and *lsr* in *E. coli* and *Salmonella*. LsrR is a known transcriptional regulator that responds to the binding of QS signal AI-2 (Taga *et al.*, 2003; Xavier and Bassler, 2005b). We suggest that the binding of phospho-AI-2 competes favorably with the binding of unhphosphorylated AI-2 and that the interchange between AI-2 and phospho-AI-2 represents a switching mechanism for the affected genes. LsrR is transcribed in its own monocistronic operon with the kinase LsrK in a manner divergent from that of the *lsr* operon (Wang *et al.*, 2005c). It is therefore distinct from the uptake genes and can operate independently. AI-2 is internalized and phosphorylated by the Lsr/LsrK complex. We suggest this is the predominant mode of AI-2 entry into the cells and, by phosphorylation, prevents AI-2 efflux. However, AI-2 is taken up by Lsr mutants. Also, intracellular AI-2 by deletion of *ydgG* has been noted (Herzberg *et al.*, 2006), and AI-2 is synthesized by non-LuxS/Pfs pathways (Li *et al.*, 2006). All are consistent with the existence of AI-2 (or its anomers) inside cells. An *lsrR* mutation leads to deficient LsrR expression and identification of LsrR-regulated genes. An *lsrK* mutation results in a lack of imported phospho-AI-2 (Wang *et al.*, 2005c), which is the principal signal for de-repression of the *lsr* operon. We suggest that unphosphorylated AI-2 participates as a specific regulator of LsrR activity and that this mode of activity is the dominant feature of LsrR-mediated QS. We also suggest that the phosphorylated AI-2 acts as a "trigger" to terminate AI-2-mediated cellular processes and initiate the recycling of AI-2 through the *lsr* operon, which thus serves as the interconnect between the signaling process and a metabolic function. The metabolic functions of LuxS were described previously (Wang *et al.*, 2005c).

We prefer this signaling modality for AI-2 mediated QS for two reasons: first, QS signals have been shown to be global regulators, and hence, cells must have a purpose for importing and processing AI-2; second, *lsrR* and *lsrK* belong to an AI-2-mediated regulon (Wang *et al.*, 2005c). Thus, it is not surprising that *E. coli* utilizes the product of this operon to

globally control the cellular phenotype. Our transcriptome results agree with this model, since most of the LsrRK-regulated genes are related to the cell's secretion systems (e.g. *flu* and *yheE*), biofilm formation (e.g. *wza*), transcriptional regulators (e.g. *mhpR*, *tdcA*, and *envR*), sRNAs or riboregulators (e.g. DsrA), and regulatory proteins for stress responses and nutrient depletion (e.g. *adiY*, *glnK*, and *cspF*). Indeed, AI-2 is perhaps a global regulator of *E. coli* (Xavier and Bassler, 2003) only when it is coupled to another regulator, as LuxP in *Vibrio harveyi*.

Our observation of biofilm phenotypes in *lsrR* and *lsrK* mutants cooperates with this model: AI-2 probably binds with LsrR to mediate biofilm architecture and formation by coordinately regulating interactions of biofilm-related genes, including the colanic acid synthesis regulator *wza* and the *flu* gene. Equally importantly, both of the genes affecting the phenotypes and the phenotypic outcomes were altered identically for the *lsrR* and *lsrK* strains. These findings suggest an intimate coordination between *lsrR* and *lsrK*, as shown in Fig. 8.6. The influence of these genes on biofilm structure has already been elucidated: their QS dependence is shown here for the first time.

Finally, our study provides the first evidence that sRNAs interact with QS regulators in *E. coli* K-12. A riboregulator, RsmZ, was found to control biofilm formation and type III secretion in *Pseudomonas aeruginosa* (Ventre *et al.*, 2006). Previous reports conclusively demonstrated that four sRNAs are intimately involved in the QS networks of *V. harveyi* and *V. cholerae* and act through the RNA chaperone Hfq (Lenz *et al.*, 2004). This finding is the first to suggest the convergence of an *E. coli* QS signaling system onto the Hfq/LuxO transduction process of *V.harveyi* and suggests yet one more modality for which bacterial autoinducer signal transduction occurs.

8.5 Experimental procedures

8.5.1 Bacterial strains and growth conditions

E. coli K-12 strain W3110 (F^-, λ^-, in *rrnD-rrnE*) was obtained from the Genetic Stock Center (New Haven, CT). Details of its kanamycin resistant isogenic mutants used in this study, W3110 Δ*lsrR* and W3110 Δ*lsrK* were described elsewhere previously (Wang *et al.*, 2005b). *E. coli* strain ZK2686 (W3110 Δ(*argF-lac*)*U169*) and its isogenic *agn43* mutant ZK2692 (ZK2686 *agn43*::*cam*) were kindly provided by R. Kolter (Danese *et al.*, 2000a). Luria Bertani broth (LB) contains 5 g/L yeast extract (Difco), 10 g/L bacto tryptone (Difco), and 10 g/L NaCl. Cultures of *E. coli* (wild-type, Δ*lsrR*, and Δ*lsrK* mutants) grown overnight in LB, were diluted to an optical density at 600 nm of 0.03 in LB and subsequently incubated at 30°C and 250 rpm in two 50-mL shake flasks. When the cultures reached the appropriate OD_{600} (2.4), the cells were harvested for RNA extraction.

8.5.2 RNA isolation, cDNA generation, and microarray processing

Total RNA was isolated using RNeasy Mini kits (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized and labeled according to the manufacturer's suggestions for *E. coli* Antisense genome arrays (Affymetrix Inc., Santa Clara, CA). Further preparation, hybridization, and scanning were carried out as previously described (Wang *et al.*, 2005c). Reverse transcription (RT)-PCR was also performed as previously reported (Wang *et al.*, 2005c), except that an Applied Biosystems 7300 real-time PCR system (Applied Biosystems) was used. 16S rRNA was used for normalizing all reactions; its transcript levels showed minimal variation between wild-type and mutant cells (data not shown).

8.5.3 Microarray data analysis

Microarray data were analyzed with the Affymetrix Microarray Suite software 5.1 (Affymetrix Inc., Santa Clara, CA) and four-comparison survival method (Chen *et al.*, 2000). The fluorescence of each array was normalized by scaling total chip fluorescence intensities to a common value of 500. For each growth condition, two independent experimental cell cultures (wild-type) were compared with two independent control groups (Δ*lsrR* or Δ*lsrK* mutant), and four comparisons were made. The change (*n*-fold) for each gene was calculated by dividing the signal intensity for these two mutants by the signal intensity for the wild-type. The reported values for the change (*n*-fold) are the averages of the four comparisons. Genes with consistent increases or decreases in all comparisons were determined and used for the analysis. However, the induced genes with absent calls of the array signal in the experimental groups and the repressed genes with absent calls of the array signal in the control groups were eliminated. Determinations of functional categories were based on the *E. coli* K-12 MG1655 database from TIGR (http://www.tigr.org/tigr-scripts/CMR2/gene_table.spl?db=ntec01).

8.5.4 SEM

Cells were collected and gently washed three times with Millonig's phosphate buffer (pH 7.3) (centrifugation at 2,000 *g* for 10 min) and fixed with 2% glutaraldehyde (1 h at room temperature and 9 h at 4°C). Cells were collected with 0.2 μL filters, and residual glutaraldehyde was washed out using Millonig's phosphate buffer three times before cells were further fixed in 1% OsO_4. The filters were then dehydrated with ethanol (70%, 95%, and 100%). The filters were fully dehydrated in a Denton vacuum freezer (Denton DCP-1 critical-point dryer) and coated with Ag-Pd (Denton DV 502/503 vacuum evaporator). Coated filters were

examined by scanning electron microscopy (SEM) at the Biological Ultrastructure Laboratory of the University of Maryland (College Park, MD).

8.5.5 Autoaggregation assay

Autoaggregation assays were performed as described previously (Hasman *et al.*, 1999), with slight modifications. Cultures grown overnight were adjusted to the same optical densities, and 10 mL of each culture was placed into a 15 mL Falcon tube and kept on ice. At each time point, 100 µL samples were taken from each tube, 1 cm from the top, and transferred into new tubes containing 1 mL 0.9% NaCl for measuring optical densities.

8.5.6 Flow cell biofilm experiments and image analysis

E. coli K-12 W3110 cells constitutively expressing green fluorescent protein via pCM18 were streaked onto LB agar plates with erythromycin (300 µg/mL) and grown in the same medium overnight. *E. coli* K-12 W3110 Δ*lsrK*:Kan^r/pCM18 and *E. coli* K-12 W3110 Δ*lsrR*:Kan^r/pCM18 were streaked onto LB agar plates with erythromycin (300 µg/mL) and kanamycin (50 µg/mL) and then grown overnight in the same medium. Cultures grown overnight were diluted into LB and erythromycin (300 µg/mL) to reach an OD_{600} of 0.05. The flow cell (Domka *et al.*, 2006) was inoculated for 2 h at 30°C with 200 mL of these cells, and fresh medium was then added at a flow rate of 10 mL/h for 49 h. The number of cells in the culture after 2 h of inoculation was 1.4×10^5 to 3.2×10^5 cells/mL. For the wild-type strain, biofilm formation was not significant at 24 h, so only images at 49 h were taken for all three strains. Green fluorescent protein was visualized by excitation with an Ar laser at 488 nm (emission, 510 to 530 nm) using a TCS SP5 scanning confocal laser microscope with a 63 x HCX PL FLUOTAR L dry objective with a correction collar and a numerical aperture of 0.7

(Leica Microsystems, Manheim, Germany). Color confocal flow cell images were converted to gray scale using an image converter (Neomash Microsystems, Wainuiomata, Wellington, New Zealand). Biomass, substratum coverage, surface roughness, and mean thickness were determined using COMSTAT image-processing software (Domka *et al.*, 2006) written as a script in Matlab 5.1 (TheMathWorks) and equipped with image processing toolbox. Tresholding was fixed for all image stacks. At each time point, nine different positions were chosen for microscope analysis, and 225 images were processed for each position. Values are means of data from the different positions at the same time point, and standard deviations were calculated based on these mean values for each position. Reconstructed three-dimensional images were obtained using IMARIS (BITplane, Zurich, Switzerland). Twenty-five pictures were processed for each three-dimensional image.

CHAPTER IX

CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

Nine previously uncharacterized genes of *Pseudomonas aeruginosa* as plant-pathogenicity determinants were identified. Seven genes were identified in the study of *P. aeruginosa* PAO1 virulence factors and poplar tree response in the rhizosphere (PA1385, PA2146, PA2462, PA2463, PA2663, PA4150, and PA4295) (Chapter III). One gene (*pyrF*) was identified in "Uracil influences quorum sensing and biofilm formation in *P. aeruginosa* and fluorouracil is an antagonist" (Chapter VI) and the last one (PA0939) was identified in Appendix B. We also found that *P. aeruginosa* is a pathogen for poplar trees and barley.

We performed the first whole-transcriptome analysis of *P. aeruginosa* in the rhizosphere and the first-transcriptome analysis of poplar trees. The DNA microarray analysis revealed that colonization of *P. aeruginosa* in the poplar rhizosphere induced 183 genes, including the genes for virulence (type III secretion, hemolysis, immunity, and attachment) and repressed 419 genes including the genes for attachment, chemotaxis, flagella, motility, and protein secretion. Through microarrays and by using transposon mutants in *in vivo* and in *in vitro* experiments, seven genes were characterized for poplar or barley pathogenesis.

PA2663, a hypothetical protein discovered through our microarray data and plant pathogenesis experiments, induces biofilm formation. Through the DNA microarray analysis, we discovered the PA2663 protein induces the *psl* operon and pyoverdine synthesis. PA2663 is the first protein found to control the *psl* operon of *P. aeruginosa*. The PA2663 protein induces quorum-sensing (QS) phenotypes, PQS, and elastase production. In addition, the PA2663 protein represses the expression of an anti-terminator, PA0939. The PA0939 protein represses

biofilm formation and induces swimming and swarming motility (Appendix B). PA0939 protein also induces the virulence of *P. aeruginosa* for barley germination; therefore, one new plant pathogenicity-related protein was discovered.

The effects of indole and 7-hydroyxindole on virulence of *P. aeruginosa* were also examined. Indole and 7-HI repressed the expression of genes that are induced by the addition of QS molecules, C_4-HSL and 3-oxo-C_{12}-HSL. Corroborating the microarray results, indole and 7-HI repressed the production of pyocyanin, rhamnolipid, PQS, and pyoverdine. These results confirmed that indole and 7-HI are QS inhibitors and anti-virulence compounds. This is the first study of indole and 7-HI on *P. aeruginosa* virulence factors, and these compounds may have therapeutic potential to be used against *P. aeruginosa* infections.

By screening uracil analogs, 5-fluorouracil (5-FU) was discovered as a new biofilm inhibitor for both *P. aeruginosa* and *E. coli* strains. Detailed studies of 5-FU revealed that 5-FU also decreases the virulence factors of *P. aeruginosa* and represses the expression of virulence genes of *E. coli*. DNA microarrays and biofilm experiments revealed that 5-FU works through *E. coli* protein AriR. Hence, 5-FU may have therapeutic potential to be used against *P. aeruginosa* and *E. coli* infections.

The biofilm formation of the autoinducer-2 (AI-2) regulator mutants in *E. coli*, *lsrR*, and *lsrK* were examined with confocal scanning microscopy, IMARIS imaging, and COMSTAT analysis. It was found that *lsrR* and *lsrK* mutants lose the ability to form thick biofilms; however, their surface coverage is higher than that of the wild-type strain.

9.2 Recommendations

Although this study opened new frontiers in the understanding of virulence factors of *P. aeruginosa* and plant-bacteria interactions, there are many opportunities to broaden our knowledge of bacterial virulence factors.

9.2.1 Discovery of the mechanism of PA0939 protein for biofilm formation and motility

Through the transcriptome analysis of the PA2663 mutant versus the wild-type strain, we found that the expression of PA0939 gene was induced. The mutation in PA0939 gene induces biofilm formation and represses swimming and swarming (Appendix B). Further experiments revealed that the PA0939 protein affects barley germination (Appendix B). We have also performed DNA microarray analysis to explore the mechanism by which PA0939 represses biofilm formation in *P. aeruginosa*. However, in the DNA microarray analysis, biofilm-related genes were not differentially expressed; therefore, we could not discover how PA0939 regulates biofilm formation in *P. aeruginosa.*

To decipher the proteins through which PA0939 protein works, transposon mutagenesis may be performed with the PA0939 mutant. A double mutant library with the PA0939 mutant can be generated by using Tn*5*-*luxAB* transposition (de Lorenzo *et al.*, 1990). This transposon was previously used in *P. aeruginosa* PA14 to find attachment defective and biofilm mutants (Ramsey and Whiteley, 2004). The mutants obtained can be first screened for reduced biofilm formation in 96-well plates. Then, the mutants with reduced biofilm formation can be screened for swimming and swarming motilities and barley seed virulence. The genes that affect all four phenotypes (biofilm formation, swimming motility, swarming motility, and barley germination) of PA0939 protein can be identified by determining the position of Tn*5*-*luxAB* transposon insertion. The position of Tn*5*-*luxAB* transposon insertion can be determined by using pGEM

easy vector (Promega, Madison, WI), and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), as described previously (Ramsey and Whiteley, 2004).

9.2.2 Discovery of the mechanism of PA2463 protein for induced virulence on poplar trees

In Chapter III, we discovered that the PA2463 mutation increased poplar tree wilting by 2-fold and hemolytic activity by 4-fold. As PA2463 is next to hemolysis gene PA2462 (Winsor *et al.*, 2005), we speculated that PA2463 is a hemolysis repressor (Chapter III). We performed a microarray experiment for the PA2463 mutant in LB medium (Appendix C). However, to learn the mechanism by which the PA2463 mutant is more virulent to poplar trees, the whole-transcriptome of the PA2463 mutant must be examined upon poplar tree infection. This experiment will help in deciphering the proteins involved in pathogenesis by PA2463.

9.2.3 Discovery of the virulence factors of *E. coli* O157:H7 upon poplar tree infection

During the study of *P. aeruginosa* infection on poplar trees, we also observed that *E. coli* O157:H7 (EHEC) strains were pathogenic to poplar trees (data not shown). Since using poplar trees as a host model makes possible the elucidation of the virulence genes (Attila *et al.*, 2008a), and the fact that the pathogens conserve the virulence factors in different hosts (Rahme *et al.*, 2000), it will be intriguing to examine the whole transcriptome of EHEC upon infection of poplar trees. The EHEC poplar infection experiments can be performed as described previously (Chapter III). However, the time course of infection should be determined by analyzing the infection of EHEC on poplar trees. The genes that will be identified through the microarray data can be knocked-out (Baba *et al.*, 2006). The mutants that will be obtained might first be screened for their virulence on poplar trees and on other plants (e.g. barley). The mutants can also be screened for rhizosphere competition (Attila *et al.*, 2008a). Since, EHEC attaches to

human epithelial cells (Iimura *et al.*, 2005), EHEC mutants deficient in plant virulence and in rhizosphere competition might be examined for adhesion to human epithelial cells.

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APPENDIX A

EXPERIMENTAL PROTOCOLS

A.1 HRP minimal medium for bacterial growth in the rhizosphere

The recipe of HRP minimal medium is based on Huynh *et al.* (1989). This medium is used during the contact of bacteria with poplar trees. To 250 mL of distilled H_2O, add:

KH_2PO_4	13.75 g
K_2HPO_4	3.75 g
$(NH_4)_2SO_4$	2.5 g
$MgCl_2$	0.86 g
NaCl	0.25 g
ddH_2O	fill until 250 mL
Sucrose	6.25 g

Adjust pH to 5.5 and filter sterilize.

A.2 25% Hoagland's solution

This solution is based on Shim *et al.* (2000) and is used to grow to grow poplar trees.

To 1 L of distilled H_2O, add:

KH_2PO_4	0.21 g
$Ca(NO_3)_2$	0.16 g
$CaSO_4$	0.29 g
KNO_3	0.14 g
$MgSO_4$	0.47 g
K_2SO_4	0.16 g
KCl	7.5 mg
H_3BO_3	3.1 mg
H_2MO_4	0.2 mg

Fertilome liquid iron 5 mL

A.3 RNA isolation from poplar roots

RNA isolation is adapted (Brunner *et al.*, 2004) with minor modifications.

There are reagents and materials required apart from the Qiagen RNA isolation kit (Cat # 74903): mortar, pestle, polyvinylpyrollidone, liquid nitrogen, 5 M potassium acetate buffer (pH 6.5), 2 collection tubes (2 mL), 5 collection tubes (1.5 mL), and ethanol (100 %).

