REGULATION OF THE TYPE III SECRETION SYSTEM IN *Pseudomonas aeruginosa*

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2006

This dissertation is dedicated to my parents, Liuting Wu, Hong Wang and my wife, Chang Xu.

ACKNOWLEDGMENTS

This work was carried out at the Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, during the years 2001-2006. It is my great pleasure to thank the following persons who have taken part in this work and thus made it possible.

I owe my deepest thanks to my mentor, Dr. Shouguang Jin. His encouragement, support, and enthusiastic attitude towards research and life in general have been inspiring and have guided me during these years and have been more than I could have ever asked for. I greatly appreciate the opportunity to be part of his research team.

I would like to sincerely thank my committee, Dr. Shouguang Jin, Dr. Ann Progulske-Fox, Dr. Paul A. Gulig and Dr. Reuben Ramphal, whose insightful advice in the last 4 years has made a great difference in my research progress and in my view of being a serious scientist.

Far too many people to mention individually have assisted in so many ways during my work. They all have my sincere gratitude. In particular, I would like to thank the past and present members of the Jin laboratory, Dr. Unhwan Ha, Dr. Mounia Alaoue-El-Azher, Dr. Li Liu, Dr. Jae Wha Kim, Xiaoling Wang, Dr. Hongjiang Yang and Dan Li, for their help and advice. Especially, many thanks go to Dr. Lin Zeng and Dr. Jinghua Jia, who have given me tremendous help in my life and research since I came to America. I am also grateful to Wei Lian, M.D., for her help and suggestions these years.

I would like to thank Dr. William W. Metcalf of the University of Illinois at Urbana-Champaign for providing the transposon plasmid and related *E. coli* strains used in my work. I would like to thank Dr. Shiwani Aurora from Dr. Reuben Ramphal's lab and Dr. Hassan Badrane from Dr. Henry V. Baker's lab, who have contributed to my research and offered valuable technical support and discussions.

My final, and most heartfelt, acknowledgments must go to my family. I want to express my earnest gratitude to my parents, Liuting Wu and Hong Wang, for their unconditional love, encouragement and for always being there when I needed them most. My wife, Chang Xu, deserves my warmest thanks. She is the source of my strength. Her support, encouragement, and companionship have turned my journey through graduate school into a pleasure. For all that, she has my everlasting love.

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Requirements for the Degree of Doctor of Philosophy

REGULATION OF THE TYPE III SECRETION SYSTEM IN *Pseudomonas aeruginosa*

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May 2006

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Pseudomonas aeruginosa is an opportunistic bacterial pathogen which primarily infects patients with cystic fibrosis (CF), severe burns, or immunosuppression. *P. aeruginosa* possesses a type III secretion system (TTSS) which injects effector proteins into host cells, resulting in cell rounding, lifting, and death by necrosis or apoptosis. By screening a transposon insertional mutant library of a wild-type strain PAK, mutation in the *mucA* or *prtR* gene was found to cause repression of the TTSS.

Mutation in the *mucA* gene causes alginate overproduction, resulting in a mucoid phenotype. Comparison of global gene expression profiles of the *mucA* mutant and wild-type PAK under TTSS inducing condition confirmed the down regulation of TTSS genes and up regulation of genes involved in the alginate biosynthesis. Further analysis indicated that the repression of the TTSS in the *mucA* mutant was AlgU and AlgR dependent. Overexpression of the *algR* gene inhibited type III gene expression.

PrtR is an inhibitor of *prtN*, which encodes a transcriptional activator for pyocin synthesis genes. In *P. aeruginosa*, pyocin synthesis is activated when PrtR is degraded

during the SOS response. Treatment of a wild-type *P. aeruginosa* strain with mitomycin C, a DNA-damaging agent resulted in the inhibition of TTSS activation. A *prtR*/*prtN* double mutant had the same TTSS defect as the *prtR* mutant, and complementation by a *prtR* gene but not by a *prtN* gene restored the TTSS function. Also, overexpression of the *prtN* gene in wild-type PAK had no effect on the TTSS; thus PrtN is not involved in the repression of the TTSS. To identify the PrtR-regulated TTSS repressor, another round of Tn mutagenesis was performed in the background of a *prtR*/*prtN* double mutant. Insertion in a small gene, designated *ptrB*, restored the normal TTSS activity. Expression of *ptrB* is specifically repressed by PrtR, and mitomycin C-mediated suppression of the TTSS is abolished in a *ptrB* mutant strain. Therefore, PtrB is a newly discovered TTSS repressor that regulates the TTSS under the stress of DNA damage.

My study revealed new regulatory relationship between MucA, PrtR and the TTSS, and indicated that the TTSS might be repressed under environmental stresses.

CHAPTER 1
INTRODUCTION AND BACKGROUND

Pseudomonas aeruginosa

Basic Bacteriology

Pseudomonas aeruginosa is a versatile bacterium that is present in soil, marshes, tap water, and coastal marine habitats. It is a straight or slightly curved, gram negative bacillus (0.5-1.0 x 3-4 μm), belonging to the γ-subdivision of the *Proteobacteria*. The bacterium is defined as an obligate aerobe; however, anaerobic growth can occur when nitrate or arginine is used as an alternate electron acceptor.

The genome sequence of this microorganism was completed several years ago and is freely available to the public (www.pseudomonas.com) (124). The complete sequence of this genome was one of the largest bacterial genomes sequenced to date, with 6.3-Mbp in size encoding 5570 predicted genes (124). Most interesting is the fact that as high as 8% of the genome encodes transcriptional regulators, which is consistent with the observed bacterial adaptability to various growth environments.

Infections

P. aeruginosa causes a wide range of infections, from minor skin infections to serious and sometimes life-threatening complications. *P. aeruginosa* is also a causative agent of systemic infections in immunocompromised patients, such as those receiving chemotherapy, elderly patients, and burn victims (105, 109). Chronic bronchopulmonary infection of *P. aeruginosa* is the major cause of morbidity and mortality in cystic fibrosis (CF) patients (57).

CF Airway Infection by *P. aeruginosa*

Today, CF is one of the most common genetic disorders in Caucasian populations. Approximately 30,000 individuals are affected in the United States. CF patients bear a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) gene located on the human chromosome 7q31.2 (41, 103). CFTR functions as an apical membrane chloride channel. Due to the mutation in CFTR, little or no Cl^- is transported across the apical surface of secretory cells, which leads to an unopposed reabsorbtion of Na^+, Cl^-, and water. This results in thick mucus in a CF patient's airway. The thickened mucus provides a favorable environment for opportunistic pathogens including *P. aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia* (51). During progression of the infection, *P. aeruginosa* predominates and grows as a biofilm, which is highly resistant to antibiotics and cannot be eradicated. Most clinical isolates from CF patients overproduce an extracellular polysaccharide called alginate, resulting in a mucoid phenotype.

It is believed that the recurring infections that culminate with chronic *P. aeruginosa* colonization cause the respiratory damage in CF patients, the progressive deterioration of respiratory function, and eventually the mortality of the patient. The clinical treatment typically includes antibiotics, anti-inflammatory drugs, bronchodilators, and physical therapy (96, 99).

Antibiotic Resistance

P. aeruginosa exhibits a remarkable ability to develop resistance to multiple antibiotics. The resistance arises through an acquired and/or intrinsic mechanism.

Acquired Resistance

Acquired resistance is developed from a mutation or an acquisition of an antibiotic modification enzyme by horizontal transfer, such as β-lactamase (76, 88) and acetyl-transferases (resistance to aminoglycosides) (91, 117).

The target gene will avoid recognition of the antibiotic if mutation occurs. For example, mutations causing lipopolysaccharide changes reduce the uptake of aminoglycosides (14). Mutations in GyrA (a DNA gyrase) result in the resistance to fluoroquinolone (94). Other mutations will cause the decrease of membrane permeability (134) or up regulation of intrinsic resistant genes/systems (110).

Intrinsic Resistance

P. aeruginosa is intrinsically resistant to many antibiotics. The mechanisms include chromosomally encoded β-lactamase (76), low permeability of outer membrane and multidrug efflux systems (100). Besides these mechanisms, the biofilm mode of growth also leads to an increased antibiotic resistance (58). More of the biofilm will be discussed in the next section.

Multidrug Efflux Systems

The multidrug efflux system is a three-component channel through the inner and outer membrane which pumps out antimicrobial agents in an energy dependent manner. It contributes to the reduced susceptibility or resistance to many antibiotics such as β-lactams, aminoglycosides, tetracycline, quinolones, chloramphenicol, sulphonamides, macrolides and trimethoprim (110). Six multidrug efflux systems have been identified in *P. aeruginosa*, including MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexJK-OprM and MexGHI-OpmD. Each of them has a different substrate specificity. MexJK-OprM and MexGHI-OpmD were found to provide resistance against triclosan

(18) and vanadium (1), respectively. MexAB-OprM is constitutively expressed at a low level. In wild-type strains, the expression of MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM is repressed. Mutation in their respective regulator will lead to derepression and increased antibiotic resistance. The substrates and regulation of these efflux systems are summarized in Table 1-1.

Table 1-1 Regulation and substrates of multidrug efflux systems

Multidrug efflux system	*Regulator*	**Substrates**
MexAB-OprM	MexR (-)	β-lactams, quinolones, chloramphenicol, tetracycline, trimethoprim, sulphonamides (101)
MexCD-OprJ	NfxB (-)	Cefpirome, quinolones, chloramphenicol, erythromycin, tetracycline (118)
MexEF-OprN	MexT (+)	Imipenem, quinolones, tetracycline (68)
MexXY-OprM	MexZ (-)	Aminoglycosides, tetracycline, erythromycin (135)

-, negative regulator; +, positive regulator

Virulence Factors

P. aeruginosa harbors an arsenal of virulence factors, which enable it to establish localized, chronic colonization or systemic infection. The virulence factors include flagella, pili, extracellular toxins, quorum sensing systems, iron metabolism factors, alginate production, and a type III secretion system (5, 19, 21, 25, 58, 64, 65, 67, 87, 97, 104, 111, 121).

Flagellum

P. aeruginosa possesses a single polar flagellum which serves as a motive organelle on the bacterial surface. The flagellum consists of a basal body, hook, flagellar filament and motor. The basal body anchors the flagellum on the surface of the bacterium, the hook functions as a joint connecting the filament to the basal body, the filament functions as a propeller, and the rotation of the flagellum is generated by the

motor. By rotating the flagellum, the bacterium can move in the surrounding environment. Two types of movement depend on flagella, swimming and swarming. Swimming is a movement of bacteria in the surrounding liquid, and swarming is a surface translocation by groups of bacteria (17, 50). During the infection, flagella mediate the adhesion of *P. aeruginosa* to mucin in human airways (5, 104).

Pilus

Besides flagella, *P. aeruginosa* produces another motive organelle, the type IV pilus. A pilus is a polar filament structure, mediating attachment to host epithelial cells and a type of surface translocation called twitching motility (141). The pilus is composed of a small subunit (pilin). Pilin is synthesized in the cytoplasm as pre-pilin and translocated through the inner membrane, cell wall, and outer membrane to the surface of bacterium. During translocation, pre-pilin is cleaved to pilin and made ready to be assembled into a pilus. The pilus is able to extend and retract, resulting in surface translocation (twitching motility) (25, 87).

Extracellular Toxins

P. aeruginosa produces a variety of extracellular virulence determinants and secondary metabolites, which could cause extensive tissue damage, inflammation, and disruption of host defense mechanisms. The extracellular toxins include exotoxin A, alkaline protease, phospholipase C, elastase, hydrogen cyanide, pyocyanin, phenazine and rhamnolipid. Exotoxin A and alkaline protease are under the control of the iron metabolism system and are expressed at a much higher level under iron limited environments (19, 97). The regulation of phospholipase C is regulated by inorganic phosphate (Pi) (121). The remaining virulence factors are under the control of a quorum sensing system (65).

Quorum Sensing

P. aeruginosa possesses a signaling system for cell-cell communication, called quorum sensing. *P. aeruginosa* possesses three quorum sensing systems, known as *las*, *rhl* and PQS (pseudomonas quinolone signal). Each system contains a small molecule involved in signal communication. The *las* and *rhl* systems use acyl-homoserine lactones, C4-HSL and 3OC12-HSL, as signal molecules, respectively. The signal molecule of the PQS system is quinolone. The signal molecules are secreted into the surrounding environment, and when their concentrations reach a threshold (usually at the mid or late log phase), they can interact with their respective receptors and modulate gene expression in the population. The three quorum sensing systems can interact with each other. When the quorum sensing systems are activated, the expression of many virulence genes is up regulated, as reported previously (65, 133). Besides functioning as signal molecules, C4-HSL and 3OC12-HSL can directly modulate the host immune system. 3OC12-HSL is able to promote induction of apoptosis in macrophages and neutrophils (128). Quorum sensing is required for biofilm formation (119, 145). Therefore, quorum sensing can be a drug target for the treatment and eradication of *P. aeruginosa* infection (11, 51).

Iron Metabolism

Iron is essential for the metabolism and survival of *P. aeruginosa*. To acquire iron from the surrounding environment, *P. aeruginosa* produces and secretes iron-chelating compounds called siderophores. Two types of siderophores, pyoverdine and pyochelin, are produced by *P. aeruginosa*, with the former having much higher affinity than the latter in binding iron (III). The pyoverdine and pyochelin synthesis genes and receptors

are under the negative control of a regulator, Fur. Under iron depleted environments, the expression of these genes is derepressed (97, 132).