Add 10 µL of *β*-mercapthoethanol into 1 mL of buffer RLT (Qiagen RNA isolation kit) . Then add 0.01 g of polyvinylpyrollidone in 1 mL of buffer RLT. Use the following steps to perform the RNA isolation experiment:

1. Cut the poplar tissue with a scissor.

2. Determine the amount of poplar root for RNA isolation. Do not weigh it, weighing time causes RNA to degrade. Take around 0.2 g of poplar roots.

3. Place the weighed sample in liquid nitrogen and grind thoroughly with a mortar and a pestle.

4. Add the powder to a tube containing 1 mL of RNeasy RLT buffer containing 0.01 g of polyviniylpyrrolidone, shake for 1 minute, then homogenize it with a vortex for 30 seconds.

5. Add 0.4 volume of 5 M potassium acetate (pH 6.5), and mix it by inversion and incubate on ice for 15 min.

6. Centrifuge at 12,000 rpm at 4°C.

7. Transfer the supernatant to a new 1.5 mL microcentrifuge tube.

8. Add 0.5 volume of the solution of ethanol (100 %) to the cleared lysate. Afterwards, mix the solution immediately by pipetting.

9. Add 650 µL of the sample onto the RNeasy mini column (Cat# 74903) placed in a 2 mL collection tube including any precipitate that may form. Please note, that if the volume exceeds

700 µL, load aliquots successively onto the RNeasy column and centrifuge again. Centrifuge the tube for 15 s at greater or equal to 8,000 *g*. and discard the flow through.

10. Add 700 µL of Buffer RW1 to the RNeasy column. Centrifuge the tube for 15 s at greater or equal to 8,000 *g* and discard the flow through.

11. Transfer the RNeasy column into a new 2 mL collection tube. Pipet 500 µL of buffer RPE (Qiagen RNA isolation kit) onto the RNeasy column. Centrifuge the tube for 15 s at greater or equal to 8,000 *g*. Discard the flow through.

12. Add another 500 µL of Buffer RPE onto the RNeasy column. Centrifuge the tube for 15 s at ≥ 8,000 *g*. Discard the flow through.

Optional: Place the RNeasy column in a new 2 mL centrifuge tube and centrifuge in a microcentrifuge at full speed for 1 min.

13. Transfer the RNeasy column to a new 1.5 mL centrifuge tube and pipet 30 µL of Rnase free water directly onto the RNeasy silica-gel membrane. Centrifuge the tube for 1 min at ≥ 8,000 *g*.

14. Pipet another 30 µL of Rnase free water onto the RNeasy silica-gel membrane and centrifuge the tube for 1 min at ≥ 8,000 *g*. Vortex the solution.

15. Place the microcentrifuge tube which contains RNA at -80°C.

16. Check the concentration and purity of RNA. The concentration and purity of RNA should be determined by measuring the absorbance at 260 nm in the spectrophotometer (Shimadzu UV-1601, Kyoto, Japan).

A.4 Phenotype microarray protocol

Use the following steps to perform the phenotype microarrays protocol (Cat# 12111, 12112, 12121, 12131, 12141, 12181, 12182, 12183) (Biolog, Hayward, CA):

1. Streak the strains on a BUG agar + blood plate and grow overnight.

2. Subculture a second time (streak a single colony) on BUG agar + blood plates.

3. Remove the cells from the plates using a sterile swab and transfer into a sterile tube containing inoculating fluid, IF-O, (1.2 X). Set the OD_{600} of 0.065. This is equal to 85% transmitter in the Biolog turbidimeter.

Before working with IF-0, practice with water to have an idea of how many colonies will be equal to OD_{600} of 0.065.

4. Preparation of the plates:

PM1 – PM2A Plates:

To inoculate two 96-well plates, use the following steps:

(i) Add 17.6 mL of IF-0 a Base (1.2 X).

(ii) Add 0.24 mL of Dye Mix D.

(iii) Add 3.76 mL of sterile DI water.

(iv) Add 2.4 mL of 85% T (OD_{600} of 0.065) bacterial suspension in IF-0 and mix completely.

The final density is 1: 10 dilution of bacterial suspension.

Inoculate PM1 and PM2A plates with 100 µL of the prepared solution in step 1 by using octapipette.

PM3-PM8 Plates:

To inoculate six 96-well plates, use the following steps:

(i) Add 66.0 mL of IF-0 a Base (1.2 X)

(ii) Add 0.90 mL of Dye Mix D

(iii) Add 0.90 mL of the carbon source (100 X).

(iv) Add 13.2 mL of sterile DI water.

(v) Add 9.0 mL of 85% T (OD_{600} of 0.065) bacterial suspension in IF-0 and mix completely.

The final density is 1:10 dilution of bacterial suspension.

Inoculate PM3-PM8 plates with 100 µL of the prepared solution in step 1 by using an octapipette.

5. Incubate the plates at 30 or 37°C quiescently.

6. Measure the OD_{590}.

7. Find the contents of each plate in phenotype microarray folder.

A.5 Quorum sensing and pyoverdine experiments

A.5.1. PQS protocol

A.5.1.1 PQS extraction

The PQS protocol is adapted (Gallagher *et al.*, 2002; Pesci *et al.*, 1999). The chemically synthesized PQS on a thin layer chromatography (TLC) plate is shown in Fig. 5.3.

1. Adjust the overnight culture to OD_{660} of 0.02 in LB medium.

2. Incubate the culture for 24 h with shaking. Depending on the experiment conditions, time and medium can be varied.

3. Add 300 µL of the culture into 900 µL of acidified ethyl acetate culture. 600 µL of the culture can be used to see PQS more clearly on the plates. Add 0.1 mL of glacial acetic acid into 1 L ethyl acetate to obtain acidified ethyl acetate.

4. Vortex the samples for 30 sec at maximum.

5. Centrifuge the samples at maximum for 5 min.

6. Take 800 µL of an aliquot of the upper organic layer sample into a new centrifuge tube.

7. Dry the solvent with nitrogen in the fume hood. (Place the needle into the tube, sparge with nitrogen for 5 min at 386 ml/min, and dry the solvent).

8. Resuspend the PQS in about 40 µL of 1:1 mix of acidified ethyl acetate with acetonitrile or 100 % methanol.

A.5.1.2 Preparation of TLC plate

1. Incubate the TLC plates at room temperature for 30 min in 5% KH_2PO_4 with rocking.

2. Incubate at 100°C for 1 h.

3. Prepare the chamber solvent (around 100 mL).

The chamber solvent consists of 95:5 mixture of dichloromethane:methanol

4. Add the samples to the TLC plates and place in the chamber until the solvent has traveled off the top of the plate.

5. Take the pictures of the plates under the Gel Imager system. Determine and photograph the PQS levels using the Bio-Rad VersaDoc 3000 imaging system (Bio-RAD, Hercules, CA) in room 516 in Jack E. Brown Building. Try 90 sec. exposure time. To have a nice image, exposure time can be adjusted. Do not forget to use background in the imager system; because it is necessary to substract the background value of the plate.

A.5.2 Pyoverdine protocol

Prepare minimal standard succinate medium (Ren *et al.*, 2005b):

To 1 L of distilled H_2O, add:

KH_2PO_4	3 g
K_2HPO_4	6 g
$MgSO_4.7H_2O$	0.2 g
$(NH_4)_2SO_4$	1 g
$C_4H_4Na_2O_4 \cdot (H_2O)_6$	9.15 g

1. Streak the culture on a LB plate.

2. Inoculate a single colony into minimal medium and grow overnight at 30 or 37 °C.

3. Reinoculate the overnight culture to the minimal standard succinate medium to an optical density of 0.05 at 600 nm.

4. Measure the OD_{600} at different time points (2-11 hrs.). At the same time, take 1 mL of culture in a microcentrifuge tube and centrifuge at maximum for 1 min. Transfer the supernatant to a plastic cuvette and measure the OD_{405}. The blank used for both OD_{600} and OD_{405} is the minimal standard succinate medium. Pyoverdine production is calculated as OD_{405}/OD_{600}.

APPENDIX B

PA0939 PROTEIN DECREASES BIOFILM FORMATION AND INCREASES MOTILITY IN *PSEUDOMONAS AERUGINOSA* PA14

B.1 Introduction

In the whole-transcriptome analysis of PA2663 mutation, we observed that the gene expression of an antiterminator, PA0939, was induced by 2.5-fold in PA2663 mutant versus *Pseudomonas aeruginosa* PAO1 wild-type strain (Attila *et al.*, 2008b). We speculated that this protein could be related to biofilm formation. As our lab possesses 5,850 transposon mutants of *P. aeruginosa* PA14 (Liberati *et al.*, 2006), we examined the biofilm formation of the PA0939 mutant of *P. aeruginosa* PA14. The mutation in PA0939 gene increased the biofilm formation by 3.4- and 3.8-fold in LB and LB glucose media, respectively. We also discovered that the PA0939 protein increases the swimming and swarming motilities and enhances the barley seed pathogenicity of *P. aeruginosa*. We performed microarrays and found that the PA0939 protein induces the expression of pyochelin genes. However, we could not completely discover how PA0939 protein controls biofilm formation in *P. aeruginosa* PA14.

B.2 Results and discussion

B.2.1 Differential gene expression in biofilms upon inactivating PA0939 gene

To explore the mechanism of how PA0939 protein represses the biofilm formation in *P. aeruginosa* PA14, differential gene expression of PA0939 transposon mutant versus *P. aeruginosa* PA14 wild-type strain in biofilm state was performed. As PA0939 protein is an antiterminator, upon the mutation of PA0939 gene, most of the genes are repressed. Thirty-seven genes were repressed and seven genes were induced upon mutation of PA0939 gene (Table B.1. and Table B.2.).

Table B.1. Partial list of repressed genes for *P. aeruginosa* PA14 PA0939 versus *P. aeruginosa* PA14 in biofilms formed on glass wool in LB at 30°C after 7 h.

PA #	Gene name	Fold change	Descriptions	Signal value in PA0939	Detection in PA0939	Signal value in wild-type	Detection in wild-type
Transport of small molecules							
PA4218		**-9.9**	Membrane protein, probable transporter, AraJ, Arabinose efflux permease, 41% similar to beta-lactamase induction signal transducer AmpG of *E. coli*	148.6	P	1399.4	P
PA4219		**-5.3**	Membrane protein, PiuB, uncharacterized iron-regulated membrane protein	94.0	A	472.8	P
PA4220		**-7.0**	Hypothetical protein, 100% similar to probable FptB protein of *P. aeruginosa*	234.5	P	1793.8	P
PA4221	*fprA*	**-13.0**	Fe(III)-pyochelin outer membrane receptor precursor, FhuE, 54% similar to ferric pseudobactin M114 receptor protein of *Pseudomonas* sp.	239.0	P	3675.3	P
PA4222		**-9.9**	Probable ATP-binding component of ABC transporter, MdlB, ABC-type multidrug transport system, ATPase and permease components	185.1	A	1345.5	P
PA4223		**-8.6**	Membrane proteins, probable ATP-binding component of ABC transporter, MdlB, ABC-type multidrug transport system, ATPase and permease components, 48% similar to yersiniabactin uptake ABC transporter YbtP of *Yersinia pestis*	153.2	P	1654.9	P
PA4224	*pchG*	**-10.6**	Membrane proteins, pyochelin biosynthetic protein PchG, oxidoreductase (NAD-binding), involved in siderophore biosynthesis	187.6	P	2304.9	P
PA4225	*pchF*	**-52.0**	Secreted factors, pyochelin synthetase, Non-ribosomal peptide synthetase modules and related proteins	17.6	A	743.8	P
PA4226	*pchE*	**-14.9**	Secreted factors, dihydroaeruginoic acid synthetase, EntF, Non-ribosomal peptide synthetase modules and related proteins	140.3	P	2068.4	P
PA4228	*pchD*	**-13.9**	Secreted factors,pyochelin biosynthesis protein PchD, Peptide arylation enzymes	222.3	P	4596.5	P
PA4229	*pchC*	**-14.9**	Secreted factors,pyochelin biosynthetic protein PchC,	319.3	P	6104.0	P

Table B.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA0939	Detection in PA0939	Signal value in wild-type	Detection in wild-type
PA4230	*pchB*	**-22.6**	Secreted factors, salicylate biosynthesis protein PchB	296.8	P	5850.8	P
PA4231	*pchA*	**-9.9**	Secreted factors, salicylate biosynthesis isochorismate synthase, MenF, Isochorismate synthase	145.6	P	1713.2	P
PA1019	*mucK*	**-2.8**	Cis-muconate transporter MucK, AraJ, Arabinose efflux permease	41.5	A	121.3	P
PA2204		**-2.3**	Probable binding protein component of ABC transporter, ABC-type amino acid transport/signal transduction systems, periplasmic component/domain	274.5	P	698.0	P
Motility and attachment							
PA4304	*rcpA*	**-2.0**	Protein secretion, export apparatus, Flp pilus assembly protein, secretin CpaC	519.8	P	854.8	P
PA4305	*rcpC*	**-2.0**	Flp pilus assembly protein CpaB	582.3	P	654.0	P
Adaptation, protection							
PA2147	*katE*	**-4.0**	Catalase HPII	57.6	A	160.4	P
Amino acid biosynthesis and metabolism							
PA4846	*aroQ1*	**-2.1**	3-dehydroquinate dehydratase	772.5	P	2006.6	P
Central intermediary metabolism							
PA4867	*ureB*	**-2.0**	Urease beta subunit	228.3	P	470.5	P
Hypothetical, unclassified, unknown function							
PA0092		**-3.3**	Hypothetical protein	19.9	A	62.5	P
PA0131		**-2.6**	Hypothetical protein	148.4	P	334.5	P
PA0145		**-3.7**	Nucleoside 2-deoxyribosyltransferase	15.2	A	59.0	P
PA0284		**-2.1**	Hypothetical protein	477.4	P	913.8	P
PA0980		**-2.0**	Hypothetical protein	77.6	P	182.0	P
PA0991		**-2.1**	Hypothetical protein	45.4	A	99.7	P

Table B.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA0939	Detection in PA0939	Signal value in wild-type	Detection in wild-type
PA1874		**-2.0**	Hypothetical protein	2548.0	P	5400.7	P
PA1942		**-2.1**	Hypothetical protein	102.7	P	305.3	P
PA3931		**-2.1**	NlpA, ABC-type metal ion transport system, periplasmic component/surface antigen	413.3	P	1042.5	P
PA4294		**-2.1**	Flp pilus assembly protein TadG	286.7	P	392.6	P
PA4703		**-2.0**	Hypothetical protein	573.7	P	993.5	P
PA5017		**-3.7**	Predicted signal transduction protein containing a membrane domain, an EAL and a GGDEF domain	240.4	P	1025.3	P
Protein secretion, export apparatus							
PA1877		**-2.0**	Probable secretion protein, EmrA, multidrug resistance efflux pump, 45% similar to ExpD2 of Sinorhizobium *meliloti*	329.9	P	735.1	P
Putative enzymes							
PA0493		**-2.0**	Probable biotin-requiring enzyme	63.3	A	135.1	P
PA1914		**-2.8**	Conserved hypothetical protein	1040.1	P	3423.1	P
PA2379		**-2.1**	Probable oxidoreductase, CoxS, aerobic-type carbon monoxide dehydrogenase, small subunit CoxS/CutS homologs	115.8	P	252.1	P
Transcription, RNA processing and degradation							
PA5239	*rho*	**-2.0**	Transcription termination factor Rho	674	P	1375.2	P

Table B.2. Partial list of induced genes for *P. aeruginosa* PA14 PA0939 versus *P. aeruginosa* PA14 in biofilms formed on glass wool in LB at 30°C after 7 h.

PA #	Gene name	Fold change	Descriptions	Signal value in PA0939	Detection in PA0939	Signal value in wild-type	Detection in wild-type
Hypothetical, unclassified, unknown function							
PA0939		**4.9**	Rof, Transcriptional antiterminator, 57% similar to YaeO in *E. coli*	243.0	P	23.9	A
PA2441		**3.5**	Hypothetical protein	316.4	P	76.2	A
Membrane Proteins							
PA0021		**2.0**	Predicted nucleotide-binding protein involved in DNA uptake, 59% similar to Smf protein of *E. coli*	215.5	P	86.6	A
Putative Enzymes							
PA1169		**2.1**	Probable lipoxygenase	332.8	P	158.2	P
Transcriptional regulator							
PA0877		**2.8**	LysR, transcriptional regulator	201.2	P	49.7	P
Transport of small molecules							
PA0029		**2.3**	Membrane proteins, probable sulfate transporter, sulfate permease	249.6	P	88	A
PA0811		**2.1**	Membrane proteins, probable major facilitator superfamily (MFS) transporter, UhpC, Sugar phosphate permease	189.3	P	99.8	A

The most striking changes were found in the expression of pyochelin genes. The highest repressed gene was *pchF* (-52-fold). PchF encodes the peptide synthetase involved in the sequential formation of pyochelin from dihydroaeruginoate (Reimmann *et al.*, 1998). The second most repressed gene was *pchB* (-22.6-fold). *pchB* gene encodes an isochorismate-pyruvate lyase involved in the formation of salicyclic acid from isochorismate (Maurhofer *et al.*, 1998). The induction of *pchB* increased the resistance of *P. fluorescens* in tobacco against tobacco necrosis virus (Maurhofer *et al.*, 1998). PchE whose gene expression is repressed 14.9-fold, is necessary for the conversion of salicylate to dihydroaeruginoate (Reimmann *et al.*, 1998). The expression of two motility genes was also repressed, which might be the cause of the motility deficiency in PA0939 mutant. The Affymetrix genome chips are designed according to *P. aeruginosa* PAO1; however, *P. aeruginosa* PA14 genome contains 392 more predicted genes than *P. aeruginosa* PAO1 (Liberati *et al.*, 2006). The genes that are absent in PAO1 contributes the virulence of *P. aeruginosa* PA14 (Choi *et al.*, 2002). This might be the reason why we could not detect biofilm-related genes in our microarray data. PA0939 protein might regulate biofilm formation through a putative PA14-specific gene.

B.2.2 Biofilm formation of PA0939 mutant and the seven microarray related mutants

The mutation in PA0939 gene increased biofilm formation by 3.4- to 3.8-fold, in LB and LB glu media, respectively. We also examined the biofilm formation of the 7 mutants whose genes were repressed in the PA0939 mutant (Table B.1), but their proteins have not yet been characterized. Except for PA4294 protein, which decreases the biofilm formation by 1.6-fold in LB medium, none of the examined proteins altered biofilm formation.