Alginate

Alginate is an exopolysaccharide synthesised by *P. aeruginosa*. Alginate production is known to be activated by environmental stress such as high osmolarity, nitrogen limitation, and membrane perturbation induced by ethanol (10). Over production of alginate renders mucoidy to the bacterium. Most *P. aeruginosa* clinical isolates from CF patients display a mucoid phenotype (111). The function and regulation of alginate are described in the introduction section of Chapter 3.

Biofilm

During chronic infection of CF airways, *P. aeruginosa* forms a biofilm on the respiratory epithelial surface. The biofilm consists of microcolonies surrounded by alginate (58), although alginate is not essential for the biofilm formation. The formation of biofilm requires flagella, pili, and quorum sensing systems (28, 51, 58). Bacteria growing in a biofilm are much more resistant to antibiotics than when growing in planktonic mode. It is believed that the slow, anaerobic growth inside the biofilm increases the antibiotic resistance. The surrounding negatively charged alginate may function as a barrier against antibiotics, especially positively charged aminoglycosides (58). Due to the biofilm mode of growth, antibiotic treatment usually fails to eradicate the bacteria (58).

Type III Secretion System

Type III secretion systems (TTSSs) are complex protein secretion and delivery machineries existing in many animal and plant pathogens. The TTSS directly translocates bacterial effector molecules into the host cell cytoplasm, causing disruption

of intracellular signaling or even cell death (35). Components of TTSSs from a variety of gram-negative bacteria display sequence and structural similarity. Most TTSS apparatus are composed of two sets of protein rings embedded in the bacterial inner and outer membranes and a needle-like structure (102). According to the current working model, the needle forms a pole in the host cell membrane, and effector proteins are delivered through the hollow needle (49, 95, 127).

The TTSS is an important virulence factor of *P. aeruginosa*: it inhibits host defense systems by inducing apoptosis in macrophages, polymorphonuclear phagocytes, and epithelial cells (21, 64, 67). The loss of the TTSS resulted in an avirulent phenotype in a burned mouse model (59).

Function of TTSS Structural Genes

The *P. aeruginosa* TTSS machinery is encoded by 31 genes arranged in four operons on the chromosome. Several genes have been well studied for their functions and interactions.

Needle structure genes

The *P. aeruginosa* TTSS needle is primarily composed of a 9-kDa protein named PscF. Partially purified needles measured about 7 nm in width and 60-80 nm in length (98). PscF has been shown to have two intracellular partners, PscE and PscG, which prevent it from polymerizing prematurely in the cytoplasm and keep it in a secretion-prone conformation (102).

Pore forming components

In order for TTSS-containing bacteria to directly deliver effector proteins into the eukaryotic cytoplasm, a mechanism is required for the TTSS to penetrate the double phospholipid cell membrane. The pore-forming activity possessed by the TTSS is

dependent on the *pcrGVHpopBD* operon (24). Upon contact with the host cell membrane, PopB and PopD polymerize and form a ring-like structure in the membrane, through which effector proteins are tanslocated. PopB and PopD have a common cytoplasmic chaperon, PcrH, which prevents their premature aggregation (114). Another gene product of this operon, PcrV, is required for the assembly and insertion of the PopB/PopD ring into the host cell membrane (44). However, no direct interaction has been detected between PcrV and PopB/PopD (44). PcrG was found to interact with PcrV (4).

Polarization of type III translocation

When cultured mammalian cells were infected with wild type *P. aeruginosa*, TTSS effector proteins could be detected only in the eukaryotic cytoplasm, but not in the tissue culture medium (131). This phenomenon is called polarized translocation, during which PopN, PcrG and PcrV are all required. Mutation in either *popN* or *pcrG* does not affect the TTSS-related cytotoxicity against HeLa cells; however, it results in high levels of ExoS in the tissue culture medium (126).

Effector proteins

Four different effector proteins have been found in *P. aeruginosa*, ExoS, ExoT, ExoY and ExoU. However, no natural *P. aeruginosa* isolates harbor both ExoS and ExoU simultaneously. ExoS and ExoT share significant sequence homology and structural similarity, with both bearing an ADP-ribosyltransferase activity and a GTPase-activating protein activity. ExoU and ExoY have lipase and adenylate cyclase activities, respectively (6, 39, 81, 112, 125). The ADP-ribosyltransferase activity of ExoS has been shown to cause programmed cell death in various types of tissue culture cells (64, 67).

Regulation of TTSS

Expression of the TTSS regulon can be stimulated by direct contact with the host cell or by growth under a low Ca^{2+} environment (61, 131). The expression of type III-related genes is coordinately regulated by a transcriptional activator, ExsA (60). ExsA is an AraC-type DNA binding protein that recognizes a consensus sequence, TXAAAXA, located upstream of the transcriptional start site of type III secretion genes, including the *exsA* gene itself (60). Three proteins, ExsD, ExsC and ExsE, directly regulate the activity of ExsA. ExsD represses ExsA activity by directly interacting with it (89). ExsC on the other hand has the ability to interact with both ExsD and ExsE (106, 130). Under TTSS non-inducing conditions, ExsC binds to ExsE; however, when the TTSS is induced, ExsE is secreted outside of the cell by TTSS machinery. This leads to the increased level of free ExsC, which in turn binds to ExsD and releases ExsA, allowing the transcriptional activation of the TTSS (27, 106, 130). The regulation cascade of the TTSS through ExsA is summarized in Fig. 1-1.

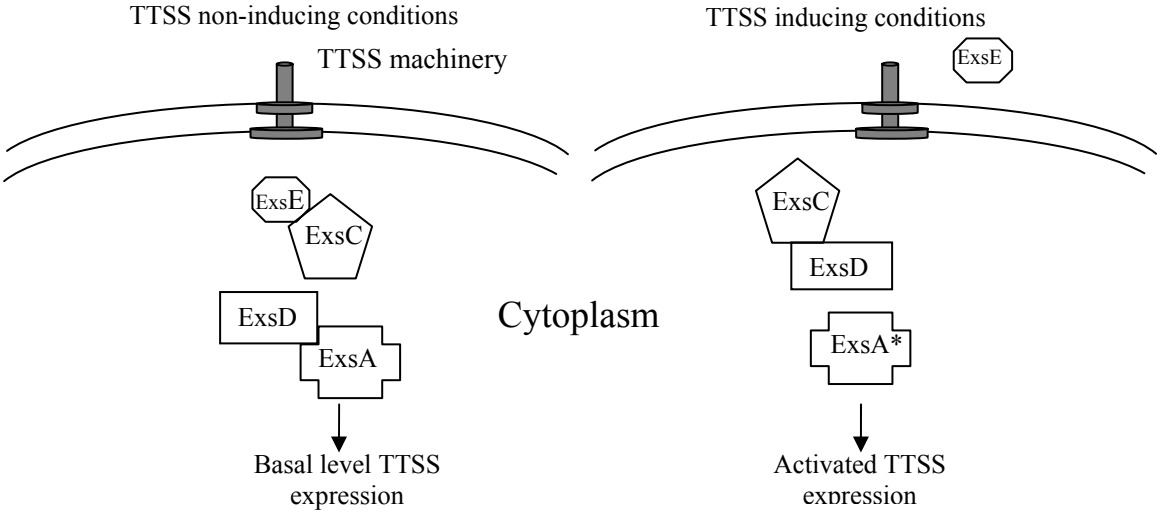

Figure 1-1. A model of the regulation of ExsA. See text for detail. *, derepressed ExsA.

Other TTSS Related Genes

In addition to genes described thus far, a number of other genes have been shown to affect the expression of type III genes, although the regulatory mechanisms are not known. Under TTSS-inducing conditions (low Ca^{2+}), the cyclic AMP level increases and a CRP homologue, Vfr, is required for TTSS activation (140). Vfr is a global regulator which mediates the activation of quorum sensing (3), twitching motility (8), type II secretion (140), and repression of flagellum synthesis (26). A novel gene, *fimL*, is also required for both TTSS and twitching motility (116, 137). Transcription of *vfr* is reduced in a *fimL* mutant, and over expression of Vfr restores both the TTSS and twitching motility, which suggests that the regulatory role of Vfr is downstream of FimL (137). Mutation in a hybrid sensor kinase/response regulator (RtsM or RetS) results in a defect in the TTSS and hyperbiofilm phenotype (42). Over expression of either Vfr or ExsA in a Δ*rtsM* mutant restores the TTSS activity (72). Furthermore, a three-component regulatory system (SadARS) is also required for both TTSS and biofilm formation in *P. aeruginosa* (69).

Some enzymes and metabolic pathways in *P. aeruginosa* are also found to be essential for the activation of TTSS. These include a periplasmic thiol:disulfide oxidoreductase (DsbA) (48), a tRNA pseudouridine synthase (TruA) (2), pyruvate dehydrogenases (AceAB) (23), and a normal histidine metabolism pathway (107). Additionally, the TTSS in *P. aeruginosa* is under the negative control of the *rhl* quorum-sensing system and the stationary-phase sigma factor RpoS (12, 56). Over expression of MexCD-OprJ or MexEF-OprN also cause the repression of the TTSS (75). Recently, our lab has demonstrated that a gene highly inducible during infection of the burn mouse model, designated *ptrA*, encodes a small protein which inhibits TTSS through direct

binding to ExsA and thus functions as an anti-ExsA factor. Expression of this gene is specifically inducible by high copper signal in vitro through a CopR/S two-component regulatory system (47). Fig. 1-2 summarizes the knowledge of the TTSS-related regulatory network in *P. aeruginosa*. The regulatory roles played by AlgR and PtrB in TTSS regulation were discovered during my doctoral research period and will be described in Chapter 2 and 3, respectively.

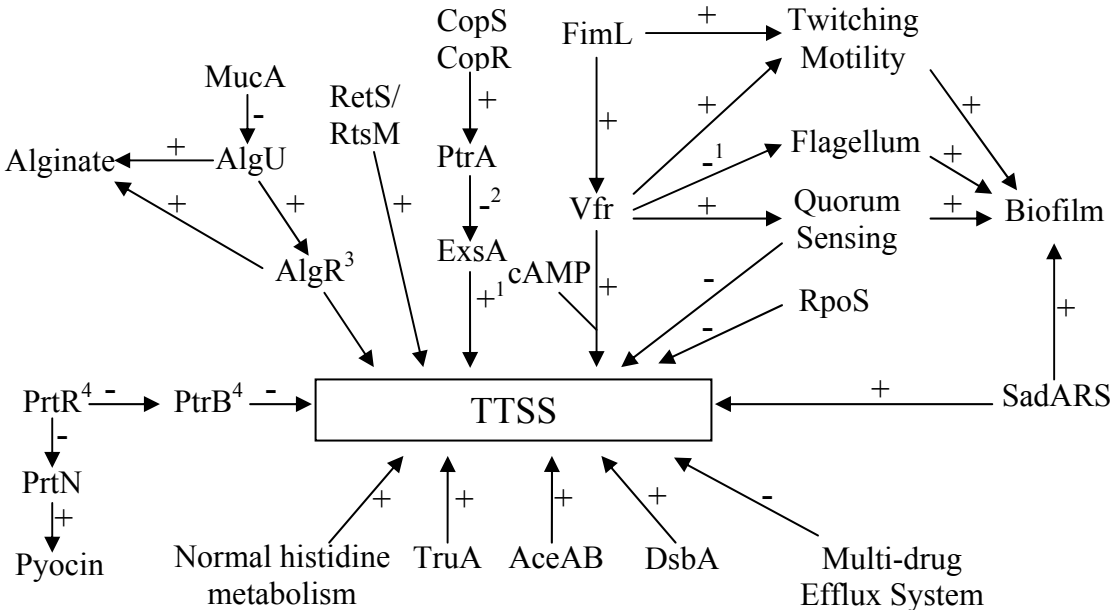

Figure 1-2. TTSS related regulatory network. See text for detail. +, positive regulation/relationship; -, negative regulation. [1], direct protein-DNA binding has been proved. [2], direct protein-protein interaction has been proved. [3], [4], this relationship was newly discovered from the work during my Ph.D. program.

CHAPTER 2
MucA-MEDIATED COORDINATION OF TYPE III SECRETION AND ALGINATE SYNTHESIS IN *Pseudomonas aeruginosa*

Introduction

Among CF patients, *P. aeruginosa* colonizes inside the thick mucus layer of the airway. In this anaerobic environment, *P. aeruginosa* overproduces the exopolysaccharide alginate and forms a biofilm which protects the bacterium from reactive oxygen intermediates and inhibits phagocytosis (51). More than 90% of *P. aeruginosa* strains isolated from CF patients show the mucoid phenotype, due to the overproduction of alginate (111). Clearly, alginate overproduction is a strategy to overcome environmental stresses. A number of stress signals trigger the overproduction of alginate, converting the bacterium to the mucoid phenotype (84).

The genes encoding enzymes for alginate synthesis form an operon (*algD* operon), and the expression of this operon is under the tight control of several regulators. The key regulatory gene of this operon is the *algU* gene (also called *algT*), included in an *algU* operon which consists of *algU-mucA-mucB-mucC-mucD*. The *algU* gene encodes a sigma factor, 22, which autoregulates its own promoter and activates many other genes, including those for alginate biosynthesis (85). The second gene in the *algU* operon, the *mucA* gene, encodes a transmembrane protein with a cytoplasmic portion binding to and inactivating AlgU (85). The third gene of the *algU* operon, the *mucB* gene, encodes a periplasmic protein, possibly sensing certain environmental signals. Upon sensing certain environmental signals, MucB transduces the signal to MucA, which in turn

releases the bound form of AlgU, resulting in activation of alginate production (85). The majority of *P. aeruginosa* isolates from the lungs of older CF patients carry mutations in the *mucA* or *mucB* gene and display a mucoid phenotype (82). In the AlgU regulon, two-component regulatory systems AlgB-FimS (78) and AlgR-AlgZ (146) and regulators AlgP (29) and AlgQ (73) are required for alginate synthesis. Among them, AlgR was also shown to be essential for *P. aeruginosa* pathogenesis (77). An *algR* mutant is less virulent than a wild-type strain in an acute septicemia infection mouse model (77). AlgR is also required for twitching motility (136, 138). Proteomic analysis of the *algR* mutant suggested that AlgR is a global regulator, affecting the expression of multiple genes (77).

In this chapter, a transposon (Tn) insertional mutant bank of a wild type *P. aeruginosa* strain, PAK, was screened for mutants that are defective in TTSS expression. I found that mutation in the *mucA* gene suppresses the expression of TTSS genes, greatly reducing the response of the TTSS to low Ca^{2+}. Furthermore, the suppression is dependent on the AlgU and AlgR functions. Comparison of global gene expression of the *mucA* mutant and wild type PAK under type III-inducing conditions confirmed the above observation. Several groups of genes have been found to be differently expressed in the *mucA* mutant and PAK, and their possible roles in TTSS expression are discussed.

Material and Methods

Bacterial Strains and Growth Conditions

Plasmids and bacterial strains used in this study are listed in Table 2-1. Bacteria were gown in Luria broth (LB) at 37°C. Antibiotics were used at the following concentrations: for *Escherichia coli*, ampicillin at 100 μg/ml, gentamicin at 10 μg/ml, tetracycline at 10 μg/ml, and kanamycin at 50 μg/ml; for *P. aeruginosa*, carbenicillin at 150 μg/ml, gentamicin at 150 μg/ml, tetracycline at 100 μg/ml, spectinomycin at 200

µg/ml, streptomycin at 200 µg/ml, and neomycin at 400 µg/ml. For ß-galactosidase assays, three single colonies of each strain were used. The overnight cultures were diluted 100-fold with fresh LB or 30-fold with LB containing 5 mM EGTA. Bacteria were grown to an optical density at 600 nm (OD_{600}) between 1.0 and 2.0 before ß-galactosidase assays (92). The data were subjected to *t*-test and $P < 0.05$ was considered as statistically significant.

Table 2-1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
BW20767/pRL27	RP4-2-Tc::Mu-1 Kan::Tn7 integrant *leu-63*::IS*10 recA1 zbf-5 creB510 hsdR17 endA1 thi uidA* (ΔMluI::*pir*)/pRL27	(71)
DH5α/λ*pir*	φ80*dlacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*/λ*pir*	(71)
P. aeruginosa strains		
PAK	Wild-type *P. aeruginosa* strain	David Bradley
PAK *exsA*::Ω	PAK with *exsA* disrupted by insertion of Ω cassette; Sp^rSm^r	(36)
PAK A44	PAK *mucA*1::Tn*5* mutant isolate; Neo^r	This study
PAK A61	PAK *mucA*2::Tn*5* mutant isolate; Neo^r	This study
PAK *mucA22*	Point mutation (ΔG440) in *mucA* gene of PAK	This study
mucA22 algU::Gm	*mucA22* with *algU* disrupted by insertion of Gm cassette; Gm^r	This study
mucA22 algR::Gm	*mucA22* with *algR* disrupted by insertion of Gm cassette; Gm^r	This study
PAK *algU*::Gm	PAK with *algU* disrupted by insertion of Gm cassette; Gm^r	This study
Plasmids		
pCR2.1-TOPO	Cloning vector for the PCR products	Invitrogen
pHW0005	*exoS* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ; Sp^r Sm^r Tc^r	(47)
pHW0006	*exoT* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ; Sp^r Sm^r Tc^r	(47)
pHW0024	*pscN* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ; Sp^r Sm^r Tc^r	(47)
pHW0032	*exsA* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ; Sp^r Sm^r Tc^r	(47)
pUCP19	Shuttle vector between *E. coli* and *P. aeruginosa*	(115)

Table 2-1. Continued

Strain or plasmid	Description	Source or reference
pWW020	*mucA* gene on pUCP19 driven by *algU* promoter; Ap^r	This study
pWW021	*mucA* gene on pUCP19 driven by *lac* promoter; Ap^r	This study
pWW025	*algU* gene on pUCP19 driven by *lac* promoter; Ap^r	This study
pMMB67EH	Low-copy-number broad-host-range cloning vector; Ap^r	(38)
pWW022	*algR* gene on pMMB67EH driven by *lac* promoter; Ap^r	This study
pEX18Tc	Gene replacement vector; Tc^r *oriT*$^+$ *sacB*$^+$	(55)
pEX18Ap	Gene replacement vector; Ap^r *oriT*$^+$ *sacB*$^+$	(55)
pPS856	Source of Gm^r cassette; Ap^r Gm^r	(55)
algU::Gm-pEX18Tc	*algU* disrupted by insertion of Gm^r cassette on pEX18Tc; Gm^r Tc^r *oriT*$^+$ *sacB*$^+$	This study
algR::Gm-pEX18Ap	*algR* disrupted by insertion of Gm^r cassette on pEX18Ap; Gm^r Ap^r *oriT*$^+$ *sacB*$^+$	This study
PAK *algR*::Gm	PAK with *algR* disrupted by insertion of Gm cassette; Gm^r	This study

Construction of Tn insertional Mutant Bank

The *P. aeruginosa* PAK strain containing the *exoT*::*lacZ* fusion plasmid (pHW0006) was grown overnight at 42°C, while *E. coli* donor strain BW20767/pRL27 was cultured to mid-log phase at 37°C. Cells of the two types of bacteria were washed with LB once to remove antibiotics in the culture medium. About 5×10^8 PAK/pHW0006 cells were mixed with 10^9 donor *E. coli* cells, and the mixture was filtered onto a sterile nitrocellulose membrane (pore size, 0.22 μm). The membrane was laid on top of nutrient agar and incubated at 37°C for 7 to 9 h before washing off the bacterial mixture from the membrane with LB. The bacterial suspension was serially diluted with LB and spread on L-agar plates containing spectinomycin at 100 μg/ml, streptomycin at 100 μg/ml, tetracycline at 50 μg/ml, neomycin at 400 μg/ml, and 20 μg

of 5-bromo-4-chloro-3-indolyl-ß-L-thiogalactopyranoside (X-Gal) per ml, with or without 2.5 mM EGTA for colony counting as well as mutant screening.

Determination of Tn Insertion Sites

To locate the Tn insertion sites of the isolated mutants, the Tn with flanking DNA was rescued as a plasmid from the mutant chromosome. Plasmid rescue was carried out as previously described (71). Briefly, genomic DNA of the Tn insertion mutants was isolated with the Wizard genomic DNA purification kit (Promega) and digested with PstI. The digested DNA was subjected to self-ligation with T4 DNA ligase and electroporated into DH5α/*pir*. Plasmids were isolated from the transformants and sequenced with primers tpnRL17-1 (5'-AAC AAG CCA GGG ATG TAA CG-3') and tpnRL13-2 (5'-CAG CAA CAC CTT CTT CAC GA-3') for the DNA flanking the two ends of the Tn. The DNA sequences were then compared with the *P. aeruginosa* genomic sequence by using BLASTN (124).

Generation of Knockout Mutants

Chromosome gene knockout mutants were generated as previously described (55). The target genes were amplified by PCR and cloned into pCR-TOPO2.1 (Invitrogen). After subcloning the PCR product into pEX18Tc or pEX18Ap, the target gene was disrupted by insertion of a gentamicin resistance cassette, leaving about 1 kb upstream and downstream of the insertion-mutation site. The plasmids were electroporated into wild-type PAK and single-crossover mutants were selected on LB plates containing gentamicin at 150 μg/ml, and tetracycline at 100 μg/ml or carbenicillin at 150 μg/ml. Double-crossover mutants were selected by plating single-crossover mutants on LB plates containing 5% sucrose and gentamicin at 150 μg/ml. In the case of the *mucA22* mutant, a 1.8-kb fragment of the *mucA* gene region was amplified from FRD1 (mucoid

strain) (78) genomic DNA, and the fragment was cloned into the HindIII site of pEX18Gm. The plasmid was transformed into *P. aeruginosa* to select for single crossover mutants on LB agar plates containing 150 μg/ml gentamicin. Single-crossover mutants were plated on L-agar plates containing 5% sucrose to select for double-crossover mutants. The double-crossover mutants were mucoid, and the introduction of the *mucA22* mutation was confirmed by sequencing of the *mucA* gene.

Plasmid Constructs for Complementation and Overexpression

Reporter fusions between the *exsA*, *exoT*, *exoS*, and *pscN* genes and promoterless *lacZ* on pDN19*lacZ* were generated by Ha *et al.* (47, 48). For *mucA* gene complementation, the *mucA* gene was amplified from PAK genomic DNA by PCR with primers MucA-1 (5'-CGG ATC CTC CGC GCT CGT GAA GCA ATC G-3') and MucA-2 (5'-TAC TGC GGC GCA CGG TCT CGA CCC ATA C-3'). The PCR product was cloned into pCR-TOPO2.1 and transformed into *E. coli* TOP10F'. The obtained plasmid was digested with *Hin*dIII-*Xmn*I and cloned into the *Hin*dIII-*Sma*I sites of pUCP19. The *mucA* gene in the resulting plasmid, pWW021, is driven by a *lac* promoter on the vector. To generate a *mucA* gene driven by the *algU* promoter, the *mucA* gene on the pCR-TOPO2.1 plasmid was subcloned into the *Bam*HI and *Xmn*I sites of pEX18Tc, resulting in *mucA*-pEX18Tc. To obtain the *algU* gene promoter, an 800-bp DNA fragment upstream of the *algU* gene open reading frame (ORF) was amplified by PCR with primers AlgT1 (5'-CCT TCG CGG GTC AGG TGG TAT TCG AAG C-3') and AlgT2 (5'-TTG GAT CCG CGC TGT ACC CGT TCA ACC A-3') and cloned into pCR-TOPO2.1. Then, this fragment was ligated into the *Eco*RI and *Bam*HI sites upstream of the *mucA* gene on the plasmid *mucA*-pEX18Tc. The obtained plasmid was digested with *Eco*RI-*Xmn*I, and the *algU* promoter and *mucA* gene ORF fragment were cloned into the

*Eco*RI-*Sma*I sites of pUCP19. On the resulting plasmid (pWW020), the *mucA* gene is driven by the *algU* promoter, and the transcriptional direction is opposite to that of the *lac* promoter on the vector. For *algR* complementation, the *algR* gene was amplified from PAK genomic DNA by PCR with primers algR1 (5'-GGT CTA GAG GCC GAG CCC CTC GGG AAA G-3') and algR2 (5'-GTG GAT CCT ACT GCT CTC GGC GGC GCT G-3'). The PCR product was initially cloned into pCR-TOPO2.1. The resulting plasmid was digested with *Cla*I, blunted ended with Klenow enzyme, and digested with *Xba*I. The *algR* gene-containing fragment was ligated into *Xba*I-*Sma*I sites of plasmid pMMB67EH, resulting in pWW022, on which the *algR* gene is driven by the *tac* promoter on the vector. For *algU* gene over expression, the *algU* gene ORF was amplified from PAK genomic DNA by PCR with primers algU1 (5'-GGG AAA GCT TTT GCA AGA AGC CCG AGT C-3') and algU2 (5'-GCT TCG TTA TCC ATC ACA GCG GAC AGA G-3'). The *algU* gene was cloned into *Hin*dIII-*Eco*RI sites of pUCP19, where the expression of the *algU* gene in the resulting plasmid pWW025 was driven by *lac* promoter on the vector.

Western Blotting

P. aeruginosa strains were cultured overnight in LB at 37°C. Bacterial cells were diluted 100-fold with fresh LB or 30-fold with LB containing 5 mM EGTA and cultured for 3.5 h. Supernatant and pellet were separated by centrifugation and mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Equal loading of the protein samples was based on the same number of bacterial cells. The proteins were transferred onto polyvinylidene difluoride membrane and probed with rabbit polyclonal antibody against ExoS (self-developed). The signal was detected by

enhanced chemiluminescence following the protocol provided by the manufacturer (Amersham Biosciences).