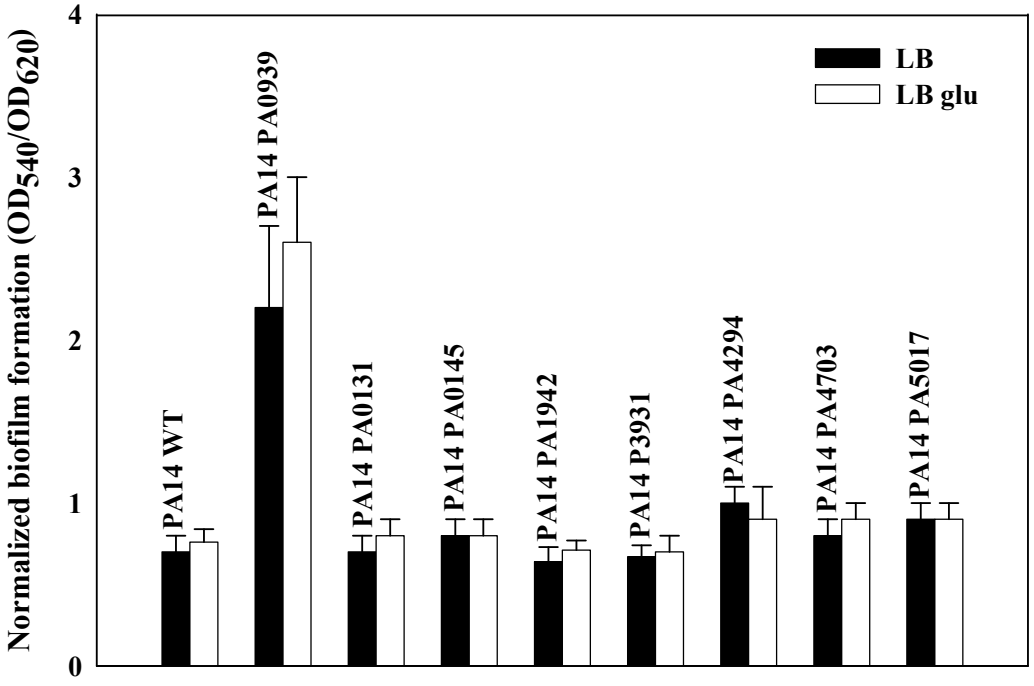

Fig. B.1. Effect of mutations in PA0939, PA0131, PA0145, PA1942, PA3931, PA4291, PA4703, and PA5017 genes on normalized biofilm formation at 30°C after 7 h in 96-well plates with LB medium (black graphs) and LB glu medium (white graphs).

B.2.3 The mutation in PA0939 gene represses swimming and swarming motility

As biofilm formation is related to motility (Wood *et al.*, 2006), we examined both swimming and swarming motility in the PA0939 mutant. The mutation in PA0939 gene decreased the swimming motility by 5-fold at 18 h and the swarming motility by 2.8-fold at 48 h. In *P. aeruginosa* PA14, an inverse relationship exists between the swarming motility and biofilm formation (Caiazza *et al.*, 2007). Our data support this relation.

B.2.4 The mutation in PA0939 gene does not change quorum sensing.

As swarming motility is related to quorum sensing phenotypes (Déziel *et al.*, 2003) and the mutation in PA0939 gene decreases swarming motility, we examined elastase, PQS, pyocyanin, pyoverdine, and rhamnolipid production in the PA0939 mutant. The PA0939 mutant increased elastase production by 1.5-fold and rhamnolipid production by 1.8-fold. PA0939 mutant did not alter PQS nor pyoverdine production and decreased the pyocyanin production by only 27%. Therefore, these results imply that PA0939 protein does not affect QS.

B.2.5 PA0939 mutant is less virulent for barley seeds

The PA0939 mutation increased the number of germinated barley seeds by 1.8-fold upon contact with *P. aeruginosa* PA14. Therefore, PA0939 protein plays an important role in barley seed pathogenesis.

In summary, we discovered that a hypothetical protein of *P. aeruginosa* PA14, PA0939, represses the biofilm formation and enhances the swimming and swarming motilities and increases virulence in barley seeds. We also examined the QS and we did not observe that the PA0939 protein affects QS. To observe the effects of the PA0939 protein on cell metabolism, phenotype microarrays may be utilized with *P. aeruginosa* PA14 PA0939 mutant versus *P.*

aeruginosa PA14 wild-type strain. As PA0939 is an antiterminator protein, to find out where this protein binds on the DNA, we could do nickel-enrichment DNA microarrays. This experiment would be done by expressing PA0939 with His_6tag as described previously (Lee *et al.*, 2007c). However, cloning of His_6tagged PA0939 into pJB866 (Blatny *et al.*, 1997) failed; therefore, we did not do this experiment. To discover the proteins through which PA0939 protein affects biofilm formation, motility, and virulence in barley germination, a double mutant library with the PA0939 transposon mutant can be generated using Tn*5*-*luxAB* transposition (de Lorenzo *et al.*, 1990). The mutants obtained from transposon mutagenesis of *P. aeruginosa* PA14 PA0939 mutant can be first screened for reduced biofilm formation in 96-well plates. Then, the mutants with reduced biofilm formation can be screened for swimming and swarming motilities and effects on barley seed virulence. The mutants that complement all four altered phenotypes (biofilm formation, swimming motility, swarming motility, and barley germination) of the PA0939 mutant can be identified by determining the position of Tn*5*-*luxAB* transposon insertion. To determine the position of transposon insertion, pGEM easy vector (Promega, Madison, WI) and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) can be used as described previously (Ramsey and Whiteley, 2004).

B.3 Experimental procedures

B.3.1 Bacterial strains, media, and growth conditions

P. aeruginosa PA14 wild-type and PA0939 mutant were obtained from Dr. Ausubel (Liberati *et al.*, 2006). The strains were grown at 30°C and 37°C. LB medium (Sambrook *et al.*, 1989) was used for the crystal violet biofilm experiment, the glass wool biofilm DNA microarrays, PQS experiments, elastase activity assays, rhamnolipid experiments, and the

swarming motility experiments. Minimal succinate medium (Ren *et al.*, 2005b) was used to measure pyoverdine production. Gentamycin (15 μg/mL) was used to select for the PA0939 mutant.

B.3.2 Verification of transposon insertion of *P. aeruginosa* mutant

To confirm the transposon insertion for the PA0939 gene inactivation, gene specific primers, PA0939-F (5'-CGAACAATCGGCACGCATCG-3') and PA0939-R (5'-TGGTCATCG TCACGCTCGGT-3') were designed to amplify the wild-type PA0939 gene. Using chromosomal DNA from *P. aeruginosa* PA14 wild-type, the PA0939 gene was amplified. This product was not amplified in the PA0939 mutant. Using the transposon specific primer GB3a (5'-TACAGTTTACGAACCGAACAGGC-3') and the gene specific primer PA0939-R and using the transposon specific primer R1 (5'-ATCGACCCAAGTACCGCCAG-3') and the gene specific primer PA0939-F, a DNA fragment corresponding to the end of the MAR2xT7 transposon and upstream flanking PA0939 was amplified from the PA0939 mutant, and as expected not from the *P. aeruginosa* PA14 wild-type. These results confirm that the transposon mutation is present in the PA0939 gene and the direction of the transposon is forward with respect to the gentamycin resistance promoter.

B.3.3 Biofilm assay

Biofilm formation was quantified in 96-well polystyrene plates as described previously (Pratt and Kolter, 1998) with 300 μL of crystal violet per well and the absorbance was measured at 540 nm. Overnight cultures were inoculated with an initial turbidity of 0.05 at 600 nm at 30°C and 37°C in both LB and in LB supplemented with 0.2% (wt/vol) glucose medium for 7 h without shaking.

B.3.4 Swimming and swarming motility

Swimming motility experiments were performed as explained previously (Sperandio *et al.*, 2002) by measuring halos on agar plates after 5 h and 18 h. Swarming motility experiments were performed in LB supplemented with 0.5% (wt/v) agar plates as described previously (Attila *et al.*, 2008b). Ten replicates (five plates from each of two independent cultures) were used to evaluate motility in each strain.

B.3.5 DNA microarrays

One mL of the overnight cultures (optical density 7.5 at 600 nm) was inoculated into 250 mL of fresh LB medium with 10 g of glass wool, and the cultures were incubated for 7 h at 30°C with shaking. Glass wool was used to increase the surface area so that RNA could be readily obtained for the microarrays. RNA was isolated from the suspension and biofilm cells as described previously (Ren *et al.*, 2004b)

The Genechip *P. aeruginosa* Genome Array (Affymetrix, P/N 900339) contains 5,500 of the 5,570 ORFs of *P. aeruginosa* (Whiteley *et al.*, 2001). cDNA synthesis, fragmentation, and hybridizations were as described previously (González-Barrios *et al.*, 2006b). Hybridization was performed for 16 h at 50°C, and the total cell intensity was scaled to an average value of 500. The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA control were used to monitor the labeling process. For each binary microarray comparison of differential genes expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 13 probe pairs (*p*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *p*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression

was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher (2-fold) than the standard deviation for the whole microarrays (Ren *et al.*, 2004a). The standard deviation was 1.7-fold. Gene functions were obtained from the *Pseudomonas g*enome database (Winsor *et al.*, 2005).

B.3.6 QS assays and pyoverdine synthesis

Elastase, rhamnolipid, PQS, and pyoverdine synthesis experiments were performed as described previously (Attila *et al.*, 2008b). The pyocyanin experiment was adapted from Essar *et al.* (1990). In brief, the strains were grown for 7 h the supernatants, extracted with chloroform and 0.2 N HCL and then analyzed spectrophotometrically. The *P. aeruginosa* PA14 *phzS* mutant was used as a negative control.

B.3.7 Barley seed pathogenicity assay

Barley seed pathogenicity was tested as described previously (Attila *et al.*, 2008a). Briefly, barley seeds were surface-sterilized in 1% sodium hypochlorite solution for 30 min, and then washed with sterilized distilled water ten times. The overnight bacteria cultures were re-grown to an optical density of 1 at 600 nm. *P. aeruginosa* cells were washed once with sterilized distilled water and twice with 1x Hoagland solution, and then resuspended to an optical density of 1.00 ± 0.03. Fifteen barley seeds were contacted with 10 mL of 1 x Hoagland solution with *P. aeruginosa*. After 3 days, the number of germinated seeds was counted.

APPENDIX C

INVESTIGATION OF PA2463 PROTEIN WITH DNA MICROARRAYS

C.1 Introduction

In our study to discern the genetic response of *P. aeruginosa* PAO1 to poplar tree roots, upon the contact of poplar trees versus glass wool, two highly induced genes of *P. aeruginosa* PAO1 were PA2459 (7.0-fold) and PA2461 (19.7-fold). These two genes are in the same operon as the PA2463 gene and the Affymetrix chip does not contain probes for PA2463 gene. Therefore, we analyzed the effect of PA2463 transposon mutant on poplar trees (Chapter III). The mutation in PA2463 gene enhanced poplar tree virulence by 2-fold and increased hemolytic activity by 4-fold. Furthermore, PA2463 mutant remained as competitive as the wild-type strain in poplar tree roots. Biofilm formation with the mutation in PA2463 gene did not show a difference in LB nor in LB glucose medium. However, the mutation in the PA2463 gene decreased the swimming motility by 30%. Here, we studied the role of PA2463 protein in biofilm formation through DNA microarrays and phenotype microarray plates. One hundred and forty-six genes were repressed and 122 genes were induced with a fold change of more than 2-fold in the PA2463 mutant versus *P. aeruginosa* PAO1 wild-type strain. We identified that nitrite reductase (*nirS*), nitric oxide reductase (*norBC*), and biofilm (*bkdA1A2B*, and *lpdV*) genes were induced, and anthranilate (*antABC*) and *chpABCD* genes were repressed in the PA2463 mutant. We also discovered that the PA2463 protein affects the growth on acetamide, *L*-pyroglutamic acid, *N*-acetyl-*D*-glucosamine, and putrescine.

C.2 Results and discussion

C.2.1 Differential gene expression in biofilms upon inactivating the PA0984 gene

To explore the mechanism by which the PA2463 protein regulates virulence in *P. aeruginosa*, differential gene expression of the PA2463 mutant versus *P. aeruginosa* PAO1 WT

Table C.1. Partial list of repressed genes more than 2-fold for *P. aeruginosa* PAO1 PA2463 versus *P. aeruginosa* PAO1 in biofilms formed on glass wool in LB at 30°C after 7 h.

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
Chemotaxis							
PA0413	*chpA*	**-4.9**	Two-component regulatory systems, motility & attachment, still frameshift probable component of chemotactic signal transduction system, chemotaxis protein histidine kinase and related kinases	232.2	P	1277.3	P
PA0414	*chpB*	**-4.6**	Probable methylesterase	123.6	P	641.1	P
PA0415	*chpC*	**-2.5**	Probable chemotaxis protein	234.0	P	430.3	P
PA0416	*chpD*	**-2.0**	Probable transcriptional regulator	88.8	P	222.7	P
Carbon compound metabolism							
PA2508	*catC*	**-2.3**	Muconolactone delta-isomerase	151.2	P	370.4	P
PA2512	*antA*	**-4.0**	Anthranilate dioxygenase large subunit, HcaE, Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases, large terminal subunit	554.2	P	2033.0	P
PA2513	*antB*	**-3.5**	Anthranilate dioxygenase small subunit, Small subunit of phenylpropionate dioxygenase	1186.5	P	3651.2	P
PA2514	*antC*	**-3.3**	Anthranilate dioxygenase reductase, UbiB, 2-polyprenylphenol hydroxylase and related flavodoxin oxidoreductases	218.8	P	758.4	P
PA0213		**-2.1**	51% similar to putative malonate decarboxylase component *MdcG* of *Klebsiella pneumoniae*	111.3	P	269.1	P
PA3589		**-2.3**	probable acyl-CoA thiolase, 67% similar to ketothiolase PhaD of *P. putida*	56.0	A	127.1	P
PA3591		**-2.0**	probable enoyl-CoA hydratase/isomerase	67.5	P	134.1	P
Adaptation, protection							
PA2385	*pvdQ*	**-2.3**	Protein related to penicillin acylase	295.8	P	775.5	P
PA2386	*pvdA*	**-2.8**	*L*-ornithine *N*-oxygenase	642.7	P	1899.4	P
PA2392	*pvdP*	**-2.1**	Pyoverdine synthesis	127.2	P	363.1	P

Table C.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA2394	*pvdN*	**-3.3**	*Pyoverdine synthesis*	417.1	P	1369.0	P
PA2395	*pvdO*	**-2.0**	Pyoverdine synthesis	432.2	P	684.1	P
PA2396	*pvdF*	**-2.1**	Secreted factors, pyoverdine synthetase, PurN, folate-dependent phosphoribosylglycinamide formyltransferase PurN	267.8	P	479.2	P
PA2397	*pvdE*	**-2.6**	Membrane proteins, transport of small molecules, pyoverdine biosynthesis protein PvdE	212.2	P	569.2	P
PA2401	*pvdJ*	**-2.3**	Non-ribosomal peptide synthetase modules and related proteins	113.3	P	233.3	P
PA2411		**-2.1**	Putative enzymes, GrsT, predicted thioesterase involved in non-ribosomal peptide biosynthesis	718.9	P	1375.5	P
PA2412		**-2.1**	64% similar to hypothetical protein MbtH of *Mycobacterium tuberculosis*	405.7	P	893.2	P
PA2413	*pvdH*	**-2.1**	*L*-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH, GabT, 4-aminobutyrate aminotransferase and related aminotransferases	360.5	P	889.3	P
PA2425	*pvdG*	**-2.6**	Predicted thioesterase involved in non-ribosomal peptide biosynthesis	194.0	P	380.2	P
PA2426	*pvdS*	**-4.6**	Sigma factor PvdS	274.3	P	1680.1	P
PA3543	*algK*	**-2.0**	Cell wall, LPS, capsule, adaptation, protection, secreted factors, alginate biosynthetic protein AlgK precursor	38.1	P	113.1	P
PA4602	*glyA3*	**-2.0**	Serine hydroxymethyltransferase	318.2	P	798.0	P
PA4468	*sodM*	**-4.3**	Superoxide dismutase	410.2	P	1742.2	P
Amino acid biosynthesis and metabolism							
PA2629	*purB*	**-2.3**	Nucleotide biosynthesis and metabolism, adenylosuccinate lyase	189.7	P	502.4	P
PA3700	*lysS*	**-2.0**	Translation, post-translational modification, degradation, lysyl-tRNA synthetase	343.5	P	708.8	P
PA3769	*guaA*	**-2.1**	Nucleotide biosynthesis and metabolism, GMP synthase	309.8	P	719.9	P
PA5429	*aspA*	**-2.5**	Aspartate ammonia	1335.1	P	2695.2	p

Table C.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
Antibiotic resistance and susceptibility							
PA1238		**-3.5**	Probable outer membrane component of multidrug efflux pump	35.7	A	94.8	P
PA2495	*oprN*	**-2.0**	Membrane proteins, transport of small molecules, multidrug efflux outer membrane protein OprN precursor	85.0	P	158.6	P
Biosynthesis of cofactors, prosthetic groups and carriers							
PA0672	*hemO*	**-2.6**	Heme oxygenase	189.5	P	438.0	P
Central intermediary metabolism							
PA2393		**-3.5**	Probable dipeptidase precursor	297.8	P	1088.4	P
Cell wall, LPS, capsule							
PA3984	*lnt*	**-2.0**	Translation, post-translational modification, degradation, apolipoprotein *N*-acyltransferase	98.4	P	213.5	P
PA4479	*mreD*	**-2.0**	Cell division, rod shape-determining protein MreD	444.1	P	727.6	P
PA4480	*mreC*	**-2.3**	Cell division, rod shape-determining protein MreC	320.7	P	736.3	P
DNA replication, recombination, modification and repair							
PA3725	*recJ*	**-2.0**	Single-stranded-DNA-specific exonuclease RecJ	178.8	P	376.8	P
Energy metabolism							
PA1317	*cyoA*	**-2.5**	Cytochrome o ubiquinol oxidase subunit II, Heme/copper-type cytochrome/quinol oxidases, subunit 2	119.1	P	379.0	P
PA1320	*cyoD*	**-2.0**	Cytochrome o ubiquinol oxidase subunit IV	49.5	P	66.4	P
PA2714		**-2.0**	Probable molybdopterin oxidoreductase	116.4	P	198.9	P
PA4470	*fumC1*	**-4.6**	Fumarate hydratase	411.7	P	1789.6	P
PA4471		**-3.7**	Hypothetical protein	217.4	P	770.2	P
Hypothetical, unclassified, unknown function							
PA0047		**-2.1**	Hypothetical protein	108.8	P	187.5	P
PA0647		**-2.0**	Hypothetical protein	91.5	P	174.7	P