RNA Isolation and Microarray Analysis

For RNA isolation, three single colonies of PAK and the isogenic mutant PAK*mucA22* were each inoculated into 3 ml of LB and grown overnight. PAK and PAK*mucA22* were subcultured into LB containing 5 mM EGTA. PAK started with an OD_{600} of 0.03, and the *mucA22* mutant started with an OD_{600} of 0.06. After 3 to 4 h of culture, bacteria were harvested at an OD_{600} of 1.0 to 1.2. Total RNA was isolated using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. The purity and quantity were determined by spectrometry and electrophoresis. Fifteen micrograms of RNA of each sample was used for cDNA synthesis. cDNA fragmentation and biotin terminal labeling were carried out as instructed (Affymetrix). The experiments were performed in triplicate. Microarray analysis was performed with the Affymetrix GeneChip *P. aeruginosa* genome array. The experimental procedure followed the manufacturer's instructions. Data were acquired and analyzed with Microarray Suite version 5.0 (Affymetrix). Significance analysis of microarrays (129) was used to detect differentially expressed ORFs. Then, a cutoff of 5% false discovery rate (FDR) was chosen to analyze the data.

Results

Activation of the TTSS Requires a Functional *mucA* Gene.

To identify *P. aeruginosa* genes that affect the expression of TTSS, a Tn insertion mutant bank was constructed in PAK containing an *exoT*::*lacZ* (transcriptional fusion) reporter plasmid (pHW0006) (see Materials and Methods). On plates containing X-Gal and EGTA, the density of the blue color of each colony indicated the expression level of

the *exoT* gene in that particular Tn insertion mutant. To identify optimal screening conditions, combinations of different concentrations of X-Gal and EGTA were tested. In the presence of 20 μg of X-Gal/ml and 2.5 mM EGTA, wild-type PAK and the type III-defective PAK*exsA* mutant harboring pHW0006 showed the greatest visual difference in colony color (blue) and thus these concentrations were adopted for the screening conditions. The mutant cells were grown on the screening plates, and we looked for colonies with lighter blue color. About 40,000 Tn insertion mutants were screened. Among four colonies with lighter blue color, two of them showed a mucoid phenotype and the other two had Tn inserted in a *prtR* gene. The relationship between PrtR and TTSS will be discussed in Chapter 3. The two mucoid mutants were picked to test their TTSS activity by ß-galactosidase assay. As shown in Fig. 2-1A, the *exoT* gene promoter activity was three- to fourfold lower in the mutants than in the parent strain PAK/pHW0006. To confirm this observation, the *exoT*::*lacZ* reporter plasmid was cured from the Tn insertion mutants by passage in the absence of antibiotic selection and a *pscN*::*lacZ* reporter plasmid (pHW0024) was reintroduced. The resulting strain was subjected to a ß-galactosidase assay. The assay results shown in Fig. 2-1D indicated that the expression of the *pscN* gene was also repressed in these mucoid mutants under both TTSS-inducing and -noninducing conditions. Similar results were also obtained by introducing *exsA*::*lacZ* (pHW0032) and *exoS*::*lacZ* (pHW0005) reporter plasmids and testing ß-galactosidase activities (Fig. 2-1B and C), confirming that the two Tn mutants were indeed defective in TTSS expression.

The Tn and flanking DNA were rescued from the mutant strains and subjected to sequencing analysis (see Materials and Methods). Sequencing results showed that the Tn

was inserted into two different positions in the *mucA* gene in these two mutants, explaining the mucoid phenotype of the isolates.

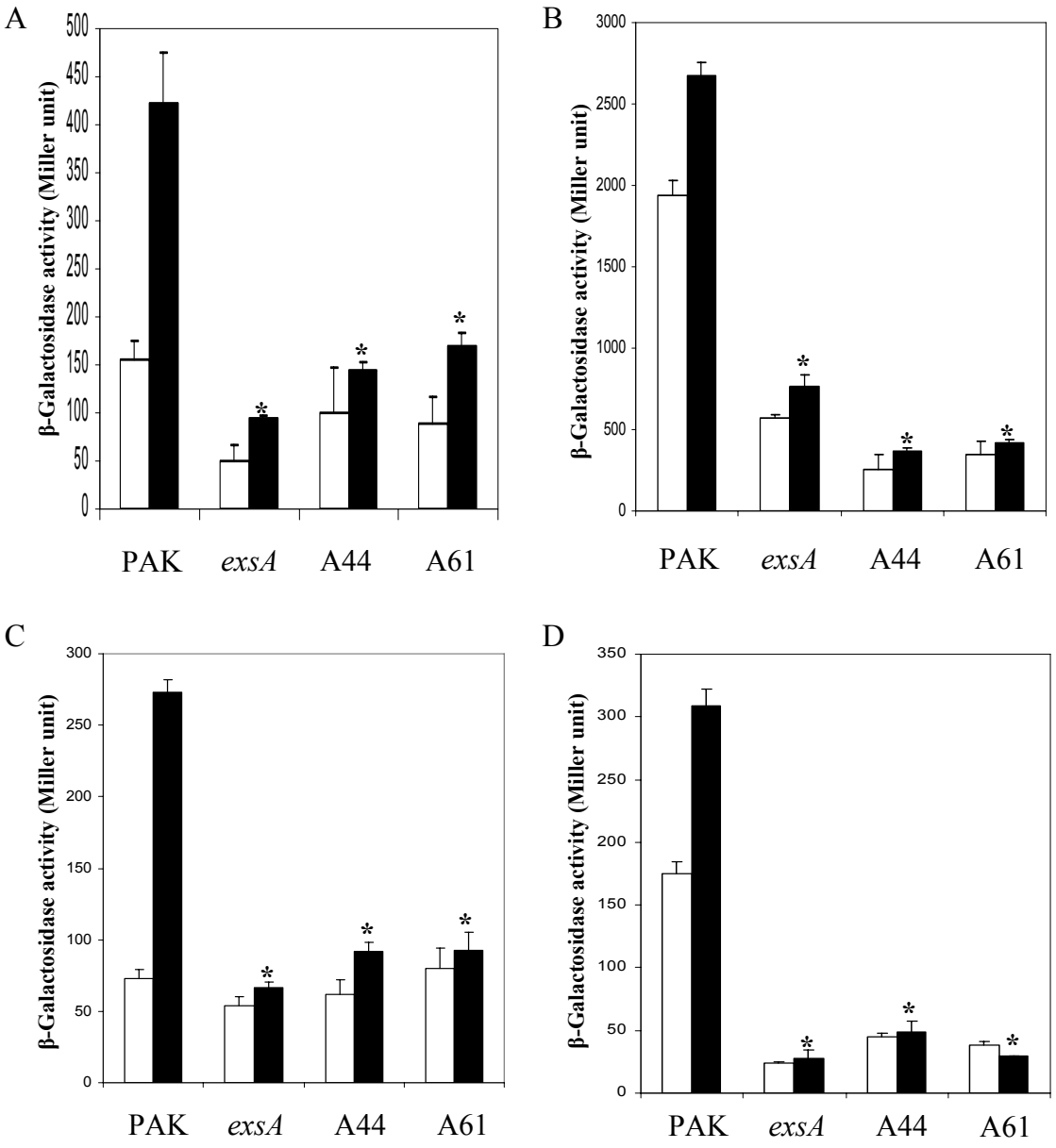

Figure 2-1. Expression of type III secretion genes in Tn insertional mutants of *mucA*. PAK, PAK*exsA*, and *mucA* mutants A44 and A61 harboring pHW0006 containing *exoT*::*lacZ* (A), pHW0032 containing *exsA*::*lacZ* (B), pHW0005 containing *exoS*::*lacZ*(C), or pHW0024 containing *pscN*::*lacZ* (D) were tested for ß-galactosidase activities. Bacteria were grown in LB (white bars) or LB containing 5 mM EGTA (black bars) to an OD600 of 1 to 2 before ß-galactosidase assays. Each assay was done in triplicate, and the error bars indicate standard deviations. *, $P < 0.001$, compared to the values in PAK.

Mutation in the *mucA* gene is commonly observed among *P. aeruginosa* isolates from CF patients, such as *mucA22*, where a nucleotide G was deleted within five G residues between positions 429 and 433 of the *mucA* coding region, causing protein truncation (13, 111). The identical *mucA22* mutant was constructed in the background of PAK by allelic replacement with a *mucA* fragment amplified from *P. aeruginosa* FRD1 (78), which bears the *mucA22* mutation (see Materials and Methods). Expression of the effector genes *exoS* and *exoT* in the resulting mutant strain PAK*mucA22* was compared to that in PAK by Western blot analysis of the secreted and cell-associated proteins. A polyclonal anti-ExoS antibody was used in the western blot experiment; however, it also cross-recognizes ExoT due to a high sequence homology between the ExoS and ExoT proteins. As shown in Fig. 2-2A, expression of ExoS and ExoT in the resulting PAK*mucA22* was greatly reduced in comparison to that in wild-type PAK when grown under type III-inducing conditions. Reporter plasmids pHW0032 (*exsA*::*lacZ*) and pHW0005 (*exoS*::*lacZ*) were further introduced into PAK*mucA22* and tested for ß-galactosidase activity. Similar to the original isolates of the *mucA* Tn insertional mutants, expression of the *exsA* and *exoS* genes in PAK*mucA22* was almost nonresponsive to low Ca^{2+}, compared to a three- to fourfold induction in the wild-type PAK background (Fig. 2-2B and C). Upon complementation of the PAK*mucA22* mutant with the *mucA* gene in pUCP19, either driven by the *algU* promoter (pWW020) or *lac* promoter (pWW021), expression of the *exsA* and *exoS* genes in the resulting strains was restored to the wild-type level (Fig. 2-2C). These results clearly demonstrate that expression of the TTSS genes requires a functional *mucA* gene.

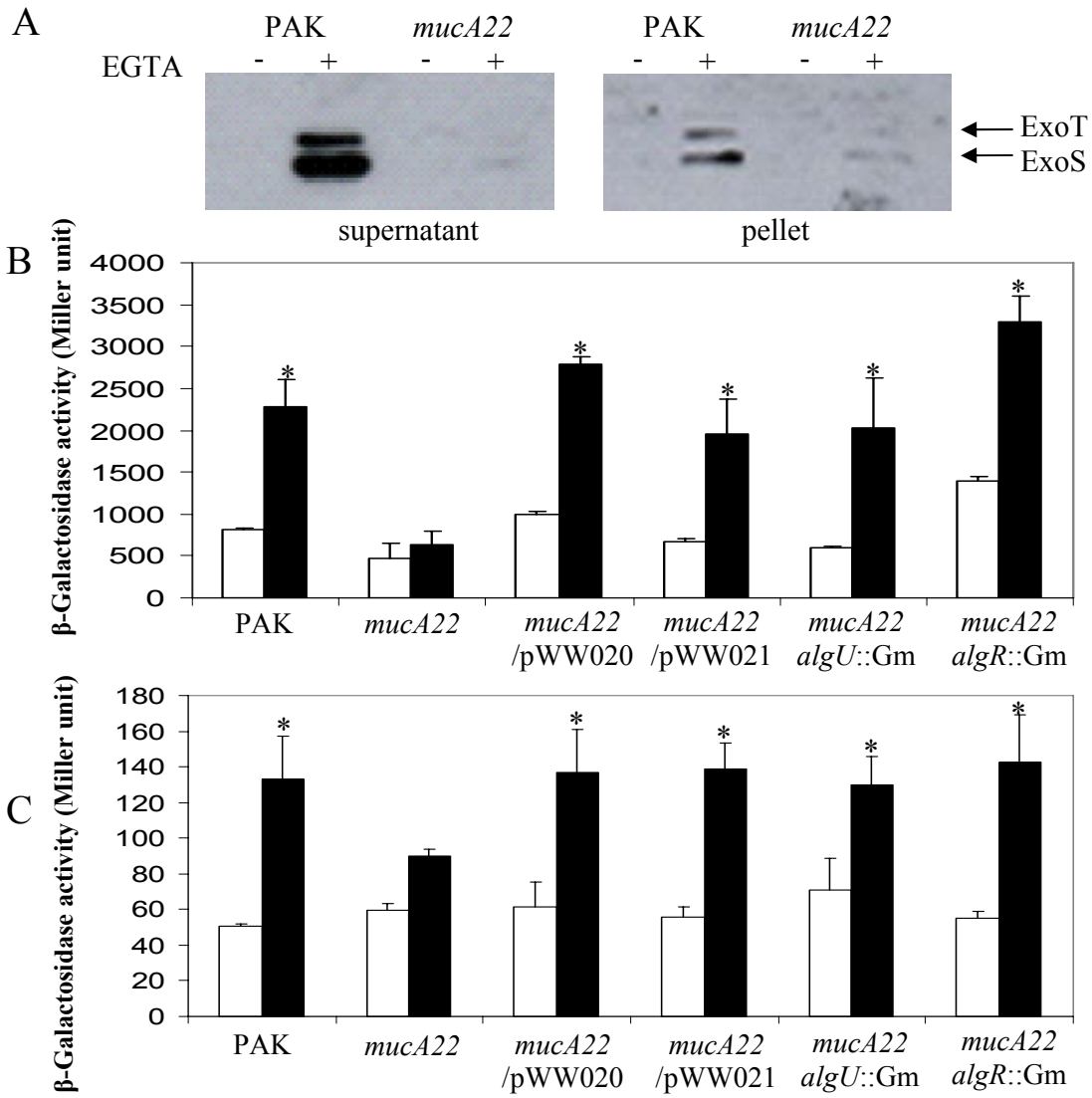

Figure 2-2. Expression and secretion of ExoS protein. (A) Comparison of cellular and secreted forms of ExoS in strains PAK and PAK*mucA22* grown in LB or LB plus 5 mM EGTA. Supernatants and pellets from equivalent bacterial cell numbers were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and immunoblotted with anti-ExoS antibody. Both ExoS and ExoT are indicated by the arrow. Anti-ExoS polyclonal antibody also recognizes ExoT due to high homology between these two proteins. (B) Expression of *exsA*::*lacZ* (pHW0032) in the backgrounds of PAK, PAK*mucA22*, with or without the *mucA* clone driven by an *algU* promoter (pWW020) or *lac* promoter (pWW021), PAK*mucA22algU*::Gm and PAK*mucA22algR*::Gm (C) Expression of *exoS*::*lacZ* (pHW0005) in the same backgrounds as described above. Bacteria were grown to an OD600 of 1 to 2 in LB with (black bars) or without (white bars) EGTA before ß-galactosidase assays. *, $P < 0.05$, compared to the values in *mucA22*.

Microarray Analysis of Gene Expression in the *mucA* Mutant

To further understand the mechanism of MucA-mediated regulation of TTSS genes, global gene expression profiles were compared between PAK*mucA22* and its wild-type parent strain PAK grown under TTSS-inducing conditions. Previously, a microarray analysis compared global gene expression patterns between mucoid (*mucA* mutant) and wild-type *P. aeruginosa* under non-TTSS-inducing conditions (32). Under these conditions, the TTSS activity in both strains was low; thus, no obvious effect of the *mucA* gene on the TTSS was observed.

Results of our gene array analysis were consistent with the published data (32, 33); genes under the control of AlgU are up regulated in the PAK*mucA22* mutant background compared to those in wild-type PAK, including genes for alginate biosynthesis (operon PA3540-3551) and regulation (Table 2-2). Also up regulated was operon, PA4468-4471, which includes the *sodM* gene (PA4468) encoding manganese superoxide dismutase, whose production is known to be higher in mucoid than that in nonmucoid *P. aeruginosa* (54), and the *fumC* gene (PA4470) encoding a tricarboxylic acid cycle enzyme fumarase C, which is essential for alginate production (53). Their results validated our gene array data.

Table 2-2. Expression of AlgU regulon genes in PAK*mucA22* (examed in microarray)

Group and ID no.	Name	Function	Fold change in *mucA22* vs wild type**
Alginate biosynthesis genes			
PA3540	*algD*	Alginate biosynthesis	64.2*
PA3541	*alg8*	Alginate biosynthesis	29.9*
PA3542	*alg44*	Alginate biosynthesis	28.9*
PA3543	*algK*	Alginate biosynthesis	81.2*
PA3544	*algE*	Alginate biosynthesis	47.9*
PA3545	*algG*	Alginate biosynthesis	38.0*

Table 2-2. Continued

Group and ID no.	Name	Function	Fold change in *mucA22* vs wild type**
PA3546	*algX*	Alginate biosynthesis	86.0*
PA3547	*algL*	Alginate biosynthesis	43.7*
PA3548	*algI*	Alginate biosynthesis	55.2*
PA3549	*algJ*	Alginate biosynthesis	27.2*
PA3550	*algF*	Alginate biosynthesis	70.5*
PA3551	*algA*	Phosphomannose isomerase	38.7*
Alginate biosynthesis regulatory genes			
PA0762	*algU*	Sigma factor	2.6*
PA0763	*mucA*	Anti-sigma factor	2.4*
PA0764	*mucB*	Negative regulator for alginate biosynthesis	1.3
PA5261	*algR*	Alginate biosynthesis; two-component system	1.5
PA5483	*algB*	Alginate biosynthesis; two-component system	2.0*
PA5484	*kinB*	Two-component sensor	2.1
Genes known to be up regulated in *mucA* mutants			
PA0059	*osmC*	Osmotically inducible protein	3.8*
PA0376	*rpoH*	Sigma factor	1.3
PA4876	*osmE*	Osmotically inducible lipoprotein	3.0*
PA5489	*dsbA*	Thiol:disulfide interchange protein	1.3

*, FDR<5%. **, Expression data is presented as fold change in *mucA22* relative to wild-type PAK.

Meanwhile, the expression levels of *exoS*, *exoT*, *exoY*, and other T3SS-related genes were clearly down regulated in the *mucA* mutant background compared to those in wild-type PAK under TTSS-inducing conditions (Table 2-3), which confirmed our ß-galactosidase assay and the Western blotting results. However, no significant changes in

the expression of the *exsA* gene and a few other TTSS genes were observed. A previous gene array study also showed that expression of the *exsA* gene and the exsD-pscL operon is relatively nonresponsive to Ca^{2+} depletion (140), yet a clear difference in the ß-galactosidase activities could be observed when PAK harboring *exsA*::*lacZ* (pHW0032) was grown in LB with or without EGTA. Similarly, we have seen differences in the ß-galactosidase activities between PAK(pHW0032) and PAK*mucA22*(pHW0032) under type III-inducing conditions without observing such differences in gene array data, suggesting possible involvement of posttranscriptional control of the *exsA* gene.

Table 2-3. Expression of TTSS-related genes in PAKmucA22 (examed in microarray)

ID no.	Gene	Function	Fold change in *mucA22* vs wild type**
PA0044	*exoT*	Exoenzyme T	–2.0*
PA2191	*exoY*	Adenylate cyclase	–1.3
PA3841	*exoS*	Exoenzyme S	–2.1*
PA1707	*pcrH*	Regulatory protein	–1.4
PA1708	*popB*	Translocator protein	–1.6
PA1709	*popD*	Translocator outer membrane protein	–1.5
PA1718	*pscE*	Type III export protein	–1.4
PA1719	*pscF*	Type III export protein	–1.5

*, FDR<5%. **, Expression data is presented as fold change in *mucA22* relative to wild-type PAK.

From the microarray analysis, genes that are differentially expressed more than threefold between PAK*mucA22* and PAK are listed in Tables 2-4 and 2-5. A number of genes known to be inducible under iron deprivation was also elevated in the *mucA22* mutant, including the sigma factor PvdS and genes regulated by PvdS for pyoverdine synthesis (53), the operon PA4468-4471 (53), and the probable two-component regulatory genes PA1300 and PA1301, encoding the extracytoplasmic function sigma-70 factor and a transmembrane sensor, respectively (97). Compared to the global gene expression profile of PAK grown under TTSS inducing or noninducing conditions, none

of the above genes seem to be affected by Ca^{2+} depletion (140). The mechanism by which these genes are activated is not clear.

Table 2-4. Genes up regulated in PAK*mucA22** (examed in microarray)

ID no.[a]	Gene	Function	Fold change in *mucA22* vs wild type**	TSB[b] (fold)	LB[b] (fold)
PA0059	*osmC*	Adaptation, protection	3.75	1.23	–1.29
PA2386	*pvdA*	Adaptation, protection	3.89	–1.50	1.07
#PA2397	*pvdE*	Adaptation, protection, membrane proteins, transport of small molecules	3.92	–3.00	–1.99
PA2401		Adaptation, protection	3.06	–1.00	–5.74
PA4468	*sodM*	Adaptation, protection	5.60	–1.30	1.02
PA2018		Antibiotic resistance and susceptibility, membrane proteins, transport of small molecules	3.88	–1.50	1.65
PA2019		Antibiotic resistance and susceptibility, transport of small molecules	4.16	–1.50	–1.36
PA1985	*pqqA*	Biosynthesis of cofactors, prosthetic groups, and carriers	2.99	–1.10	–1.24
PA1988	*pqqD*	Biosynthesis of cofactors, prosthetic groups, and carriers	3.18	–1.50	–1.28
PA1989	*pqqE*	Biosynthesis of cofactors, prosthetic groups, and carriers	3.00	1.44	–1.34
#PA2414		Carbon compound catabolism	5.17	–3.00	–1.29
PA3158	*wbpB*	Cell wall, LPS, and capsule; putative enzymes	5.76	–1.20	–1.08
PA0102		Central intermediary metabolism	3.38	–2.40	–1.15
PA2393		Central intermediary metabolism	3.27	–1.70	–4.76
PA2717	*cpo*	Central intermediary metabolism	4.75	1.21	–1.72
PA4470	*fumC1*	Energy metabolism	5.81	1.05	–1.62
PA5491		Energy metabolism	2.97	–1.30	1.08
#PA0320		Hypothetical	3.86	–7.80	–1.43
PA0586		Hypothetical	5.10	1.61	1.96
PA0587		Hypothetical	4.57	1.11	1.64

Table 2-4. Continued

ID no.[a]	Gene	Function	Fold change in *mucA22* vs wild type**	TSB[b] (fold)	LB[b] (fold)
PA0588		Hypothetical	4.57	1.12	1.64
PA0613		Hypothetical	3.60	1.14	–1.55
#PA0737		Hypothetical	10.80	1.70	–1.00
PA0807		Hypothetical	3.97	1.21	–1.52
PA0990		Hypothetical	3.45	2.31	–1.48
PA1245		Hypothetical; membrane proteins	5.12	–1.10	–1.20
PA1323		Hypothetical	4.21	1.94	2.25
#PA1471		Hypothetical	12.4	1.46	1.07
#PA1784		Hypothetical	6.71	1.33	–1.31
PA1852		Hypothetical	3.34	1.90	–1.03
PA2159		Hypothetical	3.56	1.44	–2.46
PA2161		Hypothetical	2.95	1.50	–4.92
#PA2167		Hypothetical	9.54	1.16	–1.06
PA2168		Hypothetical	3.20	–1.20	–2.80
#PA2172		Hypothetical	3.86	–1.90	–1.99
PA2176		Hypothetical	7.30	1.79	2.10
PA2403		Hypothetical; membrane proteins	4.96	–1.30	–1.52
PA2404		Hypothetical; membrane proteins	5.83	1.27	–1.69
#PA2405		Hypothetical	10.00	1.10	–5.91
PA2406		Hypothetical	5.79	–1.10	–1.22
PA2412		Hypothetical	4.56	–1.30	–2.72
PA2485		Hypothetical	5.16	–1.20	5.51
PA2486		Hypothetical	6.30	1.17	1.41
PA2562		Hypothetical	3.30	1.27	2.38
PA3274		Hypothetical	5.30	1.61	–2.16
PA4154		Hypothetical	3.63	1.49	1.08
#PA4469		Hypothetical	8.78	–1.30	–1.64
PA4471		Hypothetical	3.00	–1.00	–3.49
PA5182		Hypothetical; membrane proteins	4.81	1.29	2.11
PA5183		Hypothetical; membrane proteins	3.88	1.20	2.02
PA5212		Hypothetical	3.24	1.32	1.83
PA2409		Membrane proteins, transport of small molecules	4.21	–1.20	–1.46
PA4876	*osmE*	Membrane proteins, adaptation, protection	3.00	1.36	1.27
PA2407		Motility and attachment	4.73	–1.20	–1.61

Table 2-4. Continued

ID no.[a]	Gene	Function	Fold change in *mucA22* vs wild type**	TSB[b] (fold)	LB[b] (fold)
PA2385		Putative enzymes	3.19	1.05	–2.36
PA2394		Putative enzymes	3.56	1.00	–1.75
PA2402		Putative enzymes	3.18	–1.70	–2.00
PA2413		Putative enzymes	5.97	1.46	–2.32
PA4785		Putative enzymes	4.51	1.70	–1.98
PA0724		Related to phage, transposon, or plasmid	3.48	1.83	–2.39
PA1300		Transcriptional regulators	3.66	–1.70	–1.13
PA2426	*pvdS*	Transcriptional regulators	4.39	–1.40	–1.38
PA2408		Transport of small molecules	4.09	2.08	–3.14
PA3049	*rmf*	Translation, posttranslational modification, degradation	6.81	2.03	1.38
PA3188		Transport of small molecules	3.08	4.04	15.76
PA5470		Translation, posttranslational modification, degradation	3.31	–1.50	1.15
#PA2398	*fpvA*	Transport of small molecules	6.65	–2.10	–1.51

*, genes with FDR<5% and changes greater than threefold. **, Expression data is presented as fold change in *mucA22* relative to wild-type PAK. [a] #, up regulated in *mucA* mutant compared to PAK, but down regulated in PAK under type III-inducing conditions versus noninducing conditions, and vice versa. Not included are those known to be affected by the growth medium, such as those varied in TSB versus LB (140). [b] Change in gene expression in PAK grown under TTSS inducing conditions versus PAK grown under TTSS noninducing conditions (140). Bacteria were grown in TSB or LB.