Table C.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA0881		**-2.1**	PrpD, Uncharacterized protein involved in propionate catabolism	163.4	M	215.0	P
PA0939		**-2.5**	Hypothetical protein, transcriptional antiterminator, 57% similar to YaeO (termination factor) of *E. coli*	22.9	A	66.8	P
PA1259		**-3.5**	Uncharacterized conserved protein	45.8	A	148.3	P
PA1267		**-4.0**	Hypothetical protein, DadA, Glycine/D-amino acid oxidases	47.8	A	173.6	P
PA1325		**-5.7**	Hypothetical protein, ketosteroid isomerase homolog	22.3	A	125.7	P
PA1332		**-2.3**	Hypothetical protein	85.2	P	166.5	P
PA1360		**-2.1**	Membrane proteins, predicted permease, DMT superfamily	83.0	P	175.2	P
PA1428		**-4.0**	Conserved hypothetical protein, RimI, Acetyltransferases	14.0	A	109.4	P
PA1536		**-2.0**	Conserved hypothetical protein	54.3	P	119.4	P
PA1909		**-2.0**	Hypothetical protein, PiuB, Uncharacterized iron-regulated membrane protein	80.3	A	178.5	P
PA2033		**-2.8**	Hypothetical, unclassified, unknown	120.7	P	350.4	P
PA2034		**-2.6**	Hypothetical, unclassified, unknown	95.6	P	245.5	P
PA2283		**-2.1**	Hypothetical protein, HtpX, Zn-dependent protease with chaperone function	68.5	A	136.0	P
PA2292		**-2.0**	Hypothetical, unclassified, unknown	68.0	P	191.2	P
PA2293		**-2.6**	Hypothetical, unclassified, unknown	244.8	P	558.1	P
PA2384		**-2.0**	Hypothetical protein, Fur, Fe2+/Zn2+ uptake regulation proteins	567.3	P	1319.1	P
PA2389		**-2.3**	Conserved hypothetical protein, AcrA, membrane-fusion protein	243.5	P	669.5	P
PA2452		**-2.6**	Hypothetical, unclassified, unknown	272.3	P	695.8	P
PA2502		**-2.5**	Hypothetical protein, Leucine-rich repeat (LRR) protein	88.6	M	201.3	P
PA2630		**-2.0**	Conserved hypothetical protein, 57% similar to hypothetical protein YcfD of E. coli	106.3	P	210.2	P
PA2650		**-2.0**	Hypothetical, unclassified, unknown, UbiE, Methylase involved in ubiquinone/menaquinone biosynthesis	83.7	P	185.6	P

Table C.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA2759		**-2.1**	Hypothetical protein	50.1	P	150.3	P
PA3436		**-2.0**	Hypothetical protein	143.6	P	325.2	P
PA3449		**-2.8**	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	35.3	A	147.2	P
PA3530		**-2.1**	Conserved hypothetical protein, Bacterioferritin-associated ferredoxin	281.8	P	598.8	P
PA3720		**-2.1**	Hypothetical protein	176.9	P	412.2	P
PA3783		**-2.0**	Hypothetical, unclassified, unknown, PncA, Amidases related to nicotinamidase	135.7	P	293.1	P
PA3964		**-2.1**	Hypothetical protein	49.5	P	116.9	P
PA3980		**-2.0**	Conserved hypothetical protein, MiaB, 2-methylthioadenine synthetase	319.6	P	616.5	P
PA4105		**-2.0**	Hypothetical, unclassified, unknown	46.5	P	126.9	P
PA4371		**-2.6**	Hypothetical protein, predicted thiol oxidoreductase	221.2	P	533.8	P
PA4467		**-3.0**	Membrane proteins, hypothetical, predicted divalent heavy-metal cations transporter	97.3	P	336.0	P
PA4469		**-5.0**	Hypothetical protein	597.7	P	2904.7	P
PA4485		**-2.0**	Conserved hypothetical protein	69	P	140	P
PA4540		**-2.1**	FhaC, Hemolysin activation/secretion protein, 44% similar to HxuB of *Haemophilus influenzae*	597.7	P	2904.7	P
PA4570		**-3.0**	Hypothetical, unclassified, unknown	77.7	M	115.8	P
PA4627		**-2.0**	53% similar to putative enzyme YjjT of *E. coli*, RsmC, 16S RNA G1207 methylase RsmC	224.0	P	627.0	P
PA4686		**-2.1**	Smc, chromosome segregation ATPases	272.5	P	650.7	P
PA4708		**-2.0**	53% similar to hemin binding protein HemT of *Yersinia enterocolitica*, ChuT, ABC-type hemin transport system, periplasmic component	281.6	P	140.0	P

Table C.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
Membrane proteins							
PA0702		**-2.5**	Hypothetical protein, Sterol desaturase	53.5	A	112.9	P
PA2091		**-2.0**	Hypothetical protein, arabinose efflux permease	71.3	A	196.1	P
PA5469		**-2.1**	Conserved hypothetical protein, TerC, Membrane protein TerC, possibly involved in tellurium resistance	70.1	M	140	P
PA5568		**-2.1**	Conserved hypothetical protein, 58% similar to a 60 KD inner-membrane protein YidC of *E. coli*	772.3	P	1650.8	P
Protein secretion, export apparatus							
PA1867		**-2.3**	Hypothetical protein, 46% similar to secretion protein XcpP *of P. aeruginosa*	62.3	P	139.5	P
PA4243	*secY*	**-2.0**	Membrane proteins, secretion protein SecY	424	P	1171.4	P
Putative Enzymes							
PA0883		**-2.5**	Probable acyl-CoA lyase beta chain, citrate lyase beta subunit	58.6	A	143.1	P
PA1185		**-2.3**	Probable glutathione *S*-transferase	81.5	P	197.4	P
PA1919		**-3.3**	Probable radical-activating enzyme, PflA, Pyruvate-formate lyase-activating enzyme	25.4	A	91.1	P
PA2402		**-2.0**	Probable non-ribosomal peptide synthetase, Non-ribosomal peptide synthetase modules and related proteins	275.9	P	653.8	P
PA4709		**-2.5**	Probable hemin degrading factor, HemS, Putative heme degradation protein	290.8	P	700.6	P
PA4724		**-2.0**	Probable aminoacyl-transfer RNA synthetase	115.4	P	154.3	P
Transcriptional regulators							
PA1300		**-3.3**	Probable sigma-70 factor, ECF subfamily, RpoE, DNA-directed RNA polymerase specialized sigma subunit, sigma24 homolog	160.5	P	568.8	P
PA1301		**-3.0**	Membrane protein, probable transmembrane sensor, Fe^{2+}-dicitrate sensor, membrane component, 43% similar to FecR of *E. coli*	122.8	P	539.6	P
PA1911		**-2.0**	Membrane protein, probable transmembrane sensor	144.5	P	285.8	P

Table C.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA1980		**-2.8**	Two-component regulatory systems, CitB, Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain	57.3	A	105.6	P
PA2056		**-2.5**	Probable transcriptional regulator	38.6	A	100.3	P
PA2376		**-2.1**	CitB, Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain	50.3	P	119.7	P
PA2468		**-2.0**	Probable sigma-70 factor, ECF subfamily	73.3	P	177.9	P
PA2686	*pfeR*	**-2.1**	Two-component regulatory systems, OmpR, Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain	96.3	P	228.5	P
PA3409		**-2.1**	Membrane protein, probable transmembrane sensor, FecR, Fe^{2+}-dicitrate sensor, membrane component	149.6	P	157	P
PA3410		**-2.6**	Probable sigma-70 factor, ECF subfamily, RpoE, DNA-directed RNA polymerase specialized sigma subunit, sigma24 homolog	146.5	P	330.2	P
PA3899		**-2.3**	Probable sigma-70 factor, ECF subfamily, DNA-directed RNA polymerase specialized sigma subunit, sigma24 homolog	126.3	P	296.9	P
PA4227	*pchR*	**-2.0**	Transcriptional regulator PchR	264.7	P	579.3	P
PA4895		**-2.5**	Membrane proteins, probable transmembrane sensor	98.2	P	187	P
Translation, post-translational modification, degradation							
PA3169		**-2.0**	Probable initiation factor 2 subunit, predicted translation initiation factor 2B subunit, eIF-2B alpha/beta/delta family	260.5	P	583.8	P
PA3600		**-2.5**	Conserved hypothetical protein, 74% similar to ribosomal protein L36 of *Guillardia theta*	119.5	P	209.5	P
PA4247	*rplR*	**-2.0**	50S ribosomal protein L18	1058.2	P	3018.9	P
PA4248	*rplF*	**-2.0**	50S ribosomal protein L6	1827.9	P	3743.3	P
PA4261	*rplW*	**-2.0**	50S ribosomal protein L23	1415.1	P	2810	P
PA4262	*rplD*	**-2.1**	Transcription, RNA processing and degradation, 50S ribosomal protein L4	1508.8	P	3349.7	P

Table C.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA4672		**-2.6**	Peptidyl-tRNA hydrolase	158.5	P	445.3	P
Transport of small molecules							
PA0206		**-2.0**	Probable ATP-binding component of ABC transporter, 62% similar to ATP-binding component of spermidine/putrescine transport PotA of *E. coli*	155.8	P	275.8	P
PA0471		**-2.1**	Two-component regulatory systems, membrane proteins, probable transmembrane sensor, 99% similar to transmembrane sensor FiuR of *P. aeruginosa*	255.6	P	589.4	P
PA1144		**-2.6**	Membrane proteins, probable major facilitator superfamily (MFS) transporter	24.1	A	75.9	P
PA1147		**-2.5**	Membrane proteins, probable amino acid permease, amino acid transporters	88.3	P	267.6	P
PA1361		**-2.3**	Membrane proteins, NorM, Na^+-driven multidrug efflux pump	49.6	P	94.5	P
PA1682		**-2.1**	Membrane proteins, probable MFS metabolite transporter	67.8	A	149.8	P
PA1783	*nasA*	**-2.1**	NarK, Nitrate/nitrite transporter	34.2	A	103.1	P
PA2061		**-2.1**	Probable ATP-binding component of ABC transporter, 69% similar to YefJ, putativeATP-binding component of a transport system of *E. coli*	22.6	A	94.5	P
PA2252		**-2.0**	Probable AGCS sodium/alanine/glycine symporter	66.7	P	120.4	P
PA2314		**-2.1**	Membrane proteins, probable major facilitator superfamily (MFS) transporter, AraJ, Arabinose efflux permease	93.4	P	178.8	P
PA2390		**-2.6**	Membrane proteins, probable ATP-binding/permease fusion ABC transporter	244.8	P	558.1	P
PA2391	*opmQ*	**-2.0**	Membrane proteins, probable outer membrane protein precursor	166.2	P	398.2	P
PA2398	*fpvA*	**-2.0**	Ferripyoverdine receptor	882.8	P	1728.8	P
PA2408		**-2.0**	Probable ATP-binding component of ABC transporter, 51% similar to zinc transport protein ZnuC of *E. coli*	378.9	P	827.6	P

Table C.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA2500		**-2.0**	Membrane proteins, probable major facilitator superfamily (MFS) transporter	59.2	A	109.2	P
PA2558		**-2.1**	Membrane proteins, probable transport protein, 58% similar to Mg^{2+} transport-associated protein MgtC of *Salmonella typhimurium*	51.3	A	120.5	P
PA2563		**-2.5**	Membrane proteins, probable sulfate transporter	46.1	P	109.3	P
PA3210	*trkH*	**-2.0**	Membrane proteins, potassium uptake protein TrkH	57.1	P	126.4	P
PA3407	*hasAp*	**-2.0**	heme acquisition protein HasAp	65.3	A	112.7	P
PA3522		**-2.0**	Membrane proteins, probable Resistance-Nodulation-Cell Division (RND) efflux transporter, AcrB,	47.6	A	77.1	P
PA3936		**-2.3**	Membrane proteins, probable permease of ABC taurine transporter,	81.1	P	206.3	P
PA4628	*lysP*	**-2.1**	Membrane protein, lysine-specific permease, amino acid transporters	104.6	P	134.4	P
PA4706		**-2.0**	Probable ATP-binding component of ABC transporter, ABC-type hemin transport system, ATPase component	217.7	P	336.9	P
PA4710	*phuR*	**-4.6**	Haemoglobin uptake outer membrane receptor PhuR precursor, CirA, Outer membrane receptor proteins	182.7	P	793.5	P
PA5548		**-2.1**	Probable major facilitator superfamily (MFS) transporter, AraJ, Arabinose efflux permease	90.0	P	210.2	P

Table C.2. Partial list of induced genes more than 2-fold for *P. aeruginosa* PAO1 PA2463 versus *P. aeruginosa* PAO1 in biofilms formed on glass wool in LB at 30°C after 7 h.

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
Energy Metabolism							
PA0509		**4.6**	Biosynthesis of cofactors, prosthetic groups and carriers	690.9	P	350.3	P
PA0510		**4.6**	Biosynthesis of cofactors, prosthetic groups and carriers, probable uroporphyrin-III c-methyltransferase	1298.9	P	347.9	P
PA0511	*nirJ*	**4.0**	Biosynthesis of cofactors, prosthetic groups and carriers, heme d1 biosynthesis protein NirJ	1119.6	P	238.1	P
PA0512		**3.3**	Biosynthesis of cofactors, prosthetic groups and carriers, hypothetical, unclassified, unknown	1467.3	P	381.1	P
PA0513		**5.7**	Biosynthesis of cofactors, prosthetic groups and carriers, transcriptional regulators, probable transcriptional regulator, 77% similar to NirG protein *P. stutzeri*	861.1	P	297.1	P
PA0514	*nirL*	**5.3**	Biosynthesis of cofactors, prosthetic groups and carriers, 77% similar to NirL protein of *P. stutzeri*	761.5	P	156.7	P
PA0515		**6.5**	Biosynthesis of cofactors, prosthetic groups and carriers, transcriptional regulators	1164.0	P	256.1	P
PA0516	*nirF*	**6.5**	Biosynthesis of cofactors, prosthetic groups and carriers, heme d1 biosynthesis protein NirF	2330.6	P	341.7	P
PA0517	*nirC*	**8.0**	Biosynthesis of cofactors, prosthetic groups and carriers, probable c-type cytochrome precursor	1383.4	P	197.8	P
PA0518	*nirM*	**13.9**	Biosynthesis of cofactors, prosthetic groups and carriers, cytochrome c-551 precursor	2320.3	P	286	P
PA0519	*nirS*	**21.1**	Nitrite reductase precursor	5463.4	P	376.3	P
PA0520	*nirQ*	**3.3**	Central intermediary metabolism, regulatory protein NirQ	6896.6	P	313.2	P
PA0523	*norC*	**10.6**	Nitric-oxide reductase subunit C	967	P	300.7	P
PA0524	*norB*	**10.6**	Nitric-oxide reductase subunit B	1278.1	P	92.5	P
PA0525		**3.0**	Probable dinitrification protein NorD	660.4	P	67.3	P

Table C.2. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA0526		**3.7**	Hypothetical, unclassified, unknown	665.7	P	207.3	P
PA0794		**3.0**	Probable aconitate hydratase	1812.3	P	470.6	P
PA0918		**2.1**	Cytochrome b561	842.9	P	436.0	P
PA3391	*nosR*	**2.0**	Membrane proteins, regulatory protein NosR	309.2	P	193.0	P
PA3392	*nosZ*	**6.5**	Nitrous-oxide reductase precursor	1111.9	P	237.5	P
PA3393	*nosD*	**3.0**	NosD, nitrous oxidase accessory protein	382.0	P	145.1	P
PA3394	*nosF*	**2.1**	Transport of small molecules, NosF protein	311.4	P	195.8	P
PA4587	*ccpR*	**2.1**	Cytochrome c551 peroxidase precursor	2036.2	P	1039.1	P
Amino acid biosynthesis and metabolism							
PA2247	*bkdA1*	**2.5**	2-oxoisovalerate dehydrogenase	10925.7	P	4485.2	P
PA2248	*bkdA2*	**2.3**	2-oxoisovalerate dehydrogenase	6907.0	P	3030.2	P
PA2249	*bkdB*	**2.6**	Branched-chain alpha-keto acid dehydrogenase	4373.2	P	1690.3	P
PA2250	*lpdV*	**2.1**	Energy metabolism, lipoamide dehydrogenase-Val	4417.3	P	2073.6	P
PA5098	*hutH*	**3.3**	Histidine ammonia-lyase	835.3	P	255.7	P
PA5100	*hutU*	**2.5**	Urocanase, Urocanate hydratase	2766.1	P	1129.8	P
Adaptation, protection							
PA2279	*arsC*	**2.1**	Transport of small molecules, ArsC protein	1163.3	P	590.7	P
Biosynthesis of cofactors, prosthetic groups and carriers							
PA1985	*pqqA*	**2.0**	Pyrroloquinoline quinone biosynthesis protein A	513.4	P	283.2	P
Carbon compound catabolism							
PA0796	*prpB*	**2.3**	Fatty acid and phospholipid metabolism, central intermediary metabolism,	3160.9	P	1433.1	P
PA0887	*acsA*	**8.0**	Central intermediary metabolism, acetyl-coenzyme A synthetase	5942.6	P	687.9	P
PA1949	*rbsR*	**2.1**	Transcriptional regulators, ribose operon repressor RbsR	1733.4	P	752.9	P

Table C.2. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA1950	*rbsK*	**2.0**	Ribokinase	1234.0	P	600.3	P
PA2000		**3.5**	Carbon compound catabolism, probable CoA transferase	7260.2	P	2315.5	P
PA2001	*atoB*	**3.0**	Central intermediary metabolism, Fatty acid and phospholipid metabolism	5187.5	P	1535.9	P
PA2007	*maiA*	**2.5**	Maleylacetoacetate isomerase	2010.9	P	937.4	P
PA2008	*fahA*	**2.3**	Fumarylacetoacetase	6202.4	P	2967	P
PA2009	*hmgA*	**2.5**	Homogentisate 1,2-dioxygenase	4863.5	P	1841.6	P
PA2011	*liuE*	**2.5**	3-hydroxy-3-methylglutaryl-CoA lyase	1598.5	P	829.2	P
PA2012	*liuD*	**2.1**	Methylcrotonyl-CoA carboxylase, alpha-subunit	1782.3	P	881.9	P
PA2013	*liuC*	**2.1**	Putative 3-methylglutaconyl-CoA hydratase	3651.8	P	1542.4	P
PA2014	*liuB*	**2.0**	Methylcrotonyl-CoA carboxylase, beta-subunit	5506.9	P	2464.1	P
PA2015	*liuA*	**2.3**	Putative isovaleryl-CoA dehydrogenase	11548.2	P	4926.5	P
PA3366	*amiE*	**2.5**	Aliphatic amidase	2689.9	P	978	P
PA3568		**4.0**	Putative Enzymes, probable acetyl-coa synthetase	998.2	P	236.8	P
PA3569	*mmsB*	**4.0**	3-hydroxyisobutyrate dehydrogenase, leucine and isoleucine degradation	4036.8	P	891.7	P
PA3570	*mmsA*	**3.3**	Amino acid biosynthesis and metabolism, methylmalonate-semialdehyde dehydrogenase	10111.0	P	3096.3	P
PA3710		**2.5**	Probable GMC-type oxidoreductase	1482.0	P	585.7	P
Central intermediary metabolism							
PA0795	*prpC*	**2.6**	Citrate synthase 2	5268.3	P	1830.3	P
PA1999		**3.3**	Carbon compound catabolism, probable CoA transferase,	9454.9	P	3793.4	P
PA4880		**2.8**	Probable bacterioferritin	4897.2	P	1501	P
PA5352		**2.8**	Hypothetical, unclassified, unknown	2001.8	P	749	P