Table 2-5. Genes down regulated in PAK*mucA22** (examed in microarray)

ID no.[a]	Gene	Function	Fold change in *mucA22* vs wild type**	TSB[b] (fold)	LB[b] (fold)
PA3450		Adaptation, protection	–3.5	1.84	1.17
PA2138		DNA replication, recombination, modification, and repair	–3.2	–2.50	–3.19
PA0523	*norC*	Energy metabolism	–3.0	–1.70	–1.7
#PA3445		Hypothetical	–3.9	2.66	1.59
#PA3446		Hypothetical	–5.1	1.36	1.57
PA3931		Hypothetical	–3.8	1.91	1.22
PA0281	*cysW*	Membrane proteins, transport of small molecules	–3.5	1.18	1.13
PA0282	*cysT*	Membrane proteins, transport of small molecules	–3.0	–1.10	–1.07
PA1601		Putative enzymes	–3.5	1.35	–1.44
PA3444		Putative enzymes	–5.1	1.11	–1.51

Table 2-5. Continued

ID no.[a]	Gene	Function	Fold change in *mucA22* vs wild type**	TSB[b] (fold)	LB[b] (fold)
PA1246	*aprD*	Secreted factors (toxins, enzymes, alginate); protein secretion-export apparatus	–3.4	2.05	–2.64
PA1312		Transcriptional regulators	–3.1	–1.10	–1.55
PA3927		Transcriptional regulators	–5.1	–1.70	–1.67
PA0198	*exbB1*	Transport of small molecules	–5.5	2.71	–3.68
PA0280	*cysA*	Transport of small molecules	–6.0	–1.00	–1.18
PA2204		Transport of small molecules	–4.7	1.16	–1.16

*, genes with FDR<5% and changes greater than threefold. **, Expression data is presented as fold change in *mucA22* relative to wild-type PAK. [a] #, up regulated in *mucA* mutant compared to PAK but down regulated in PAK under type III-inducing conditions versus noninducing conditions and vice versa. Not included are those known to be affected by the growth medium, such as those varied in TSB versus LB (140). [b] Change in gene expression in PAK grown under TTSS inducing conditions versus PAK grown under TTSS noninducing conditions (140). Bacteria were grown in TSB or LB.

TTSS Repression in the *mucA* Mutant is AlgU Dependent.

MucA is an anti-sigma factor which represses the activity of AlgU (σ^{22}). In the *mucA* mutant, AlgU is derepressed and activates the expression of genes for alginate synthesis, resulting in a mucoid phenotype. AlgU can also activate the expression of itself and downstream genes (*mucA-B-C-D*) in the same operon. To determine the role of AlgU in the repression of TTSS in the *mucA* mutant, the *algU* gene was knocked out in the background of PAK*mucA22*, resulting in a PAK*mucA22algU*::Gm double mutant. Under TTSS inducing conditions, expression of the *exsA* and *exoS* genes in this double mutant was similar to that in the wild-type (Fig. 2-2B and C), indicating that AlgU is required for the TTSS repression in the *mucA* mutant. An *algU*::Gm mutant was further generated in the background of PAK, and TTSS activity in the resulting mutant was compared with that in PAK. As shown in Fig. 2-3, expression of the *exsA* and exoS genes was the same in the PAK*algU*::Gm mutant and wild-type PAK under both TTSS

inducing and noninducing conditions, suggesting that the basal level of AlgU in wild-type *P. aeruginosa* does not play a significant role in the regulation of TTSS genes.

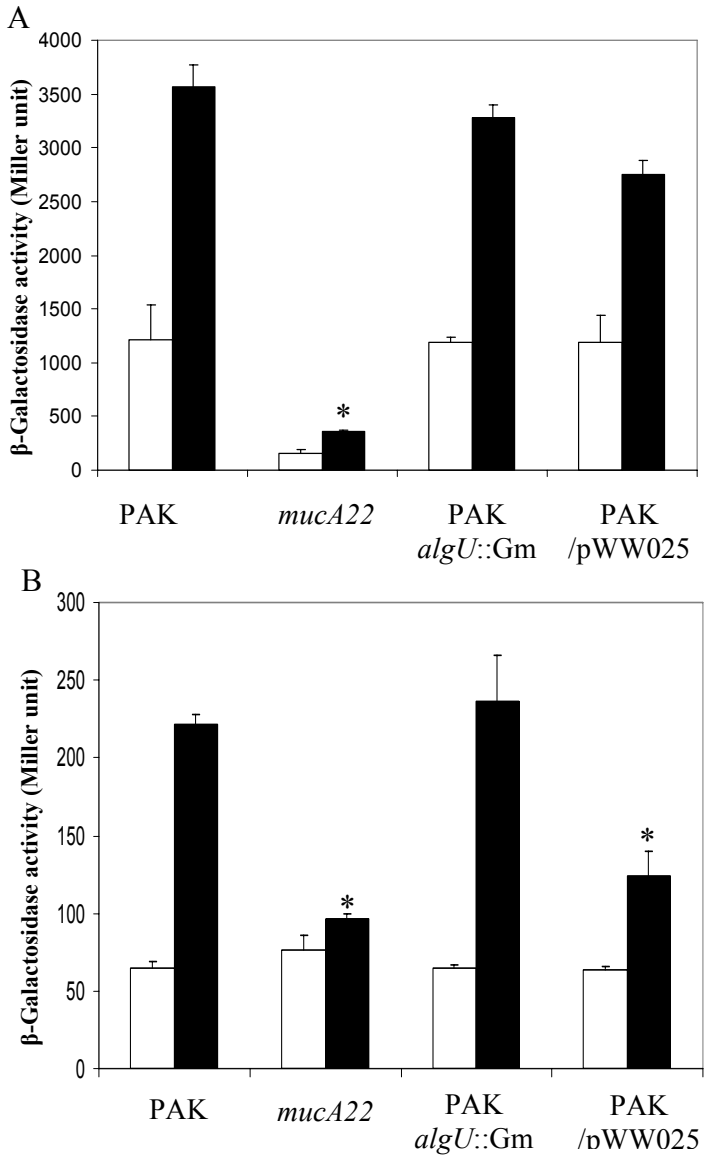

Figure 2-3. Expression of *exsA*::*lacZ* (A) and *exoS*::*lacZ* (B) in strains PAK, PAK*mucA22*, PAK*algU*::Gm, and PAK harboring *algU* overexpression plasmid pWW025. Bacteria were grown in LB (white bars) or LB plus 5 mM EGTA (black bars) to an OD_{600} of 1 to 2 before ß-galactosidase assays. *, $P<0.05$, compared to the values in PAK.

When the *algU* gene was overexpressed in wild-type PAK by introducing pWW025, the TTSS activity was partially repressed under type III-inducing conditions

(Fig. 2-3B). Since AlgU mediates the activation of the *algU-mucA* operon, an extra copy of *algU* also increased the expression of its repressor MucA; thus, overexpression of the *algU* gene could not repress TTSS expression to the level seen in the *mucA* mutant.

AlgR has a Negative Regulatory Function on the TTSS

algR is a regulatory gene required for alginate synthesis and is under the control of AlgU (78, 146). To investigate the role of AlgR in the regulation of TTSS, the *algR* gene was knocked out in the background of PAK*mucA22*. In the PAK*mucA22algR*::Gm double mutant, the expression of the *exsA* and *exoS* genes was restored to that of the wild type (Fig. 2-2A and B), suggesting that the repression of TTSS in the *mucA* mutant is also AlgR dependent. To test the function of AlgR on TTSS in wild-type *P. aeruginosa*, an *algR*::Gm mutant was generated in the PAK background. The expression of the *exoS* gene was consistently higher in the resulting PAK*algR*::Gm mutant than in PAK under both type III inducing and noninducing conditions (Fig. 2-4B). However, the expression of the *exsA* gene was similar in the PAK*algR*::Gm mutant and wild-type PAK. Complementation of the *algR* mutant with an *algR*-expressing clone (pWW022) decreased *exsA* and *exoS* expression under both type III inducing and noninducing conditions (Fig. 2-4). However, higher expression of *algR* induced by increasing the amount of isopropyl-ß-D-thiogalactopyranoside (IPTG) could not further decrease *exsA* and *exoS* expression (Fig. 2-4). These results indicate that AlgR has a negative regulatory effect on the TTSS, but the up regulation of AlgR alone might not be sufficient to repress TTSS activity to the level seen in the *mucA* mutant. It is likely that in the *mucA* mutant, *algR* gene expression is activated by AlgU, which in turn represses TTSS activity.

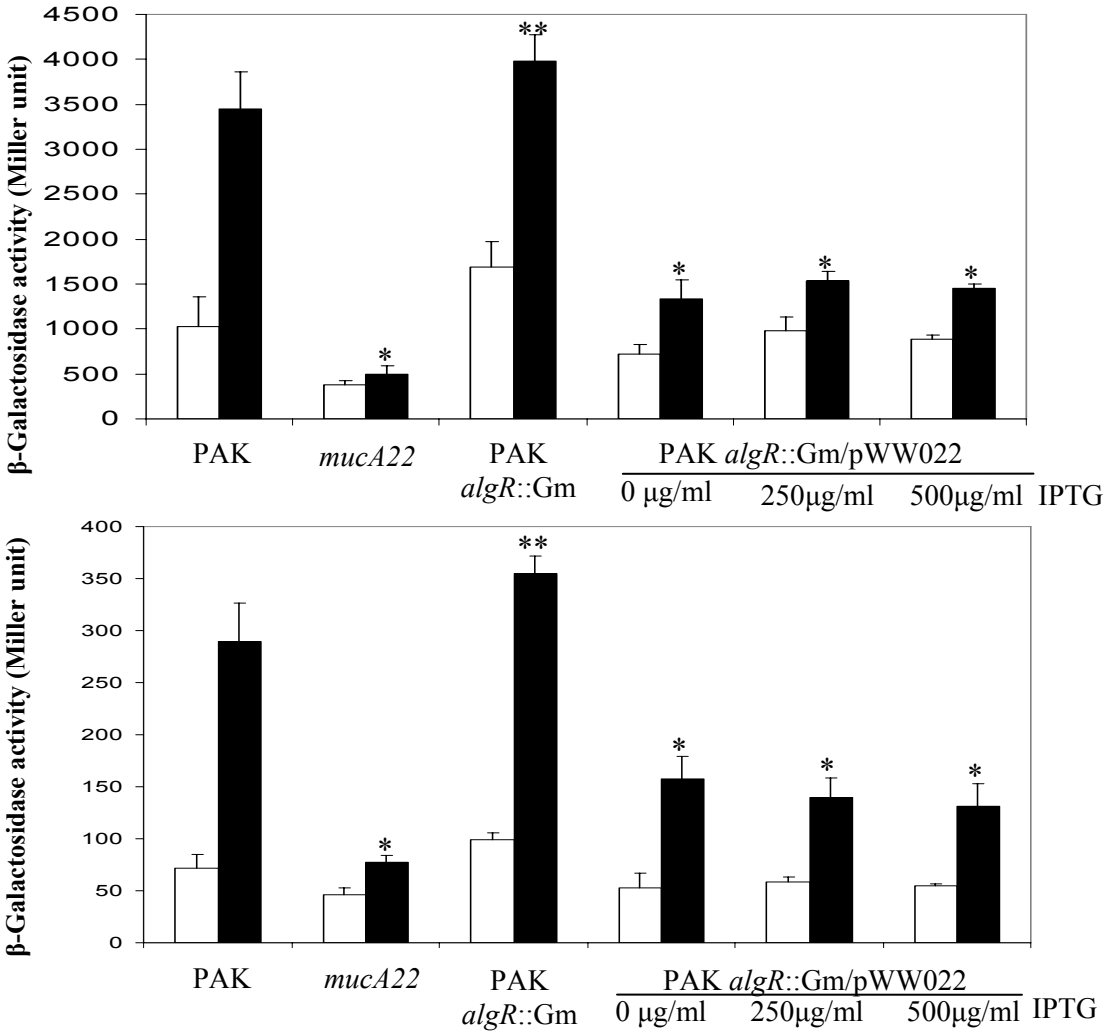

Figure 2-4. Expression of *exsA*::*lacZ* (A) and *exoS*::*lacZ* (B) in the backgrounds of PAK, PAK*mucA22*, PAK*algR*::Gm, and PAK*algR*::Gm complemented with *algR*-expressing plasmid pWW022. For *algR* gene complementation, various concentrations of IPTG were added into the culture medium as indicated. Bacteria were grown in LB (white bars) or LB plus 5 mM EGTA (black bars) to an OD_{600} of 1 to 2 before ß-galactosidase assays. *, $P < 0.05$, compared to the values in PAK; **, $P < 0.01$, compared to the values in *mucA22*.

Discussion and Future Directions

The Expression of *exsA* in the *mucA* Mutant

TTSS is an important virulence determinant for *P. aeruginosa*: it inhibits the host defense system by inducing apoptosis in macrophages, polymorphonuclear phagocytes, and epithelial cells. In our screen for mutants with lower TTSS activities, *mucA* mutants were found defective in *exoT* expression under type III-inducing conditions.

Furthermore, the basal promoter activity of the type III master regulatory gene *exsA* was decreased two- to threefold in the *mucA* mutant compared to that in wild-type PAK, suggesting that the down regulation of TTSS genes occurs through repression of ExsA. Since ExsA is an autoactivator (60), the repression could be on the transcriptional or posttranscriptional level. Our microarray results showed that the transcript level of *exsA* in the *mucA* mutant was similar to that in wild-type PAK under type III-inducing conditions, which suggested that the activity of ExsA might be repressed at the posttranscriptional level. However, the data from *exsA*::*lacZ* reporter plasmid indicates that the promoter activity of *exsA* gene is much lower in the *mucA* mutant (Fig.2-1B). Real-time PCR may be necessary to precisely determine the mRNA levels of *exsA* gene. Further study is required to clarify the mechanism of *exsA* gene regulation.

The Regulatory Pathway of AlgU Regulon

MucA is a transmembrane protein, with its cytoplasmic domain binding to and repressing the sigma factor AlgU. Mutation in the *mucA* gene leads to derepression of AlgU, which in turn activates genes for alginate synthesis as well as others, such as *dsbA*, *oprF*, *osmE*, and *rpoH* (32, 80). In the *mucA* mutant, not only the sigma factor AlgU but also AlgQ, an anti-σ^{70} factor, are activated (31), thus posing the possibility that sigma factor competition by AlgU and AlgQ effectively decreases the availability of σ^{70}-containing RNA polymerase for the expression of TTSS related genes (62). However, the observation that AlgR, an AlgU-dependent transcriptional activator, is required for the TTSS suppression makes it unlikely that sigma factor competition leads to the type III gene suppression; instead, an AlgR-dependent repressor is likely involved. AlgR is a global regulator, affecting expression of multiple genes. Proteomics analysis of an *algR*::Gm mutant showed that more than 17 proteins were up regulated and 30 proteins

were down regulated (77). In the present study, AlgR was also found to mediate the repression of type III secretion genes. In the PAK*algR*::Gm mutant background, expression of the *exoS* gene was higher than in wild-type PAK and, when complemented by an algR gene clone, expression of *exsA* and *exoS* genes decreased to about 50% of that seen in wild-type PAK (Fig. 2-4). The inability to suppress TTSS genes to the level seen in the *mucA* mutant by pWW022 was possibly due to a lower level of expression of the *algR* gene from pWW022 than that in the PAK*mucA* background, in which *algR* is activated through the MucA-AlgU pathway. pMMB67HE is a low-copy-number plasmid (38), and the *tac* promoter is not as strong a promoter in *P. aeruginosa* as it is in *E. coli*. AlgR is a DNA binding protein which binds to the promoter regions of *algD* (93) and *hcnA* (hydrogen cyanide synthesis gene) (15). It is possible that AlgR represses *exsA* expression by directly binding to the promoter region of the *exsCEBA* operon. The protein-DNA binding can be tested by gel-shift assay and the *algD* promoter can be used as a positive control. Alternatively, other regulatory genes might be involved in the repression of TTSS. Further study is needed to understand this observation.

We propose a model for TTSS repression in the *mucA* mutant (Fig. 2-5). With the activation of AlgU, the regulatory genes *algP*, *algQ*, *algB*, and *algR* are activated, which up regulates the expression of the *algD* operon. AlgR is required for TTSS repression in the *mucA* mutant, but whether the repression function is directly on ExsA or not is unclear. The involvement of other regulatory genes (*algP*, *algQ*, and *algB*) in TTSS regulation awaits further study.

The TTSS Activity in *P. aeruginosa* CF Isolates

During chronic infection of CF patient airways, *P. aeruginosa* overproduces alginate and forms a biofilm (58). Alginate production is known to be activated by high

osmolarity, nitrogen limitation, and membrane perturbation induced by ethanol (10); thus, the high salt concentration in the CF patient airway might be a signal for the overproduction of alginate. The biofilm mode of growth can help the bacterium survive in hostile environments and also render resistance against macrophages and polymorphonuclear cells (58).

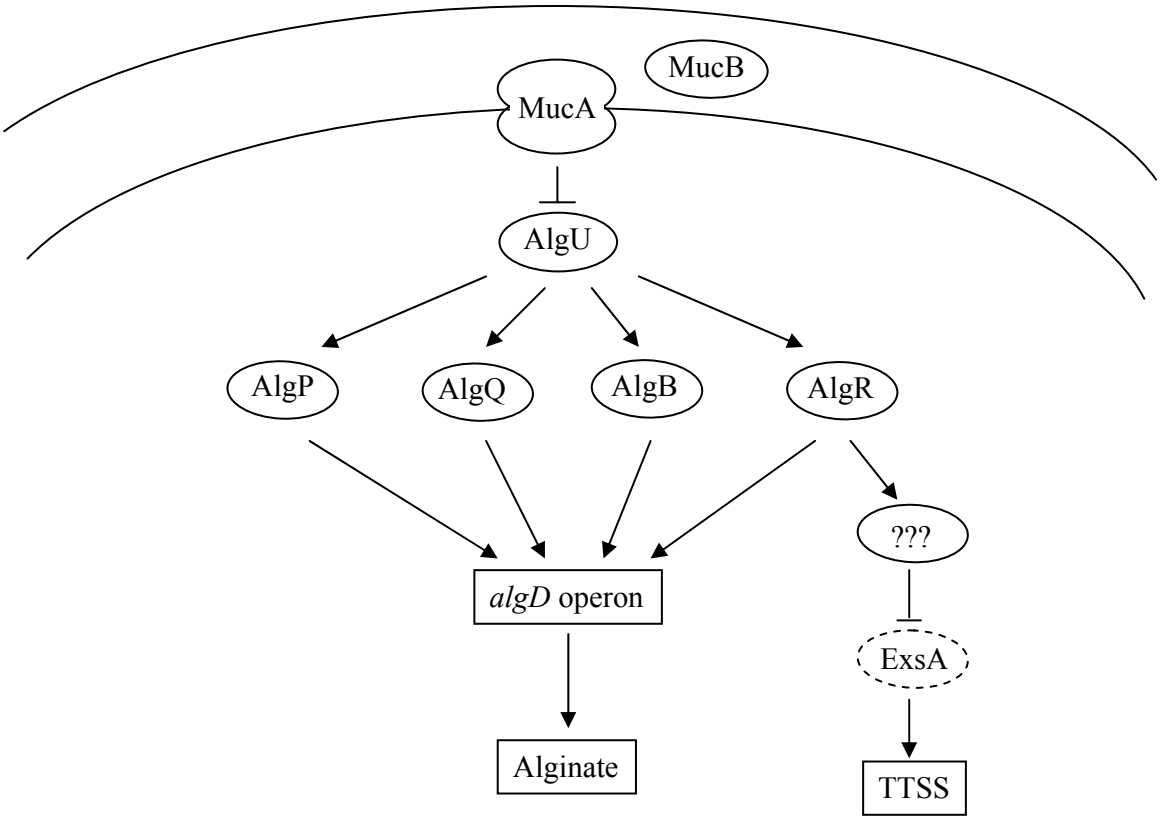

Figure 2-5. Proposed model of MucA-mediated coordination of alginate production and TTSS expression. MucA is a transmembrane protein, with its cytoplasmic portion binding and inhibiting the sigma factor AlgU. Upon sensing of certain environmental stress signals by the periplasmic MucB, it signals MucA through the periplasmic domain to release the bound AlgU. Free AlgU is required for the expression of downstream transcriptional activators AlgP, AlgQ, AlgB, and AlgR, all of which contribute to the optimal expression of the *algD* operon, encoding enzymes for the synthesis of alginate. AlgR, on the other hand, also activates downstream genes which are responsible for the suppression of the type III secretion genes.

Our experimental data suggest that bacteria have evolved a mechanism to turn off TTSS when they need to synthesize alginate to overcome environmental stress. Such

coordinated regulation of two energy-expensive processes is likely to render to the bacterium a survival advantage under environmental stress conditions. In addition, when the bacteria are surrounded by alginate, no intimate contact can be established between the bacteria and host cells. Under this circumstance, the TTSS needle can not reach the host cell membrane, which renders the TTSS unnecessary. This might be another reason to turn off TTSS while over producing alginate. Indeed, a majority of *P. aeruginosa* isolates from CF patients at a late stage in the disease displays the mucoid phenotype (34, 111) and are defective in type III gene expression (22). In a previous report, introduction of the wild-type *exsA* gene into type III secretion-defective clinical isolates restored type III secretion (22). However, our attempts to restore TTSS gene expression in 10 mucoid CF isolates by introducing a *mucA* gene clone failed, although all of the transformants were reverted back to the nonmucoid phenotype. It is possible that those mucoid clinical isolates may harbor additional mutations in the TTSS genes.

Genes Differently Expressed in the *mucA* Mutant and Isogenic Wild-type PAK

Known TTSS regulators include ExsA, Vfr, CyaA/B, ExsD, ExsC and ExsE (27, 60, 89, 106, 130, 140). Recently, DsbA and AceAB were also found to be necessary for the expression of TTSS. AceA and -B are subunits of pyruvate dehydrogenase, suggesting that metabolic imbalance influences the expression of TTSS (23, 107). DsbA is a periplasmic thiol-disulfide oxidoreductase and was shown to affect TTSS expression, twitching motility, and intracellular survival of *P. aeruginosa* upon infection of HeLa cells (48, 80). Interestingly, the *dsbA* gene is up regulated in the *mucA* mutant background, and its expression was shown to be regulated by AlgU (80). However, the role of DsbA on the TTSS is believed to be through its general effect on protein disulfide

bond formation in the periplasm, and up regulation of this gene may not be related to the MucA-AlgU-AglR-mediated suppression of the TTSS.

From the microarray analysis of the *mucA* mutant and wild-type strain under TTSS inducing conditions, alginate synthesis genes and genes known to be under the control of AlgU were up regulated, while TTSS genes were down regulated in the *mucA* mutant (Tables 2-2 and -3). In addition, pyoverdine synthesis genes as well as an operon, PA4468-4471, which might be under the control of Fur (54), were up regulated in the *mucA* mutant under TTSS-inducing conditions (Table 2-4). These findings are consistent with published results, in which mucoid *P. aeruginosa* strains produced higher levels of pyoverdine, pyochelin, manganese superoxide dismutase (PA4468), and fumarase (PA4470) than wild-type strains (52) (53). However, pyochelin synthesis genes were not seen up regulated in our microarray data. The mechanism by which these genes are up regulated in the *mucA* mutant background is not known.

The *mucA* gene mutation-mediated suppression of the TTSS genes requires AlgR, which is a transcriptional regulator; thus, it is likely that AlgR may repress TTSS genes or an AlgR-regulated repressor mediates the suppression of TTSS genes. To identify such candidate genes from the gene array data, I initially identified genes that were differentially expressed in the *mucA* mutant compared to wild-type PAK under type III inducing conditions. The selected genes include those that were up regulated in the *mucA* mutant compared to PAK under type III inducing conditions but were down regulated in PAK under type III inducing conditions versus noninducing conditions, and vice versa. I further eliminated those known to be affected by the growth medium, such as those with varied responses in tryptic soy broth (TSB) versus LB (140). Based on the above criteria,

13 genes were identified (Tables 2-4 and -5). For example, expression of the PA2172 gene in *mucA22* was up regulated about fourfold compared to that in wild-type PAK under TTSS inducing conditions. From published data, the expression of this gene was down regulated twofold in wild-type PAK grown under type III inducing conditions compared to that under noninducing conditions (140). Therefore, mutation in the *mucA* gene reversed the expression of PA2172 in response to the type III-inducing signal.

Among the 13 genes, *pvdE* and *fpvA* are involved in pyoverdine synthesis and absorption, respectively; PA2414 is involved in carbon compound catabolism. The remaining 10 genes are all hypothetical genes. The expression of PA0737, PA2167, PA2176, and PA4785 seems to be ExsA dependent, since in the *exsA* mutant the expression of these genes was lower than in wild-type PAK under type III inducing conditions and overexpression of *exsA* could activate expression of these genes under non-type III-inducing conditions (140). It is reasonable to hypothesize that one or more of such differentially expressed genes mediate the repression of the TTSS in the *mucA* mutant. It will be interesting to mutate each of these candidate genes in the background of PAK*mucA22* and test the TTSS activities.

Another approach to identify the TTSS repressor is to screen a random Tn library generated in the background of PAK*mucA22* for those mutants with restored wild-type TTSS activity. In those mutants, the TTSS repressor should be knocked out by the insertion of Tn. There are two potential pitfalls in this Tn mutagenesis strategy. One is that the *mucA* mutant over produces alginate which might obstruct the intimate contact between the *E. coli* donor strain and the *P. aeruginosa* recipient strain. To solve this problem, I can knock out the alginate synthesis gene, *algD*, which would render the *mucA*

mutant non-mucoid. The other problem is that, when cultured statically, *mucA* mutants tend to become non-mucoid, due to spontaneous mutations in the *algU* gene (143). During the conjugation for Tn mutagenesis, *algU* mutants may accumulate in the population. These *mucAalgU* double mutants display wild-type TTSS activity, which may lead to wrong interpretation of Tn mutated genes. It was reported that cultures containing the alternative electron acceptor nitrate may decrease the mutation rate of the *algU* gene. So during the conjugation, nitrate can be added into the nutrient agar.

In conclusion, in *mucA* mutants, the TTSS is repressed and the repression is AlgU and AlgR dependent. Most *P. aeruginosa* clinical isolates from CF patients display mucoid phenotype and are defective in the TTSS. This study provides possible explanation on the relationship between these two phenotypes and indicates that during chronic infection, *P. aeruginosa* might over produce alginate, which might function as a protection mechanism, and down regulate the TTSS, a virulence factor.

CHAPTER 3
PtrB OF *Pseudomonas aeruginosa* SUPPRESSES THE TYPE III SECRETION SYSTEM UNDER THE STRESS OF DNA DAMAGE

Introduction

As described in Chapter 2, two mutants with Tn inserted into the *prtR* gene were found to be defective in the TTSS activity. PrtR is a λCI homologue which binds to the promoter region of the *prtN* gene and inhibits its expression. PrtN is an activator of genes required for the production of a kind of bacteriocins, called pyocins. Three types of pyocins, R-, F- and S-type, have been identified. R- and F-type pyocins resemble phage tails. After they bind to their receptors, lipopolysaccharides (LPS), R-type pyocins cause a depolarization of the cytoplasmic membrane, which leads to cell death. S-type pyocins cause cell death by DNA breakdown due to their endonuclease activity (90). The uptake of most S-type pyocins occurs through ferripyoverdine receptors so that their killing activity is greatly increased when bacteria are grown under iron-limited conditions (7). The production of pyocins is induced by DNA-damaging agents, such as UV light and mitomycin C, when the bacterial SOS response is activated. Under these conditions, the RecA protein is activated and cleaves PrtR. As a result, PrtN is up regulated and actives the expression of pyocin synthesis genes (86, 90).