Table C.2. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA5353	*glcF*	**3.0**	Carbon compound catabolism, glycolate oxidase subunit GlcF	1644.4	P	521.4	P
PA5354	*glcE*	**2.5**	Carbon compound catabolism, glycolate oxidase subunit GlcE	1492.2	P	546.4	P
PA5355	*glcD*	**2.6**	Carbon compound catabolism, glycolate oxidase subunit GlcD	1958.8	P	749.6	P
PA5435		**3.0**	Probable transcarboxylase subunit	2166.4	P	641	P
PA5436		**3.3**	Probable biotin carboxylase subunit of a transcarboxylase	5151.1	P	1158	P
Chaperones and heat shock proteins							
PA3365		**2.6**	Probable chaperone	2438.2	P	1007.6	P
Chemotaxis							
PA4290		**5.3**	Adaptation, protection, probable chemotaxis transducer, 58% similar to a region of putative methyl-accepting chemotaxis protein of *Synechocystis sp*.	2716.8	P	532.9	P
DNA replication, recombination, modification and repair							
PA5348		**2.5**	Probable DNA-binding protein, HimA, Bacterial nucleoid DNA-binding protein	10056.1	P	4026.1	P
PA0105	*coxB*	**2.0**	Cytochrome c oxidase, subunit II	690.9	P	350.3	P
Fatty acid and phospholipid							
PA0447	*gcdH*	**2.0**	Amino acid biosynthesis and metabolism ; carbon compound catabolism, glutaryl-CoA dehydrogenase	4754.7	P	2077.8	P
PA2557		**2.5**	Probable AMP-binding enzyme	1592.6	P	564	P
Hypothetical, unclassified, unknown function							
PA0793		**2.6**	Hypothetical, unclassified, unknown	2546.3	P	958.3	P
PA2110		**2.0**	Hypothetical protein	2337.1	P	1149.1	P
PA2111		**2.1**	Hypothetical protein	4254.3	P	1799.4	P
PA2112		**2.0**	Conserved hypothetical protein	5863.1	P	2808.6	P

Table C.2. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA3080		**2.5**	Hypothetical, unclassified, unknown	1090.4	P	701.9	P
PA3232		**3.3**	Putative enzyme, probable nuclease	460.3	P	177.6	P
PA3233		**4.9**	Hypothetical protein	819.0	P	171.7	P
PA3234		**9.9**	Membrane protein, transport of small molecules, probable sodium solute symporter	3121.1	P	323.9	P
PA3235		**12.1**	Membrane protein, conserved hypothetical protein, 78% similar to *E. coli* YjcH(unknown function)	4447.9	P	370.3	P
PA3880		**2.0**	Conserved hypothetical protein	954.2	P	627.7	P
PA3919		**2.0**	Conserved hypothetical protein	2972.6	P	1549.1	P
PA3922		**2.3**	Conserved hypothetical protein	4474.1	P	1832	P
PA3923		**2.0**	Conserved hypothetical protein	4620	P	2455.1	P
PA5083		**3.3**	Hypothetical, unclassified, unknown	649.3	P	194	P
PA5303		**2.5**	Conserved hypothetical protein, TdcF, Putative translation initiation inhibitor, yjgF family	2259.4	P	879.9	P
Membrane proteins							
PA3079		**3.3**	Hypothetical, unclassified, unknown	449.3	P	170	P
PA3709		**3.0**	Transport of small molecules, probable major facilitator superfamily (MFS) transporter	374.5	P	158.2	P
Putative enzymes							
PA0534		**2.1**	Conserved hypothetical protein, DadA, Glycine/D-amino acid oxidases	2542.0	P	1107.2	P
PA0744		**2.1**	Probable enoyl-CoA hydratase/isomerase	3407.2	P	1879.0	P
PA0746		**2.1**	Probable acyl-CoA dehydrogenase	5020.4	P	2150.4	P
PA0747		**2.0**	Probable aldehyde dehydrogenase	2616.9	P	1291.1	P
PA1617		**2.5**	Probable AMP-binding enzyme	1505.4	P	810.2	P

Table C.2. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA1984		**3.0**	Probable aldehyde dehydrogenase	641.5	P	348.8	P
PA2552		**2.5**	probable acyl-CoA dehydrogenase	3974.3	P	1156.8	P
PA2553		**2.6**	Probable acyl-CoA thiolase	2541.4	P	1165.7	P
PA2554		**2.5**	Probable short-chain dehydrogenase, 73% similar to 3-hydroxyacyl-CoA dehydrogenase of Bos taurus	4259.9	P	1399.9	P
PA2555		**2.5**	Probable AMP-binding enzyme, Acs, Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases	2789.4	P	965.1	P
PA5084		**2.8**	Probable oxidoreductase	1083.3	P	478.1	P
PA5445		**2.0**	Probable coenzyme A transferase	132.3	P	62.5	P
Secreted factors							
PA2862	*lipA*	**2.1**	Carbon compound metabolism, lactonizing lipase precursor	552.8	P	211	P
Transcriptional regulator							
PA0048		**2.1**	Probable transcriptional regulator	2237	P	943.8	P
PA0535		**2.0**	Probable transcriptional regulator	802.1	P	404.6	P
PA1759		**2.0**	Probable transcriptional regulator	1246.8	P	684.3	P
PA3363	*amiR*	**2.0**	Carbon compound catabolism, aliphatic amidase regulator	2107.7	P	825.8	P
PA3364	*amiC*	**2.1**	Aliphatic amidase expression-regulating protein	1386.8	P	630.9	P
Transport of small molecules							
PA1946	*rbsB*	**2.6**	Binding protein component precursor of ABC ribose transporter	1317.4	P	467.7	P
PA1947	*rbsA*	**2.8**	Ribose transport protein RbsA	689.9	P	324	P
PA1948	*rbsC*	**2.6**	Membrane protein component of ABC ribose transporter	636.2	P	232.2	P
PA2113	*opdO*	**2.3**	Membrane proteins, pyroglutamate porin OpdO	4790.4	P	2186.6	P
PA2114		**2.5**	Membrane proteins, AraJ, Arabinose efflux permease	3197.2	P	1365.4	P
PA3038		**5.7**	Probable porin, 65% similar to PhaK of *P. putida*	3622.1	P	805.3	P

Table C.2. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA2463	Detection in PA2463	Signal value in wild-type	Detection in wild-type
PA3531	*bfrB*	**2.3**	Bacterioferritin	808.0	P	338.3	P
PA4223		**2.0**	Membrane proteins, probable ATP-binding component of ABC transporter	1919.0	P	744.7	P
PA4224	*pchG*	**2.0**	Membrane proteins. pyochelin biosynthetic protein PchG	1623.8	P	864.8	P
PA4500		**2.3**	Probable binding protein component of ABC transporter	1083.8	P	339.6	P
PA5082		**4.3**	Probable binding protein component of ABC transporter, 70% similar to hypothetical ybeJ of E. coli	977.0	P	191.3	P
PA5094		**2.5**	Probable ATP-binding component of ABC transporter, ProV, ABC-type proline/glycine betaine transport system, ATPase component	1450.1	P	613.3	P
PA5095		**3.0**	Membrane proteins, probable permease of ABC transporter	846.6	P	275.2	P
PA5096		**3.0**	Probable binding protein component of ABC transporter	531.6	P	143.3	P
PA5097		**2.3**	Membrane proteins, probable amino acid permease	538.7	P	256.9	P
PA5099		**2.1**	Membrane proteins. probable transporter, CodB, Purine-cytosine permease and related proteins	398.1	P	242.8	P
PA5153		**2.3**	Probable periplasmic binding protein, ABC-type amino acid transport/signal transduction systems, periplasmic component/domain	3119.5	P	1269.5	P
PA5167		**2.1**	Membrane proteins, probable c4-dicarboxylate-binding protein	2301.4	P	1061.9	P

was performed. One hundred and twenty-two genes were induced and 146 genes were repressed in the PA2463 mutant greater than 2.0-fold. (Table C.1 and Table C.2).

The most induced genes were PA0519 (*nirS*) and PA0518 (*nirM*), by 21.1- and 13.9-fold. The surrounding genes, from PA0509 to PA0526, were also induced in the PA2463 mutant more than 3.3-fold. These genes are related to energy metabolism (Winsor *et al.*, 2005). *norB* and *norC* (PA0523 and PA0524) were induced by 10.6-fold. The *nir* and *nor* operon are involved in the reduction of nitrite and nitric oxide in *P. aeruginosa* (Arai *et al.*, 1999). Δ*norBC* mutant induces biofilm dispersal in *P. aeruginosa* (Barraud *et al.*, 2006).

Among the induced genes, the 2-oxoisovalerate dehydrogenase operon (*bkdA1A2B*, and *lpdV*) was also induced. The *bkdA1A2B* operon was induced in developing biofilms and in stationary phase planktonic cultures (Waite *et al.*, 2006), and this operon is next to the *psl* operon (Winsor *et al.*, 2005). Hence, this operon is important for biofilm formation in *P. aeruginosa*. The *arsC* gene was induced by 8-fold, this homolog of the *ars* operon in *E. coli* contributes to increased resistance to arsenic and antimony (Cai *et al.*, 1998).

The mutation in the PA2463 gene repressed *chpABCD* genes by 2.0- to 4.9-fold. The *chp* operon regulates twitching motility in *P. aeruginosa* (Mattick, 2002); hence, the decrease in swimming motility of PA2463 mutant might be due to the repression of the *chp* operon. Moreover, *chpA* mutant kills *Drosophila* more slowly than wild-type *P. aeruginosa* strain (D'Argenio *et al.*, 2001). Also anthranilate genes (*antABC*) were down-regulated from 3.3- to 4.0-fold. Anthranilate is involved in the production of PQS (Urata *et al.*, 2004). To learn more about the relation of the PA2463 protein and PQS production, PQS production should be measured in the PA2463 mutant. Also, 11 genes of the pyoverdine operon were repressed upon the inactivation of PA2463.

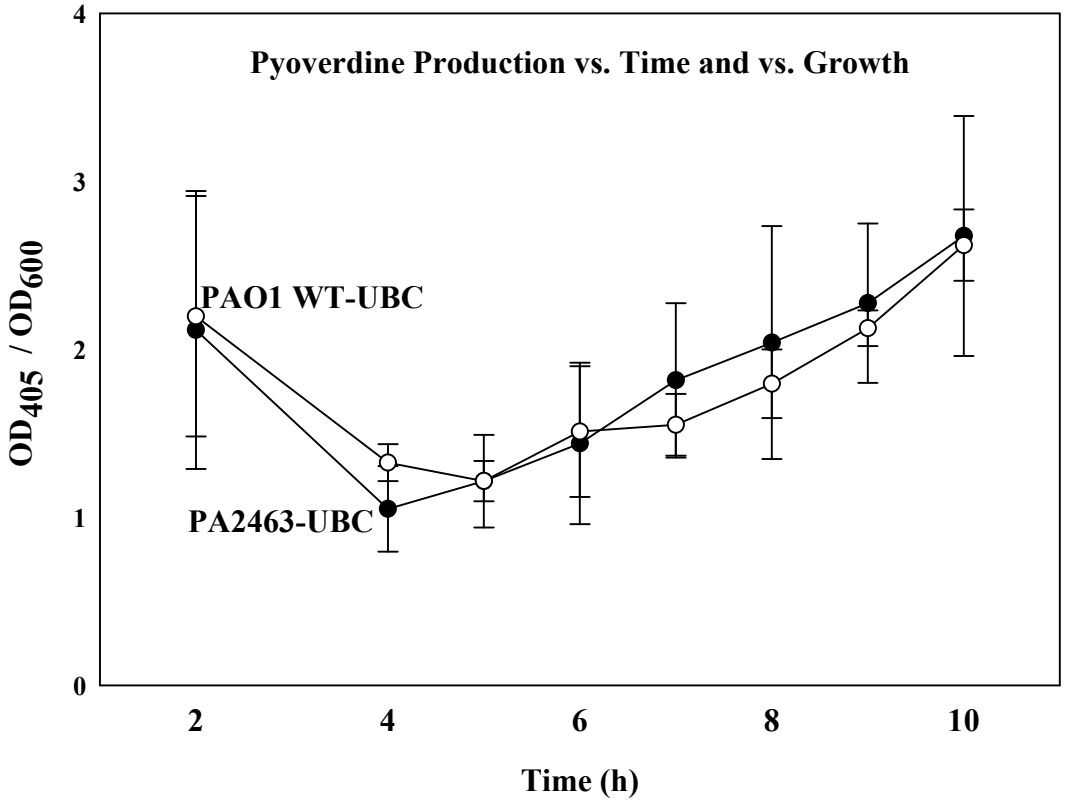

Fig. C.1. Effect of the mutation in PA2463 gene on cell density-normalized pyoverdine production. Pyoverdine production was normalized by measuring OD at 405 nm as explained in methods and normalized by dividing to the growth OD at 600 nm.

C.2.2 The mutation in the PA2463 gene does not alter pyoverdine production

As in LB medium biofilm cells, the PA2463 mutation repressed the expression of 11 pyoverdine genes. We measured the pyoverdine production in PA2463 mutant in iron deficient minimal medium (Fig. C.1). The mutation in PA2463 gene did not alter the pyoverdine production. This might have two causes; (i) the fold changes of the pyoverdine genes were around 2-fold, so the repression was low and it was not enough to affect the pyoverdine production, (ii) the DNA microarrays were done in LB medium and in biofilm cells, and the PA2463 protein may only regulate pyoverdine production under these conditions and not in minimal iron deficient medium.

C.2.3 PA2463 protein affects cell phenotypes

We explored the impact of the PA2463 protein using phenotype arrays. The mutation in the PA2463 gene affected growth on carbon and nitrogen sources such as acetamide, putrescine, *L*-cysteine, *L*-pyroglutamic acid, and *N*-acetyl-*D*-glucosamine (Table C.3). The highest increase was in *L*-pyroglutamic acid (10-fold). Utilization of *L*-pyroglutamic acid as a carbon source increased phenazine production, a virulence factor in *P. aeruginosa* and also in *P. chlororaphis* PCL1391 (van Rij *et al.*, 2004); hence, *L*-pyroglutamic acid might be involved in the induced virulence of PA2463 mutant in poplar trees and hemolytic activity (Attila *et al.*, 2008a). The mutation in the PA2463 gene increased growth in acetamide, when acetamide was used in the presence of energy metabolism inhibitors, non-mucoid *P. aeruginosa* cells became mucoid (Kim *et al.*, 1998), and the PA2463 mutation altered the gene expression of 29 energy metabolism genes (Table C.1. and Table C.2.). Therefore, PA2463 protein may be involved in the mucoidy of *P. aeruginosa*. The mutation in PA2463 gene decreases growth on putrescine (1.8-fold) as a carbon source. Putrescine is found in the outer membrane of *E. coli* and *Salmonella*

Table C.3. Metabolic phenotype based on Biolog arrays upon inactivating PA2463 versus *P. aeruginosa* PAO1 WT at 24 h at 30°C.

Compound	OD_{590} PA2463 /OD_{590} wild type	Physiological role	Pathway
Putrescine	0.56	Carbon source	Urea cycle and metabolism of amino groups, ABC transporters
N-acetyl-*D*-glucosamine	5.0	Carbon source	
Acetamide	3.33	Carbon source	
L-pyroglutamic acid	10.0	Carbon source	
L-cysteine	0.56	Nitrogen source	Cysteine metabolism
Glu-Val	0.29	Peptide nitrogen source	
Asp-Trp	0.27	Peptide nitrogen source	
Asp-Val	0.40	Peptide nitrogen source	
Gly-Val	0.23	Peptide nitrogen source	
Gly-His	0.37	Peptide nitrogen source	
His-Leu	0.20	Peptide nitrogen source	
Leu-Glu	0.00	Peptide nitrogen source	
Leu-Ile	0.24	Peptide nitrogen source	
Lys-Arg	0.23	Peptide nitrogen source	
Phe-Ile	0.24	Peptide nitrogen source	
Pro-Phe	0.22	Peptide nitrogen source	
Tyr-Leu	0.38	Peptide nitrogen source	
Val-Ala	0.37	Peptide nitrogen source	
Val-Tyr-Ala	0.24	Peptide nitrogen source	
Phe-Val	0.37	Peptide nitrogen source	
Val-Phe	0.38	Peptide nitrogen source	

typhimurium (Koski and Vaara, 1991) and is a signal essential for swarming motility of *Proteus mirabilis* (Sturgill and Rather, 2004). The concentration of putrescine is correlated to the biofilm level in *Yersinia pestis* (Patel *et al.*, 2006); hence, putrescine might be important in the repression of the PA2463 mutation (Attila *et al.*, 2008a). The mutation in the PA2463 gene also enhanced the growth on *N*-acetyl-*D*-glucosamine by 5-fold. *N*-acetylglucosamine is a constituent of the polysaccharide produced by the *psl* locus of *P. aeruginosa* (Ma *et al.*, 2006) and is required for *Staphylococcus aureus* virulence (Kropec *et al.*, 2005); so this increase virulence in poplar tree wilting assays and hemolytic activity of PA2463 mutant may be related to the altered metabolism of *N*-acetyl-*D*-glucosamine.

C.3 Experimental procedures

C.3.1 Bacterial RNA isolation and DNA microarrays

The strains used in this study are listed in Table 3.3. Bacterial RNA isolation and DNA microarray experiments were conducted as described previously (Attila *et al.*, 2008b). The wild-type and the PA2463 mutant strains were grown in LB (wild-type strain) and LB with 50 μg/mL tetracycline (PA2463 mutant). One mL of the overnight cultures (optical density 7.5 at 600 nm) was inoculated into 250 mL of fresh LB medium with 10 g of glass wool and the cultures were incubated for 7 h at 30°C with shaking. The cell pellets from glass wool and the total RNA were prepared as described previously (Attila *et al.*, 2008b).

The Genechip *P. aeruginosa* Genome Array (Affymetrix, P/N 900339) contains 5,500 of the 5,570 ORFs of *P. aeruginosa* (Whiteley *et al.*, 2001). cDNA synthesis, fragmentation, and hybridizations were as described previously (González-Barrios *et al.*, 2006b). Hybridization was performed for 16 h at 50°C, and the total cell intensity was scaled to an average value of 500.

The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA control were used to monitor the labeling process. For each binary microarray comparison of differential genes expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 13 probe pairs (*p*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *p*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher (2-fold) than the standard deviation for the whole microarrays (Ren *et al.*, 2004a). The standard deviation for all the genes was 1.8. Gene functions were obtained from the *Pseudomonas g*enome database (Winsor *et al.*, 2005).

C.3.2 Pyoverdine production

Pyoverdine production assays were performed as described previously (Attila *et al.*, 2008b). Briefly, overnight cultures were grown in minimal succinate medium and were diluted to a turbidity of 0.05 at 600 nm in fresh medium and one milliliter of the culture was centrifuged at each time point to determine the pyoverdine concentration by measuring the absorbance at 405 nm (Stintzi *et al.*, 1998). The pyoverdine concentrations were normalized by dividing by the cell density.

C.3.3 Phenotype microarrays

Phenotype microarrays were performed as described previously (Attila *et al.*, 2008b), and the strains were incubated in the inoculation fluid (IF-O) buffer on the phenotype microarray plates at 30°C for 24 h.

APPENDIX D

INVESTIGATION OF PA1385 PROTEIN WITH DNA MICROARRAYS

D.1 Introduction

PA1385 gene is one of the seven novel virulence genes we discovered in our rhizosphere study (Attila *et al.*, 2008a). PA1385 is a glycosyl transferase protein; it encodes polysaccharide biosynthesis involved in biofilm formation (Jackson *et al.*, 2004; Winsor *et al.*, 2005). The mutation in the PA1385 gene decreased biofilm formation by 30% in LB glu medium (Attila *et al.*, 2008a). The PA1385 gene is in an operon (PA1385-PA1391) that has less G+C content than the surrounding genes (Stover *et al.*, 2000). This operon, including the PA1385 gene, contains four genes encoding glycosyl transferases (Stover *et al.*, 2000). In *Pseudomonas aeruginosa,* glycosyl transferases are involved in lipopolysaccharide biosynthesis (Potvin *et al.*, 2003). Glycosyl transferases are important for the virulence of pathogenic bacteria; a direct relationship between a glycosyl transferase and hemolysis has been shown in *Streptococcus agalactiae* (Forquin *et al.*, 2007), and in *Burkholderia pseudomallei*, a mutation in the glycosyl transferase gene renders it less virulent (Reckseidler *et al.*, 2001). In our rhizosphere study (Chapter III), the mutation in the PA1385 gene also decreased virulence in both plant models and deteriorated the rhizosphere competitiveness by 2.5-fold. Inactivation of PA1385 gene also decreased hemolytic activity by 50%. Also, expression of the PA1385 gene was induced in microarrays of the PA2663 mutant versus the wild-type strain (Chapter IV). To learn more about the PA1385 protein and the genetic basis of reduction in virulence, we used DNA microarrays to study the gene expression of the *P. aeruginosa* PAO1 PA1385 mutant versus the wild-type strain in biofilms. We found that the PA1385 protein induces expression of pyoverdine genes (*pvdA*, *pvdH*, and *pvdN*), pyochelin genes (*pchE*, *pchR*, and *pchD*), and a quorum sensing gene, *rhlA*; and represses the expression of *cup* genes, type II and type III secretion genes.

D.2 Results and discussion

D.2.1 Differential gene expression in biofilms upon inactivating the PA1385 gene

To explore the mechanism by which PA1385 protein induces the virulence in two plant hosts and hemolytic activity in *P. aeruginosa*, differential gene expression in *P. aeruginosa* PAO1 PA1385 mutant versus *P. aeruginosa* PAO1 wild-type was performed. In this experiment, 1,294 genes were induced and 197 genes were repressed in the PA1385 mutant greater than 1.8-fold. The fold changes, however, were not high; the highest induced gene was PA3897 at 6.5-fold, and the lowest repressed gene was PA4247 at -3.5-fold. The partial list of the induced and repressed genes is shown in Table D.1 and Table D.2.

The mutation in the PA1385 gene strikingly repressed the expression of two siderophore genes (*pchERD* and *pvdAHN*). PvdA (*L*-ornithine N^5-oxygenase) converts ornithine into N^5-formyl-N^5-hydroxyornithine (Lamont and Martin, 2003; Visca *et al.*, 1994). PvdH is an aminotransferase (Vandenende *et al.*, 2004), and PvdN is involved in twin arginine translocation (Voulhoux *et al.*, 2006). The twin arginine translocation system plays an important role in the export of phospholipases, iron uptake, anaerobic respiration, osmotic stress defense, motility, and biofilm formation (Ochsner *et al.*, 2002a). PchE is involved in the conversion of salicylic acid to dihydroaeruginoic acid (Cornelis and Matthijs, 2002). PchR is also involved in carbon metabolism, stress response, and pathogenesis (Michel *et al.*, 2005). The *pvd* operon encodes proteins used in pyoverdine synthesis, and *pch* operon encodes proteins used in pyochelin synthesis. As the mutation in PA1385 gene decreases expression of these two operons, to determine the effect of PA1385 protein, pyoverdine and pyochelin production should be measured in the PA1385 mutant.

Table D.1. Partial list of repressed genes for *P. aeruginosa* PAO1 PA1385 versus *P. aeruginosa* PAO1 in biofilms formed on glass wool in LB at 30°C after 7 h.

PA #	Gene name	Fold change	Descriptions	Signal value in PA1385	Detection in PA1385	Signal value in wild-type	Detection in wild-type
Adaptation, protection							
PA2386	*pvdA*	**-2.5**	*L*-ornithine *N*-oxygenase	763.9	P	1899.4	P
PA2394	*pvdN*	**-2.5**	Pyoverdine synthesis	561.0	P	1369.0	P
PA2413	*pvdH*	**-2.1**	*L*-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH	564.6	P	889.3	P
Transport of small molecules							
PA4226	*pchE*	**-2.1**	Dihydroaeruginoic acid synthetase	571.5	P	1143.3	P
PA4227	*pchR*	**-1.2**	Transcriptional regulator PchR	399.8	P	579.3	P
PA4228	*pchD*	**-2.5**	Pyochelin biosynthesis protein PchD	829.6	P	1798.3	P
Energy metabolism							
PA1552		**-2.3**	Probable cytochrome c	460.1	P	1061.9	P
PA1581	*sdhC*	**-2.1**	Succinate dehydrogenase	875.3	P	2142.4	P
PA1582	*sdhD*	**-1.8**	Succinate dehydrogenase	1244.8	P	2519.8	P
PA1583	*sdhA*	**-2.3**	Succinate dehydrogenase	1010.7	P	2385.6	P
PA1584	*sdhB*	**-2.0**	Succinate dehydrogenase	804.2	P	1920.7	P
PA1585	*sucA*	**-2.5**	2-oxoglutarate dehydrogenase	593.1	P	1481.4	P
PA1586	*sucB*	**-2.0**	Dihydrolipoamide succinyltransferase	1282.5	P	2922.2	P
PA1587	*lpdG*	**-2.3**	Amino acid biosynthesis and metabolism, lipoamide dehydrogenase	625.4	P	1444.6	P
PA1588	*sucC*	**-2.6**	Succinyl-CoA synthetase beta chain	1526.1	P	4183.9	P
PA1770	*ppsA*	**-2.6**	Carbon compound catabolism, central intermediary metabolism, phosphoenolpyruvate synthase	440.2	P	1670.1	P
PA2951	*etfA*	**-2.1**	Electron transfer flavoprotein alpha-subunit	1225.7	P	2133.9	P
PA4470	*fumC1*	**-2.3**	Fumarate hydratase	741.9	P	1789.6	P
PA5555	*atpG*	**-2.1**	ATP synthase gamma chain	2110.0	P	4570.5	P

Table D.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA1385	Detection in PA1385	Signal value in wild-type	Detection in wild-type
PA5556	*atpA*	**-2.8**	ATP synthase alpha chain	1268.2	P	3823.1	P
PA5557	*atpH*	**-2.5**	ATP synthase delta chain	1748.7	P	5080.3	P
PA5558	*atpF*	**-2.1**	ATP synthase B chain	3475.2	P	7514.7	P
PA5559	*atpE*	**-2.1**	Atp synthase C chain	3219.5	P	6262.1	P
Secreted Factors (toxins, enzymes, alginate)							
PA3479	*rhlA*	**-2.3**	Rhamnosyltransferase chain A	879.3	P	2621.3	P
Amino acid biosynthesis and metabolism							
PA5429	*aspA*	**-3.3**	Aspartate ammonia-lyase	1056.2	P	2695.2	P
Antibiotic resistance and susceptibility							
PA0807	*ampDh3*	**-2.1**	57% similar to hypothetical protein AmpD of *E. coli*	2245.1	P	5937.5	P
Cell wall, LPS, capsule							
PA3645	*fabZ*	**-2.1**	Fatty acid and phospholipid metabolism, 3-hydroxymyristoyl-acyl carrier protein dehydratase	546.0	P	1088.5	P
Central intermediary metabolism							
PA2393		**-2.8**	Probable dipeptidase precursor	453.2	P	1088.4	P
Fatty acid and phospholipid metabolism							
PA2968	*fabD*	**-2.1**	Malonyl-CoA-(acyl-carrier-protein) transacylase	248.9	P	449.1	P
Hypothetical, unclassified, unknown function							
PA0615		**-2.1**	Hypothetical protein	4575.1	P	9858.1	P
PA0909		**-2.1**	Related to phage, transposon or plasmid, membrane proteins	626.3	P	2003.8	P
PA0910		**-2.8**	Hypothetical protein	1678.8	P	4851.7	P
PA0911		**-2.5**	Hypothetical protein	2020.5	P	4976.4	P
PA2406		**-2.3**	Hypothetical protein	280.7	P	762.0	P

Table D.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA1385	Detection in PA1385	Signal value in wild-type	Detection in wild-type
PA2412		**-2.1**	Hypothetical protein	451.8	P	893.2	P
PA2971		**-2.5**	Hypothetical protein	377.4	P	996.2	P
Nucleotide biosynthesis and metabolism							
PA3637	*pyrG*	**-2.1**	CTP synthase	820.1	P	1835.5	P
Protein secretion, export apparatus							
PA4403	*secA*	**-2.1**	Secretion protein SecA	485.0	P	1037.9	P
Putative enzymes							
PA3001		**-2.6**	Probable dipeptidase precursor	913.4	P	2485.5	P
Related to phage, transposon, or plasmid							
PA0617		**-2.3**	Probable bacteriophage protein	3895.0	P	9661.6	P
PA0618		**-2.5**	Probable bacteriophage protein	4604.6	P	12835.5	P
PA0619		**-2.1**	Bacteriophage P2-related tail formation protein	5530.9	P	12053.3	P
PA0620		**-2.1**	Phage-related tail fibre protein	5291.2	P	12840.6	P
PA0624		**-2.5**	Hypothetical protein	5864.4	P	12633.2	P
PA0625		**-2.5**	Mu-like prophage protein	2522.6	P	6943.2	P
PA0626		**-2.8**	Phage protein U	3827.1	P	5304.9	P
PA0627		**-2.5**	P2-like prophage tail protein X	3877.1	P	9497.2	P
PA0628		**-2.0**	Hypothetical protein	6228.2	P	12280.8	P
PA0629		**-3.0**	Predicted chitinase	2886.6	P	8894.2	P
PA0630		**-2.8**	Hypothetical protein	3418.8	P	9400.9	P
PA0631		**-2.8**	Hypothetical protein	2436.8	P	6471.2	P
PA0632		**-2.8**	Hypothetical protein	2157.4	P	6628.2	P
PA0633		**-2.0**	Hypothetical protein	8721.6	P	17648.8	P
PA0634		**-2.5**	Hypothetical protein	3213.3	P	8858.6	P

Table D.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA1385	Detection in PA1385	Signal value in wild-type	Detection in wild-type
PA0635		**-2.8**	Hypothetical protein	4433.9	P	10252.6	P
PA0636		**-1.9**	Hypothetical protein	7564.2	P	14551.7	P
PA0637		**-2.8**	Phage-related protein	2980.7	P	7711.3	P
PA0638		**-2.5**	Probable bacteriophage protein	4827.4	P	12465.3	P
PA0639		**-3.0**	Predicted metal-dependent protease of the PAD1/JAB1 superfamily	4011.4	P	10857.4	P
PA0640		**-2.8**	Phage-related protein, tail component	1198.0	P	3547.1	P
PA0641		**-3.0**	Phage-related protein, tail component	2662.6	P	8887.4	P
PA0642		**-1.4**	Hypothetical protein	717.5	P	1081.4	P
PA0643		**-2.1**	Hypothetical protein	1295.0	P	3555.8	P
PA0644		**-2.1**	Hypothetical protein	2246.2	P	5039.0	P
PA0645		**-2.8**	Hypothetical protein	1985.5	P	4296.2	P
Transcriptional regulators							
PA0612	*ptrB*	**-3.0**	Repressor, DnaK suppressor protein	1345.5	P	5304.9	P
Transcription, RNA processing and degradation							
PA3743	*trmD*	**-2.1**	tRNA (guanine-N1)-methyltransferase	449.7	P	1074.2	P
PA3744	*rimM*	**-2.1**	16S rRNA processing protein	577.2	P	1272.7	P
PA3745	*rpsP*	**-2.6**	DNA replication, recombination, modification and repair, 30S ribosomal protein S16	783.7	P	2004.4	P
PA4740	*pnp*	**-2.1**	Polyribonucleotide nucleotidyltransferase	686.5	P	1001.1	P
Translation, post-translational modification, degradation							
PA0579	*rpsU*	**-2.1**	30S ribosomal protein S21	498.2	P	1121.5	P
PA2619	*infA*	**-2.1**	Initiation factor	306.4	P	547.0	P
PA3162	*rpsA*	**-2.5**	30S ribosomal protein S1	1041.1	P	2646.8	P
PA3655	*tsf*	**-2.3**	Elongation factor Ts	403.8	P	737.8	P

Table D.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA1385	Detection in PA1385	Signal value in wild-type	Detection in wild-type
PA3656	*rpsB*	**-2.6**	30S ribosomal protein S2	822.1	P	2186.9	P
PA4238	*rpoA*	**-3.0**	DNA-directed RNA polymerase alpha chain	1255.9	P	4215.5	P
PA4239	*rpSD*	**-2.6**	30S ribosomal protein S4	617.0	P	1787.2	P
PA4240	*rpsK*	**-3.0**	30S ribosomal protein S11	875.8	P	2523.4	P
PA4241	*rpsM*	**-3.0**	30S ribosomal protein S13	671.4	P	1936.3	P
PA4242	*rpmJ*	**-2.8**	50S ribosomal protein L36	839.6	P	2486	P
PA4243	*secY*	**-2.8**	Membrane proteins, protein secretion/export apparatus	366.9	P	1171.4	P
PA4244	*rplO*	**-2.8**	50S ribosomal protein L15	1016.6	P	2611.3	P
PA4245	*rpmD*	**-2.8**	50S ribosomal protein L30	662.9	P	1872.9	P
PA4246	*rpsE*	**-2.6**	30S ribosomal protein S5	1142.5	P	3199.7	P
PA4247	*rplR*	**-3.5**	50S ribosomal protein L18	868.3	P	3018.9	P
PA4248	*rplF*	**-3.3**	50S ribosomal protein L6	1055.7	P	3743.3	P
PA4249	*rpsH*	**-2.6**	30S ribosomal protein S8	1596.4	P	4500.3	P
PA4252	*rplX*	**-2.6**	50S ribosomal protein L24	611.4	P	1189.5	P
PA4253	*rplN*	**-2.6**	50S ribosomal protein L14	1457.9	P	3534.9	P
PA4254	*rpsQ*	**-2.6**	30S ribosomal protein S17	2145.5	P	5495.8	P
PA4255	*rpmC*	**-2.5**	50S ribosomal protein L29	377.4	P	511.4	P
PA4256	*rplP*	**-2.3**	50S ribosomal protein L16	1231.1	P	3420.7	P
PA4257	*rpsC*	**-2.5**	30S ribosomal protein S3	921.7	P	2228.5	P
PA4258	*rplV*	**-2.6**	50S ribosomal protein L22	1328.6	P	3455.6	P
PA4259	*rpsS*	**-2.3**	30S ribosomal protein S19	905.6	P	2310.1	P
PA4260	*rplB*	**-2.3**	50S ribosomal protein L2	2507.3	P	5208.8	P
PA4261	*rplW*	**-2.1**	50S ribosomal protein L23	1497.3	P	2810	P
PA4262	*rplD*	**-2.5**	50S ribosomal protein L4	1189.2	P	3349.7	P

Table D.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA1385	Detection in PA1385	Signal value in wild-type	Detection in wild-type
PA4263	*rplC*	**-2.5**	50S ribosomal protein L3	1409.9	P	3751.5	P
PA4264	*rpsJ*	**-2.6**	Transcription, RNA processing and degradation, 30S ribosomal protein S10	1801.3	P	4417.1	P
PA4266	*fusA1*	**-2.6**	Elongation factor	971.9	P	2846.2	P
PA4268	*rpsL*	**-2.8**	30S ribosomal protein S12	955.4	P	2970.8	P
PA4270	*rpoB*	**-2.5**	DNA-directed RNA polymerase beta chain	496	P	1461.3	P
PA4271	*rplL*	**-2.1**	50S ribosomal protein L7/L12	1186.2	P	1609.5	P
PA4272	*rplJ*	**-2.6**	50S ribosomal protein L10	1117.4	P	2235	P
PA4273	*rplA*	**-2.6**	50S ribosomal protein L1	821.5	P	2129.5	P
PA4274	*rplK*	**-2.5**	50S ribosomal protein L11	1670.2	P	4175.2	P
PA4433	*rplM*	**-3.3**	50S ribosomal protein L13	636.3	P	2534.2	P
PA4568	*rplU*	**-2.3**	50S ribosomal protein L21	1422.5	P	3319.6	P
PA4671		**-2.5**	Adaptation, protection, probable ribosomal protein L25	692.8	P	1582.3	P
PA4935	*rpsF*	**-2.3**	30S ribosomal protein S6	905.6	P	1619.9	P
PA5316	*rpmB*	**-2.1**	50S ribosomal protein L28	1100	P	2087.1	P
PA5569	*rnpA*	**-2.1**	Ribonuclease P protein component	1086.5	P	2386.7	P
PA5570	*rpmH*	**-2.1**	50S ribosomal protein L34	341.7	P	827.6	P

Table D.2. Partial list of induced genes for *P. aeruginosa* PAO1 PA1385 versus *P. aeruginosa* PAO1 in biofilms formed on glass wool in LB at 30°C after 7 h.

PA #	Gene name	Fold change	Descriptions	Signal value in PA1385	Detection in PA1385	Signal value in wild-type	Detection in wild-type
Motility and attachment							
PA0499		**3.0**	Chaperones and heat shock proteins, probable pili assembly chaperone	379.7	P	101.7	P
PA0992	*cupC1*	**3.0**	Fimbrial subunit CupC1	545.7	P	169.2	P
PA0993	*cupC2*	**3.5**	Chaperones and heat shock proteins, chaperone CupC2	298.7	P	78.0	P
PA4084	*cupB3*	**3.0**	Usher CupB3, probable fimbrial biogenesis usher protein	308.0	P	107.6	P
PA4549	*fimT*	**3.5**	Type 4 fimbrial biogenesis protein	331.5	P	104.3	P
Protein secretion, export apparatus							
PA1705	*pcrG*	**3.3**	Regulator in type III secretion	330.8	P	133.1	P
PA2673		**3.0**	Probable type II secretion system protein	293.1	P	105.4	P
PA2674		**3.0**	Probable type II secretion system protein	379.6	P	154.6	P
PA2675		**3.0**	Probable type II secretion system protein	302.1	P	111.6	P
Amino acid biosynthesis and metabolism							
PA1393	*cysC*	**3.3**	Adenosine 5'-phosphosulfate (APS) kinase, central intermediary metabolism, nucleotide biosynthesis and metabolism	199.8	P	67.5	P
PA5417	*soxD*	**3.0**	Sarcosine oxidase delta subunit	430.4	P	132.2	P
Antibiotic resistance and susceptibility							
PA1129		**3.0**	Related to phage, transposon, or plasmid, probable fosfomycin resistance protein	184.8	P	139.6	P
Biosynthesis of cofactors, prosthetic groups and carriers							
PA4891	*ureE*	**3.0**	Urease accessory protein UreE	237.7	P	112.3	P
Carbon compound catabolism							
PA0227		**3.0**	Probable CoA transferase, subunit B	490.8	P	175.5	P

Table D.2. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA1385	Detection in PA1385	Signal value in wild-type	Detection in wild-type
PA1384	*galE*	**5.3**	UDP-glucose 4-epimerase, nucleotide biosynthesis and metabolism, central intermediary metabolism	156.8	P	32.0	P
PA1385		**3.7**	Cell wall, LPS, capsule, probable glycosyl transferase	79.4	P	24.1	A
PA1386		**3.3**	Probable ATP-binding component of ABC transporter	344.7	P	99.2	P
PA1983	*exaB*	**3.7**	Energy metabolism, cytochrome c, mono- and diheme variants	219.1	P	63.0	P
Hypothetical, unclassified, unknown function							
PA0264		**3.0**	Hypothetical protein	406.7	P	125.2	P
PA0466		**3.0**	Hypothetical protein	642.5	P	208.0	P
PA0498		**3.0**	Hypothetical protein	136.1	P	54.8	P
PA0673		**3.0**	Hypothetical protein	507.1	P	158	P
PA0697		**3.0**	Hypothetical protein	314.4	P	117.4	P
PA0823		**4.0**	Hypothetical protein	445.2	P	112.2	P
PA0874		**4.0**	Hypothetical protein	136.1	P	45.8	P
PA0980		**3.0**	Hypothetical protein	134.1	P	54.1	P
PA0981		**3.3**	Hypothetical protein	67.4	P	22.9	A
PA1362		**3.0**	Hypothetical protein	721.9	P	258.7	P
PA1936		**3.3**	Hypothetical protein	240.4	P	96.4	P
PA2036		**4.0**	Hypothetical protein	116.9	P	36.3	A
PA2037		**3.5**	Hypothetical protein	151.0	P	34.8	P
PA2228		**3.7**	Hypothetical protein, *β*-lactamase class C and other penicillin binding proteins	84.4	P	17.7	A
PA2292		**3.3**	Hypothetical protein	494.6	P	191.2	P
PA2636		**3.0**	Hypothetical protein	314.5	P	100.1	P
PA2935		**4.6**	Hypothetical protein	144.3	P	28.2	P

Table D.2. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA1385	Detection in PA1385	Signal value in wild-type	Detection in wild-type
PA3218		**3.0**	Hypothetical protein	395.4	P	110.0	P
PA3229		**3.3**	Hypothetical protein	454.8	P	142.1	P
PA3412		**3.5**	Hypothetical protein	289.2	P	123.9	P
PA3501		**3.0**	Hypothetical protein	347.3	P	119.9	P
PA3884		**3.3**	Hypothetical protein	310.9	P	88.1	P
PA4071		**3.0**	Hypothetical protein	307.3	P	118.7	P
PA4146		**3.3**	Hypothetical protein	562.4	P	205.5	P
PA4674		**4.0**	Hypothetical protein	200.2	P	55.2	P
PA4683		**4.0**	Hypothetical protein	147.5	P	60.2	A
PA4823		**3.3**	Hypothetical protein	614.2	P	178.3	P
PA4881		**3.0**	Hypothetical protein	777.6	P	260.0	P
PA4882		**2.0**	Hypotheticall protein	336.2	P	149.1	P
PA4883		**3.3**	Hypothetical protein, thiosulfate reductase cytochrome B subunit	329.8	P	84.8	P
PA5385		**3.3**	Predicted thioesterase	554.8	P	167.7	P
PA5480		**3.3**	Hypothetical protein	304.5	P	101.3	P
Membrane Proteins							
PA0013		**3.3**	Hypothetical protein, 77% similar to hypothetical protein YhhQ in *E. coli*	232.4	P	77.2	P
PA1230		**3.0**	Hypothetical protein	438.4	P	171.0	P
PA3897		**6.5**	Membrane proteins ; hypothetical	140.3	P	22.2	A
PA5566		**3.5**	Hypothetical protein	544.2	P	153.6	P
Putative Enzymes							
PA2355		**3.0**	Probable FMNH2-dependent monooxygenase	415.3	P	155.1	P
PA3389		**3.0**	Probable ring-cleaving dioxygenase	394.0	P	137.9	P

Table D.2. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA1385	Detection in PA1385	Signal value in wild-type	Detection in wild-type
PA3498		**3.0**	Probable oxidoreductase	227.5	P	76.6	P
Transcriptional regulator							
PA5403		**4.0**	Probable transcriptional regulator	189.2	P	86.2	P
Transport of small molecules							
PA0198	*exbB*	**3.0**	Transport protein ExbB	272.8	P	113.1	P
PA0204		**3.0**	Membrane protein, probable permease of ABC transporter	463.1	P	148.5	P
PA0205		**3.5**	Membrane protein, probable permease of ABC transporter, ABC-type spermidine/putrescine transport system, permease component I	662.4	P	278.6	P
PA0326		**3.0**	Probable ATP-binding component of ABC transporter	534.5	P	164.4	P
PA0689		**3.3**	Hypothetical protein, ABC-type phosphate transport system, periplasmic component	465.1	P	88.4	P
PA0885		**3.0**	Probable C_4-dicarboxylate transporter	211.0	P	76.5	P
PA0886		**3.0**	Probable C_4-dicarboxylate transporter	444.6	P	189.6	P
PA1632	*kdpF*	**3.3**	kdpF protein	294.0	P	87.2	P
PA1971	*braZ*	**3.3**	Branched chain amino acid transporter BraZ	342.9	P	102.8	P
PA2214		**3.0**	Membrane proteins, probable major facilitator superfamily (MFS) transporter	395.3	P	119.6	P
PA2262		**3.0**	Membrane proteins, probable 2-ketogluconate transporter	179.5	P	49.3	P
PA4194		**3.0**	Membrane protein	269.4	P	129.5	P
PA4837		**3.0**	Probable outer membrane protein precursor	556.7	P	270.7	P
PA4862		**3.3**	Probable ATP-binding component of ABC transporter	402.7	P	105.7	P
PA5082		**3.0**	Probable binding protein component of ABC transporter	674.7	P	191.3	P

Dehydrogenase genes (PA1581-1588) were repressed in the PA1385 mutant. The quorum-sensing gene, *rhlA* was also repressed by 2.3-fold. *rhlA* encodes rhamnosyltransferase (Winsor *et al.*, 2005). A *rhlA,* rhamnolipid-deficient mutant lost its ability to swarm (Caiazza *et al.*, 2005) and altered the biofilm formation architecture (Davey *et al.*, 2003).

Of the induced genes, five motility and attachment genes, including *cup* operon genes, were induced. These motility genes might be the cause of the increase in swimming motility in the PA1385 mutant (Attila *et al.*, 2008a). The Cup proteins are also involved in biofilm formation and in surface adherence (Vallet *et al.*, 2001).

Although the transcriptome analysis of the PA1385 mutant showed interesting results to the PA1385 protein and siderophores and motility genes, the transcriptome analysis was not performed on live tissue so that, except type II and type III secretion genes, other virulence genes influenced by the PA1385 protein were not observed.

D.3 Experimental procedures

D.3.1 Bacterial RNA isolation and DNA microarrays

The strains used in this study are listed in Table 3.3. Bacterial RNA isolation and DNA microarray experiments were conducted as described previously (Attila *et al.*, 2008b). Briefly, the wild-type and the PA1385 mutant strains were grown in LB (wild-type strain) and LB with 50 µg/mL tetracycline (PA1385 mutant). One mL of the overnight cultures (optical density 7.5 at 600 nm) was inoculated into 250 mL of fresh LB medium with 10 g of glass wool and the cultures were incubated for 7 h at 30°C with shaking. The cell pellets from glass wool and the total RNA was prepared as described previously (Attila *et al.*, 2008b).

The Genechip *P. aeruginosa* Genome Array (Affymetrix, P/N 900339) contains 5,500 of

the 5,570 ORFs of *P. aeruginosa* (Whiteley *et al.*, 2001). cDNA synthesis, fragmentation, and hybridizations were as described previously (González-Barrios *et al.*, 2006b). Hybridization was performed for 16 h at 50°C, and the total cell intensity was scaled to an average value of 500. The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA control were used to monitor the labeling process. For each binary microarray comparison of differential genes expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 13 probe pairs (*p*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *p*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher (1.8-fold) than the standard deviation for the whole microarrays (Ren *et al.*, 2004a). The standard deviation for all the probe sets was 1.8-fold. Gene functions were obtained from the *Pseudomonas g*enome database (Winsor *et al.*, 2005).

APPENDIX E

INVESTIGATION OF PA0984 PROTEIN WITH DNA MICROARRAYS

E.1 Introduction

PA0984 is an immunity protein that encodes a bacteriocin (Winsor *et al.*, 2005). Bacteriocins produce toxins and cause cell death (Michel-Briand and Baysse, 2002). PA0984 gene expression was induced in the sugar beet root exudates (Mark *et al.*, 2005) and in our rhizosphere study (Chapter III) PA0984 gene was induced by 10.6-fold upon poplar tree contact (Attila *et al.*, 2008a). We also found that the mutation in the PA0984 gene represses rhizosphere competitiveness, and hemolytic and cytotoxic activities (Attila *et al.*, 2008a). Also, biofilm formation was decreased by 2-fold and the motility was induced by 1.5-fold upon mutation of the PA0984 gene. To investigate the genetic basis of biofilm inhibition and reduction in virulence, we used DNA microarrays to study the gene expression of *Pseudomonas aeruginosa* PAO1 PA0984 mutant versus the wild-type strain in biofilms. We found that PA0984 protein induces expression of *alg* and *pel* genes; *pel* is one of the two biofilm operons of *P. aeruginosa*.

E.2 Results and discussion

E.2.1 Differential gene expression in biofilms upon inactivating PA0984 gene

To explore the mechanism by which PA0984 protein induces biofilm formation in *P. aeruginosa*, differential gene expression of *P. aeruginosa* PA0984 mutant versus *P. aeruginosa* PAO1 wild-type strains was performed. A total of 23 genes were induced and 193 genes were repressed upon the mutation in PA0984 gene greater than 2.5-fold (Table E.1 and Table E.2). The most striking repression was observed in *pelA* gene (-17.2-fold) and also two other *pel* operon genes, *pelE* and *pelF*, were repressed by -8.0- and -2.6-fold, respectively. The *pel* operon is important for both early and late stages of biofilm formation in *P. aeruginosa* PAK strain

Table E.1. Partial list of repressed genes more than 2.5-fold for *P. aeruginosa* PAO1 PA0984 versus *P. aeruginosa* PAO1 in biofilms formed on glass wool in LB at 30°C after 7 h.

PA #	Gene name	Fold change	Descriptions	Signal value in PA084	Detection in PA0984	Signal value in wild-type	Detection in wild-type
Cell wall, LPS, capsule							
PA3064	*pelA*	**-17.2**	Part of *pelABCDEFG* cluster involved in biofilm matrix formation in *P. aeruginosa* PA14	4.5	A	79.9	P
PA3060	*pelE*	**-8.0**	Part of *pelABCDEFG* cluster involved in biofilm matrix formation in *P. aeruginosa* PA14 and ZK2870	8.5	A	127.1	P
PA3059	*pelF*	**-2.6**	Part of *pelABCDEFG* cluster involved in biofilm matrix formation in *P. aeruginosa* PA14 and ZK2870	57.7	A	243.9	P
PA3540	*algD*	**-3.0**	Adaptation, protection, secreted factors (toxins and alginate), GDP-mannose 6-dehydrogenase AlgD	44.7	A	99.0	P
PA3543	*algK*	**-3.5**	Adaptation, protection, secreted factors (toxins and alginate), alginate biosynthetic protein AlgK precursor	37.0	A	113.3	P
PA5538	*amiA*	**-2.6**	*N*-acetylmuramoyl-*L*-alanine amidase	69.7	M	181.0	P
Protein secretion, export apparatus							
PA1702		**-3.0**	Conserved hypothetical protein in type III secretion	49.7	A	134.4	P
PA1867	*xphA*	**-3.3**	46% similar to secretion protein XcpP in *P. aeruginosa*	42.7	A	139.5	P
PA1868	*xqhA*	**-4.9**	Type II secretory pathway, component PulD	26.6	A	140.2	P
Carbon compound catabolism							
PA0213		**-4.9**	Hypothetical protein	47.5	A	269.1	P
PA0214		**-2.8**	Probable acyl transferase	135.2	P	246.3	P
PA0228	*pcaF*	**-2.8**	Fatty acid and phospholipid metabolism, beta-ketoadipyl CoA thiolase PcaF	48.8	A	140.4	P
PA2507	*catA*	**-3.0**	Catechol 1,2-dioxygenase	85.4	P	217.7	P
PA2509	*catB*	**-3.0**	Muconate cycloisomerase	32.6	A	129.5	P
PA2512	*antA*	**-3.0**	Anthranilate dioxygenase large subunit	646.2	P	2033	P
PA2513	*antB*	**-2.1**	Anthranilate dioxygenase small subunit	1583.4	P	3651.2	P

Table E.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA0984	Detection in PA0984	Signal value in wild-type	Detection in wild-type
PA2514	*antC*	**-2.6**	Anthranilate dioxygenase reductase	283.1	P	758.4	P
PA3589		**-4.0**	Probable acyl-CoA thiolase	34	A	127.1	P
PA4901	mdlC	**-2.6**	Benzoylformate decarboxylase	44.2	A	94.2	P
Adaptation, protection							
PA2147	*katE*	**-2.6**	Catalase HPII	47.8	A	198.5	P
Amino acid biosynthesis and metabolism							
PA1421	*gbuA*	**-2.8**	Guanidinobutyrase	51.7	A	140.7	P
Antibiotic resistance and susceptibility							
PA1129		**-3.3**	Related to phage, transposon, or plasmid, probable fosfomycin resistance protein	20.1	A	139.6	P
PA1238		**-5.3**	Transport of small molecules, probable outer membrane component of multidrug efflux pump	12.5	A	94.8	P
PA2055		**-5.7**	Membrane proteins, transport of small molecules, probable major facilitator superfamily transporter	21.1	A	142.6	P
PA4990		**-2.6**	Membrane proteins, transport of small molecules, multidrug efflux transporter	54.2	P	120.2	P
Biosynthesis of cofactors, prosthetic groups and carriers							
PA0583		**-2.6**	7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase	92.2	P	160.1	P
PA4973	*thiC*	**-2.6**	Thiamin biosynthesis protein ThiC	72.4	P	183.5	P
PA4988	*waaA*	**-2.6**	3-deoxy-*D*-manno-octulosonic-acid (KDO) transferase	114.2	M	310.5	P
DNA replication, recombination, modification and repair							
PA2138		**-3.0**	Probable ATP-dependent DNA ligase	62.0	A	243.3	P
Energy metabolism							
PA0525		**-2.6**	Probable dinitrification protein NorD	58.1	A	207.3	P
PA2382	*lldA*	**-3.0**	*L*-lactate dehydrogenase	61.5	A	122.6	P

Table E.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA0984	Detection in PA0984	Signal value in wild-type	Detection in wild-type
PA2714		**-3.0**	Probable molybdopterin oxidoreductase	58.2	M	198.9	P
PA2715		**-8.6**	Probable ferredoxin	3.7	A	73.9	P
PA3394	*nosF*	**-3.5**	Transport of small molecules, ABC-type multidrug transport system	61.0	A	195.8	P
PA3395	*nosY*	**-1.5**	ABC-type transport system involved in multi-copper enzyme maturation, permease component	92.9	P	157.7	P
PA3396	*nosL*	**-2.8**	Predicted lipoprotein involved in nitrous oxide reduction	28.7	A	127	P
PA4772		**-2.8**	Probable ferredoxin	45.8	A	167.7	P
Fatty acid and phospholipid metabolism							
PA4813	*lipC*	**-8.6**	Lipase LipC	15.2	A	88.1	P
Hypothetical, unclassified, unknown function							
PA0238		**-2.6**	Sugar phosphate isomerases/epimerases	134.7	P	318.3	P
PA0492		**-3.0**	Hypothetical protein	38.9	A	141.0	P
PA0695		**-4.0**	Hypothetical protein	11.1	A	65.7	P
PA0697		**-17.2**	Hypothetical protein	5.2	A	117.4	P
PA0774		**-2.6**	2-polyprenyl-3-methyl-5-hydroxy-6-metoxy-1,4-benzoquinol methylase	62.2	P	292.9	P
PA0845		**-2.6**	Hypothetical protein	60.6	A	146.8	P
PA0881		**-2.6**	Hypothetical protein	77.0	A	215.0	P
PA0914		**-3.7**	Hypothetical protein	34.3	A	136.5	P
PA0957		**-17.2**	Uncharacterized protein, possibly involved in aromatic compounds catabolism	4.8	A	92.9	P
PA0977		**-3.3**	Hypothetical protein	21.0	A	63.0	P
PA1211		**-2.8**	Hypothetical protein	106.4	A	266.4	P
PA1259		**-2.6**	Hypothetical protein	53.0	A	148.3	P

Table E.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA0984	Detection in PA0984	Signal value in wild-type	Detection in wild-type
PA1267		**-3.5**	Hypothetical protein	53.4	A	173.6	P
PA1270		**-3.0**	Hypothetical protein	32.7	A	89.6	P
PA1291		**-4.0**	Hypothetical protein	43.9	A	188.0	P
PA1332		**-3.3**	Hypothetical protein	43.4	A	166.5	P
PA1743		**-2.6**	Hypothetical protein	48.6	A	136.5	P
PA1797		**-3.0**	Beta-lactamase class C and other penicillin binding proteins	27.0	A	87.4	P
PA1831		**-2.6**	Fructose-2,6-bisphosphatase	53.1	P	148.8	P
PA1921		**-3.5**	Hypothetical protein	43.5	A	166.7	P
PA1924		**-3.0**	Hypothetical protein	77.4	A	142.8	P
PA2078		**-2.8**	Hypothetical protein	32.3	A	127.1	P
PA2087		**-2.6**	Hypothetical protein	40.6	A	88.2	P
PA2136		**-2.8**	Hypothetical protein	35.1	A	133.2	P
PA2163		**-2.8**	4-alpha-glucanotransferase	106.1	A	418.2	P
PA2192		**-2.8**	Hypothetical protein	46.2	A	136.6	P
PA2207		**-4.3**	Hypothetical protein	18.6	A	100.9	P
PA2215		**-4.9**	*L*-alanine-*D*, *L*-glutamate epimerase and related enzymes of enolase superfamily	10.9	A	72.5	P
PA2245	*pslO*	**-3.0**	Part of *pslABCDEFGHIJKLMNO* cluster involved in biofilm development	32.0	A	107.3	P
PA2296		**-3.0**	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	43.8	A	138.7	P
PA2441		**-3.3**	Hypothetical protein	40.8	A	102.9	P
PA2471		**-3.0**	2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase	28.5	A	88.8	P
PA2689		**-2.8**	Predicted hydrolase of the alpha/beta superfamily	79.1	M	162.9	P

Table E.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA0984	Detection in PA0984	Signal value in wild-type	Detection in wild-type
PA2759		**-3.3**	Hypothetical protein	32.3	A	150.3	P
PA2789		**-4.9**	Hypothetical protein	22.4	A	85.5	P
PA2804		**-16.0**	Hypothetical protein	6.4	A	121.0	P
PA3036		**-3.0**	Hypothetical protein	45.9	A	147.8	P
PA3066		**-3.0**	Amidases related to nicotinamidase	34.2	A	115.3	P
PA3237		**-2.8**	Hypothetical protein	28.3	A	76.2	P
PA3505		**-2.6**	Hypothetical protein	99.4	A	262.2	P
PA4103		**-2.6**	Hypothetical protein	27.1	A	119.8	P
PA4104		**-2.8**	Hypothetical protein	146.8	P	375.7	P
PA4287		**-4.6**	Hypothetical protein	20.1	A	118.1	P
PA4445		**-2.6**	Hypothetical protein	104.0	P	315.9	P
PA4509		**-4.3**	Allophanate hydrolase subunit 2	31.0	A	134.5	P
PA4773		**-6.5**	*S*-adenosylmethionine decarboxylase	18.3	A	132.1	P
PA4871		**-2.8**	Hypothetical protein	23.0	A	85.4	P
PA4883		**-7.5**	Thiosulfate reductase cytochrome B subunit	6.7	A	84.8	P
PA5284		**-3.0**	P pilus assembly protein, pilin FimA	21.8	A	82.0	P
PA5540		**-3.0**	Hypothetical protein	97.6	P	253.9	P
Membrane proteins							
PA0013		**-2.8**	Conserved hypothetical protein	26.2	A	77.2	P
PA1232		**-2.6**	Hypothetical protein	61.0	M	199.3	P
PA1265		**-11.3**	Hypothetical protein	12.3	A	162.1	P
PA2070		**-6.1**	Outer membrane receptor proteins, mostly Fe transport	5.4	A	65.0	P
PA2091		**-2.8**	Hypothetical protein	44.3	A	196.1	P
PA2439		**-7.5**	Membrane protease subunits, stomatin/prohibitin homologs	8.5	A	68.7	P

Table E.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA0984	Detection in PA0984	Signal value in wild-type	Detection in wild-type
PA2919		**-2.6**	Hypothetical protein	55.0	A	152.0	P
PA3457		**-2.8**	Predicted permeases	59.7	A	241.1	P
PA5469		**-5.3**	Membrane protein TerC, possibly involved in tellurium resistance	21.7	A	140.0	P
Nucleotide biosynthesis and metabolism							
PA1919	*nrdG*	**-4.9**	Class III (anaerobic) ribonucleoside-triphosphate reductase activating protein, NrdG	14.9	A	91.1	P
Putative enzymes							
PA0244		**-2.8**	Shikimate 5-dehydrogenase	68.1	A	184.3	P
PA0321		**-8.6**	Probable acetylpolyamine aminohydrolase	8.5	A	85.4	P
PA0883		**-2.6**	Probable acyl-CoA lyase beta chain	61.3	A	143.1	P
PA1266		**-2.6**	Probable oxidoreductase	68.7	M	155.9	P
PA1284		**-2.6**	Probable acyl-CoA dehydrogenase	46.6	A	143.5	P
PA1303		**-3.0**	Probable signal peptidase	26.1	A	95.8	P
PA1417		**-2.8**	Thiamine pyrophosphate-requiring enzymes [acetolactate synthase, pyruvate dehydrogenase	48.6	A	118.5	P
PA1648		**-2.8**	Putative NADP-dependent oxidoreductases	46.8	A	87.7	P
PA1827		**-3.5**	Probable short-chain dehydrogenase	50.0	A	132.7	P
PA2124		**-2.8**	Choline dehydrogenase and related flavoproteins	37.0	A	145.8	P
PA2125		**-8.6**	NAD-dependent aldehyde dehydrogenases	11.1	A	99.9	P
PA2162		**-3.5**	Probable glycosyl hydrolase	32.3	A	195.2	P
PA2310		**-2.6**	Probable taurine catabolism dioxygenase	29.5	A	128.2	P
PA2347		**-2.8**	Acyl-CoA dehydrogenases	38.2	A	125.1	P
PA3498		**-12.1**	Flavodoxin reductases (ferredoxin-NADPH reductases) family 1	6.6	A	76.6	P
PA4089		**-4.3**	Probable short-chain dehydrogenase	37.1	A	205	P

Table E.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA0984	Detection in PA0984	Signal value in wild-type	Detection in wild-type
PA4148		**-3.3**	Probable short-chain dehydrogenase	51.3	A	165.2	P
PA4189		**-2.8**	Probable aldehyde dehydrogenase	45.9	A	137	P
PA5384		**-2.8**	Probable lipolytic enzyme	78.5	A	167.2	P
Transcriptional regulators							
PA0253		**-2.6**	Probable transcriptional regulator	161.7	P	309.4	P
PA1109		**-4.6**	Probable transcriptional regulator	13.4	A	62.1	P
PA1136		**-2.6**	DNA-binding HTH domain-containing proteins	100.0	P	237.2	P
PA1380		**-2.6**	AraC-type DNA-binding domain-containing proteins	43.1	A	102.6	P
PA2447		**-2.8**	Probable transcriptional regulator	92.6	P	262.9	P
PA2704		**-6.1**	AraC-type DNA-binding domain-containing	18.9	A	139.6	P
PA3900		**-2.6**	Probable transmembrane sensor	67.4	A	218.0	P
PA4895		**-3.3**	Probable transmembrane sensor	73.1	M	187.0	P
Transcription, RNA processing and degradation							
PA1704	*pcrR*	**-3.7**	Transcriptional regulator protein PcrR	38.5	A	104.3	P
Transport of small molecules							
PA0198	*exbB1*	**-2.8**	Transport protein ExbB	50.6	A	113.1	P
PA0324		**-3.5**	Probable permease of ABC transporter	29.7	A	159.9	P
PA0811		**-2.8**	Probable major facilitator superfamily (MFS) transporter	68.6	A	144.1	P
PA1108		**-4.0**	Membrane proteins, probable major facilitator superfamily (MFS) transporter	28.0	A	191.3	P
PA1260		**-7.0**	Probable binding protein component of ABC transporter	15.2	A	85.9	P
PA1282		**-2.6**	Membrane proteins, probable major facilitator superfamily (MFS) transporter	68.6	A	154.3	P
PA1419		**-2.8**	Purine-cytosine permease and related proteins	47.0	A	122.0	P

Table E.1. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA0984	Detection in PA0984	Signal value in wild-type	Detection in wild-type
PA1569		**-9.9**	Membrane proteins, probable major facilitator superfamily (MFS) transporter	3.8	A	89.0	P
PA1908		**-2.6**	Membrane proteins, probable major facilitator superfamily (MFS) transporter	42.8	A	115.2	P
PA2058		**-2.8**	Probable binding protein component of ABC transporter	26.1	A	85.4	P
PA2252		**-2.8**	Probable AGCS sodium/alanine/glycine symporter	28.7	A	120.4	P
PA2308		**-14.9**	Probable ATP-binding component of ABC transporter	4.3	A	74.8	P
PA2350		**-2.8**	Probable ATP-binding component of ABC transporter	48.7	A	264.2	P
PA3382		**-2.8**	Membrane proteins, phosphonate transport protein PhnE	47.5	A	105.4	P
PA3448		**-3.0**	Membrane proteins, probable permease of ABC transporter	39.3	A	135.3	P
PA3560	*fruA*	**-4.0**	Carbon compound catabolism, phosphotransferase system, fructose-specific IIBC component	7.7	A	136.0	P
PA3766		**-3.7**	Probable aromatic amino acid transporter	57.4	A	160.9	P
PA3933		**-4.0**	Membrane proteins, probable choline transporter	32.4	A	149.3	P
PA4160		**-4.6**	Membrane proteins, ferric enterobactin transport protein FepD	35.4	A	176.1	P
PA4220		**-4.6**	Hypothetical protein	173.8	P	795.0	P
PA4221	*fptA*	**-1.9**	Fe(III)-pyochelin outer membrane receptor precursor	628.9	P	1112.2	P
PA4222		**-2.8**	Probable ATP-binding component of ABC transporter	212.3	P	772.5	P
PA4343		**-2.8**	Membrane proteins, probable major facilitator superfamily (MFS) transporter	48.9	A	122.9	P
PA4887		**-2.8**	Probable major facilitator superfamily (MFS) transporter	91.8	P	252.4	P
Two-component regulatory systems							
PA4886		**-2.6**	Signal transduction histidine kinase	58.3	P	147.3	P

Table E.2. Partial list of induced genes more than 2-fold for *P. aeruginosa* PAO1 PA0984 vs. *P. aeruginosa* PAO1 in biofilms formed on glass wool in LB at 30°C after 7 h.

PA #	Gene name	Fold change	Descriptions	Signal value in PA0984	Detection in PA0984	Signal value in wild-type	Detection in wild-type
Motility and attachment							
PA2570	*lecA*	**2.8**	Adaptation, protection, cell wall, LPS, capsule	2121.1	P	865.6	P
Biosynthesis of cofactors, prosthetic groups and carriers							
PA1546	*hemN*	**3.0**	Oxygen-independent coproporphyrinogen III oxidase	1578.6	P	547.3	P
Cell wall, LPS, capsule							
PA3154	*wzy*	**2.5**	B-band O-antigen polymerase	174.2	P	97.5	P
PA3337	*rfaD*	**2.5**	ADP-L-glycero-D-mannoheptose 6-epimerase	927.3	P	415.7	P
Energy metabolism							
PA1556		**2.6**	Probable cytochrome c oxidase subunit	3000.5	P	1088.6	P
PA1557		**4.0**	Probable cytochrome oxidase subunit (cbb3-type)	2625	P	562.3	P
PA4571		**2.8**	Probable cytochrome c	730.8	P	278.6	P
Hypothetical, unclassified, unknown function							
PA0729		**2.5**	Hypothetical protein	455.2	P	233.7	P
PA0983		**2.6**	Hypothetical protein	603.2	P	343.2	P
PA1508		**2.3**	Hypothetical protein	464.4	P	176.4	P
PA1509		**2.3**	Hypothetical protein	925.5	P	519.1	P
PA1510		**2.5**	Hypothetical protein	329.6	P	160.2	P
PA1673		**3.3**	Hypothetical protein	1430.1	P	446.2	P
PA1942		**2.6**	Hypothetical protein	354.5	P	90.5	P
PA2805		**2.5**	Hypothetical protein	1937.5	P	682	P
PA3291		**2.5**	Hypothetical protein	624.1	P	271.1	P
PA3520		**2.8**	Hypothetical protein	2818	P	998.3	P

Table E.2. (continued)

PA #	Gene name	Fold change	Descriptions	Signal value in PA0984	Detection in PA0984	Signal value in wild-type	Detection in wild-type
PA3729		**2.5**	Hypothetical protein	1686.4	P	718.7	P
PA3732		**2.5**	Hypothetical protein	1186.9	P	627.7	P
PA3880		**2.3**	Hypothetical protein	423.7	P	154.9	P
PA4139		**2.5**	Hypothetical protein	5114.5	P	2113.5	P
PA5027		**2.5**	Hypothetical protein	1277.1	P	497.0	P
PA5088		**2.3**	Hypothetical protein	258.8	P	133.8	P
PA5303		**2.3**	Hypothetical protein	1971.9	P	879.9	P
PA5475		**2.3**	Hypothetical protein	2115.8	P	899.9	P
Putative Enzymes							
PA2119		**2.5**	Threonine dehydrogenase and related Zn-dependent dehydrogenases	869.3	P	367.9	P
Translation, post-translational modification, degradation							
PA4242	*rpmJ*	**2.6**	50S ribosomal protein L36	6837.6	P	2486.0	P
PA4250	*rpsN*	**2.5**	30S ribosomal protein S14	3189.9	P	1493.8	P

(Vasseur *et al.*, 2005). The *pel* operon consists of seven genes *pelA-F* and is named *pel* since mutants in this operon cannot form pellicles (Friedman and Kolter, 2004b). *pel* genes are necessary to form glucose-rich biofilm matrices (Friedman and Kolter, 2004b). Also, the *pslO* gene was repressed by 3-fold. The *psl* operon is important for biofilm initiation in *P. aeruginosa* (Ma *et al.*, 2006). Moreover, two alginate genes, *algD* and *algK* were repressed in DNA microarrays upon the mutation in PA0984 gene. Alginate production affects biofilm architecture of *P. aeruginosa* (Hentzer *et al.*, 2001; Stapper *et al.*, 2004) and protects *P. aeruginosa* biofilms from the human immune system (Leid *et al.*, 2005). Therefore, the mutation in the PA0984 gene represses the expression of *pel* and *alg* genes, which causes PA0984 mutants to be deficient in biofilm formation.

Three genes (PA1702, *xqhA*, and *xphA*) related to type III and type II secretion systems were repressed upon the inactivation of PA0984 gene. The type III secretion system is an important virulence of *P. aeruginosa* in acute human infections (Shen *et al.*, 2008). *xphA* and *xqhA* genes are adjacent in the chromosome (Winsor *et al.*, 2005). XphA and XqhA help to constitute a hybrid secretion system in *P. aeruginosa* by working with XcpR-Z proteins (Michel *et al.*, 2007). Three anthranilate dioxygenase genes (*antABC*) were repressed 2- to 3-fold in the microarrays. Anthranilate is a precursor for signal 2-heptyl-3-hydroxy-4-quinolone (PQS) (Urata *et al.*, 2004) that controls the *rhl* quorum-sensing pathway, virulence, and biofilm formation (Diggle *et al.*, 2006). As anthranilate is the precursor of PQS, PQS production in the PA0984 mutant should be examined. Although, it is speculative, the PA0984 protein may regulate virulence through *ant* and secretion proteins.

Of the induced genes, *lecA* gene expression was induced by 2.8-fold. LecA is regulated by *rhl* QS system (Winzer *et al.*, 2000). LecA also has a motility and attachment function (Winsor *et al.*, 2005), which may be the cause of an increase in PA0984 mutant swimming

motility. As many hypothetical genes were induced and repressed (21 genes induced more than 2.5-fold and 50 genes were repressed less than 2.5-fold) upon inactivation of the PA0984 gene, these genes also must be examined to discover how PA0984 protein regulates virulence in *P. aeruginosa.* To learn more about whether PA0984 protein affects the glucose metabolism, phenotype microarrays should be performed with PA0984 mutant versus wild-type strain.

E.3 Experimental procedures

E.3.1 Bacterial RNA isolation and DNA microarrays

The strains used in this study are listed in Table 3.3. Bacterial RNA isolation and DNA microarray experiments were conducted as described previously (Attila *et al.*, 2008b). Briefly, the wild-type and the PA0984 mutant were grown in LB (wild-type strain) and LB with 50 μg/mL tetracycline (PA0984 mutant). One mL of the overnight cultures (optical density 7.5 at 600 nm) was inoculated into 250 mL of fresh LB medium with 10 g of glass wool and the cultures were incubated for 7 h at 30°C with shaking. The cell pellets from glass wool and the total RNA were prepared as described previously (Attila *et al.*, 2008b).

The Genechip *P. aeruginosa* Genome Array (Affymetrix, P/N 900339) contains 5,500 of the 5,570 ORFs of *P. aeruginosa* (Whiteley *et al.*, 2001). cDNA synthesis, fragmentation, and hybridizations were as described previously (González-Barrios *et al.*, 2006b). Hybridization was performed for 16 h at 50°C, and the total cell intensity was scaled to an average value of 500. The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA control were used to monitor the labeling process. For each binary microarray comparison of differential genes expression, if the gene with the larger transcription

rate did not have a consistent transcription rate based on the 13 probe pairs (*p*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *p*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher (2.5-fold) than the standard deviation for the whole microarrays (Ren *et al.*, 2004a). The standard deviation for all the probe sets was 2.5-fold. Gene functions were obtained from the *Pseudomonas g*enome database (Winsor *et al.*, 2005).

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