In this Chapter, I describe a coordinated repression of the TTSS under the stress of DNA damage. The expression of TTSS genes was found to be repressed in the background of a *prtR* mutant. Further analysis eliminated the possible involvement of the *prtN* gene in the TTSS repression. A gene designated *ptrB* has been identified which is

specifically repressed by PrtR and mediates the suppression of the TTSS genes. PtrB has a prokaryotic DskA/TraR C4-type zinc-finger motif but may not directly interact with the master regulator, ExsA.

Material and Methords

Bacterial Strains and Growth Conditions

Plasmids and bacterial strains used in this study are listed in Table 3-1. Growth conditions and antibiotic concentrations are the same as described in Chapter 2.

Table 3-1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
BW20767/pRL27	RP4-2-Tc::Mu-1 kan::Tn*7* integrant *leu-63*::IS10 *recA1 zbf-5 creB510 hsdR17 endA1 thi uidA* (Δ *Mlu*I)::*pir*$^+$/pRL27	(71)
DH5α/λ*pir*	φ80*dlacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*/λ*pir*	(71)
P. aeruginosa strains		
PAK	Wild-type *P. aeruginosa* strain	David Bradley
PAK A51	PAK *prtR*::Tn*5* mutant isolate; Neo^r	This study
PAKΔ*prtNprtR*::Gm	PAK with *prtN* and *prtR* disrupted by replacement of Gm cassette; Gm^r	This study
PAK*prtN*::Gm	PAK with *prtN* disrupted by insertion of Gm cassette; Gm^r	This study
F4	PAKΔ*prtNprtR*::GmPA0612::Tn5; Gm^r Neo^r	This study
PAKΔ *prtNprtR*::GmΔ PA0612-613	PAKΔ*prtNprtR*::Gm with deletion of PA0612 and PA0613; Gm^r	This study
PAKΔ *prtNprtR*::GmΔPA0612	PAKΔ*prtNprtR*::Gm with deletion of PA0612; Gm^r	This study
PAKΔ *prtNprtR*::GmΔPA0613	PAKΔ*prtNprtR*::Gm with deletion of PA0613; Gm^r	This study
PAKΔPA0612-613	PAK with deletion of PA0612 and PA0613	This study
PAKΔPA0612	PAK with deletion of PA0612	This study
PAKΔPA0613	PAK with deletion of PA0613	This study
Plasmids		
pCR2.1-TOPO	Cloning vector for the PCR products	Invitrogen
pHW0005	*exoS* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ; Sp^r Sm^r Tc^r	(46)

Table 3-2. Continued

Strain or plasmid	Description	Source or reference
pHW0006	*exoT* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ; Sp^r Sm^r Tc^r	(46)
pUCP19	Shuttle vector between *E. coli* and *P. aeruginosa*	(47)
pEX18Gm	Gene replacement vector; Gm^r, $oriT^+$ $sacB^+$	(55)
pEX18Ap	Gene replacement vector; Ap^r, $oriT^+$ $sacB^+$	(55)
pPS856	Source of Gm^r cassette; Ap^r Gm^r	(55)
pWW031	*prtN* gene of PAK on pUCP19 driven by *lac* promoter; Ap^r	This study
pWW037	*prtR* gene of PAK on pUCP19 driven by *lac* promoter; Ap^r	This study
pWW033	*prtN* disrupted by insertion of Gm cassette on pEX18Ap; Ap^r Gm^r	This study
pWW035	*prtN* and *prtR* disrupted by replacement of Gm cassette on pEX18Ap; Ap^r Gm^r	This study
pWW048-1	PA0612 promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ; Sp^r Sm^r Tc^r	This study
pWW048-2	*exsC* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ; Sp^r Sm^r Tc^r	This study
pWW069	Deletion of PA0612 and PA0613 on plasmid pEX18Ap; Ap^r	This study
pWW070	Deletion of PA0612 and PA0613 on plasmid pEX18Gm; Gm^r	This study
pWW075	Deletion of PA0612 on plasmid pEX18Gm; Gm^r	This study
pWW076	Deletion of PA0613 on plasmid pEX18Gm; Gm^r	This study
pWW071	PA0613 open reading frame cloned into pCR2.1-TOPO; Ap^r	This study
pWW072	PA0612 open reading frame cloned into pCR2.1-TOPO; Ap^r	This study
pBT	Bait vector plasmid encoding full length bacterial phage λcI protein; Chl^r	Stratagene
pTRG	Target vector plasmid encoding RNAP-alpha subunit protein; Tc^r	Stratagene
pBT-LGF2	Interaction control plasmid containing dimerization domain of Gal4 on bait vector; Chl^r	Stratagene
pTRG-Gal 11^p	Interaction control plasmid encoding mutant form of Gal11 on target vector; Tc^r	Stratagene
pWW077	PA0612 open reading frame cloned into pTRG; Tc^r	This study
pWW078	PA0613 open reading frame cloned into pTRG; Tc^r	This study
pWW079	PA0612 open reading frame cloned into pBT; Chl^r	This study
pWW080	PA0613 open reading frame cloned into pBT; Chl^r	This study
pHW0315	*exsA* open reading frame cloned into pTRG; Tc^r	(47)
pWW081	*exsA* open reading frame cloned into pBT; Chl^r	This study

For *prtR* gene complementation, a *prtR* containing fragment was amplified from PAK genomic DNA by PCR (Table 3-2). The PCR product was cloned into pCR2.1-TOPO (Invitrogen), resulting in pTopo-*prtR*. From pTopo-*prtR*, the *prtR* gene was isolated as a *Hin*cII-*Hin*dIII fragment and cloned into *Sma*I-*Hin*dIII sites of pUCP19, resulting in pWW037, where the *prtR* gene is driven by a *lac* promoter. For *prtN* gene overexpression, *prtN* coding sequence was amplified by PCR (Table 3-2), initially cloned into pCR2.1-TOPO, and subcloned into *Hin*dIII-*Xba*I sites of pUCP19, where the expression of the *prtN* gene in the resulting plasmid, pWW031, was driven by the *lac* promoter on the vector. The promoter region of PA0612 was amplified from PAK chromosomal DNA (Table 3-2), cloned into pCR2.1-TOPO, and subcloned into *Eco*RI-*Bam*HI sites of pDN19*lacZ*, resulting in pWW048-1. For the construction of *exsC*::*lacZ* reporter plasmid, a PCR product containing *exsCEBA* (Table 3-2) was cloned into pCR2.1-TOPO. The *exsC* promoter was cut out with *Eco*RI and *Hin*cII, and subcloned into pDN19*lacZ*.

Chromosomal gene mutations were generated as described (55). A fragment containing the *prtN* and *prtR* genes was amplified by PCR using the primers PrtR1 and PrtN2. The PCR product was cloned into pCR2.1-TOPO and subcloned into *Hin*dIII-*Xba*I sites of pEX18Ap, resulting in pEX18Ap-prtNR. For construction of a *prtN prtR* double mutant, a *Sph*I fragment containing 3'-terminal sequence of *prtR* and 5'-terminal sequence of *prtN* was replaced with a gentamicin resistance cassette, resulting in pWW035. For the construction of PA0612-613, PA0612, and PA0613 mutants, a 2.4-kb fragment was amplified from PAK chromosomal DNA with primers 612-3M1 and 612-3M2 (Table 3-2), followed by cloning into pCR2.1-TOPO. A SacII fragment containing

both PA0612 and PA0613 was deleted to generate the PA0612-613 mutant. A 76-bp SacII-PstI fragment within PA0612 was removed to generate the PA0612 mutant, while a 116-bp ClaI-SacII fragment was deleted to generate the PA0613 mutant. The resulting plasmids were transformed into wild-type PAK or PAK*prtNprtR*::Gm and selected for single and double crossover mutants as described previously (55). Construction of a transposon (Tn5) insertion mutant library, plasmid rescue, and sequence analysis were conducted as described in Chapter 2.

Table 3-2. PCR primers used in this study

Gene	Amplicon size (bp)	Sequences of primers
ptrR	1,355	PrtR1: 5'-CCAGTTCGTTGGCGTGATCGGCAAGGTC-3' PrtR2: 5'-CCCTCCTGCGGCTACACGTCGTTGAGGG-3'
prtN	1,376	PrtN1: 5'-CCATGCAGCCATCCATCGCCCCTAGCAC-3' PrtN2: 5'-CCGTCGCAGCGCATGTCCATCGAATTCA-3'
ptrB (promoter)	616	lac1H: 5'-AAGCTTTCGGGCGGGATCTGGGTGCTCT-3' lacB2: 5'-TGGGATCCCCGCAGTCCTCGCAGTCTTC-3'
PA0612-3	2,417	612-3M1: 5'-AAGCTTATCTGGCGGCTGCGCATGTCCT-3' 612-3M2: 5'-CAGCATCACCGCCACGCCGCAGACAATC-3'
PA0612	240	612BT1: 5'-GCGGCCGCCACGCCAGGGAGGCTTTCCA-3' 612BT2: 5'-CTCGAGGTCGGTTCAACGGCGCTCGTGG-3'
PA0613	417	613BT1: 5'-GCGGCCGCGAAAGGAGACACGACCGTGAT-3' 613BT2: 5'-CTCGAGGGGGGACACGGTATCCGGTCCAG-3'
exsCEBA	2662	exsA1: 5′-TGCAGCTCATCCAGCAGTACACCCAGAGCCATAAC-3′ exsA3: 5′-ACAAACTGCTCGATGCGTAACCCGGCACC-3′
PA0612-3 (RT-PCR)	649	612GS1: 5'-GGATCCCCATGGCTGACCTTGCCGATCAC-3' 613BT2: 5'-CTCGAGGGGGGACACGGTATCCGGTCCAG-3'
ptrB (Q-PCR)[a]	101	Forward: 5'-GATCACGCCAACGAACTGGTC-3' Reverse: 5'-CCGCAGTCCTCGCAGTCTTCC-3'
rpsL (Q-PCR)	120	Forward: 5'-CAAGCGCATGGTCGACAAGAG-3' Reverse: 5'-ACCTTACGCAGTGCCGAGTTC-3'

RT-PCR and Quantitative Real-time PCR

Overnight cultures of bacterial cells were diluted 100-fold into fresh medium and grown to an optical density at 600 nm (OD_{600}) of 1.0. Total RNA was isolated with an RNeasy Mini kit (QIAGEN). DNA was eliminated by column digestion as described by

the manufacturer (QIAGEN). cDNA was synthesized with an iScript cDNA synthesis kit (Bio-Rad). Taq DNA polymerase from Eppendorf was used in PCRs. The cDNAs synthesized by reverse transcription-PCR (RT-PCR) were used as templates in quantitative real-time PCR. The cDNA was mixed with 5pmol of forward and reverse primers (Table 3-2) and iQ SYBR Green Supermix (Bio-Rad). Quantitative real-time PCR was conducted using the ABI Prism 7000 sequence detection system (Applied Biosystems). The results were analyzed with ABI Prism 7000 SDS software. Transcript for the 30S ribosomal protein (*rpsL*) was used as an internal standard to compensate for differences in the amount of cDNA. The mRNA levels of *ptrB* in test strains were expressed relative to that of PAK, which was set at 1.00.

Cytotoxicity Assay

HeLa cells (5×10^4) were seeded into each well of a 24-well plate. The cells were cultured in Dulbecco's modified Eagle's medium with 5% fetal calf serum at 37°C with 5% CO2 for 24 h. Overnight bacterial cultures were washed with LB and subcultured to log phase before infection. Bacteria were washed once with phosphate-buffered saline and resuspended in tissue culture medium. HeLa cells were infected with the bacteria at a multiplicity of infection (MOI) of 20. A cell lifting assay was performed after 4 h of infection. Culture medium in each well was aspirated. Cells were washed twice with phosphate-buffered saline (PBS) and stained with 0.05% crystal violet for 5 min. The stain solution was discarded, and the plates were washed twice with water. A 250-µl volume of 95% ethanol was then added into each well and incubated at room temperature for 30 min with gentle shaking. The ethanol solution with dissolved crystal violet dye was used to measure absorbance at a wavelength of 590 nm.

Application of BacterioMatch Two-hybrid System

PA0612 and PA0613 open reading frames were amplified from PAK chromosomal DNA with primers 612BT1 plus 612BT2 and 613BT1 plus 613BT2, respectively (Table 3-2). The PCR products were cloned into pCR2.1-TOPO, and each was subcloned into *Not*I-*Xho*I sites of pBT and pTRG, resulting in pWW079 (PA0612 in pBT), pWW077 (PA0612 in pTRG), pWW080 (PA0613 in pBT), and pWW078 (PA0613 in pTRG). The *exsA* open reading frame was isolated from pHW0315 (*exsA* in pTRG) as a *Not*I-*Spe*I fragment. The *Spe*I site was blunt ended and ligated into *Not*I-*Sma*I sites of pBT, resulting in pWW081 (*exsA* in pBT). Desired pairs of plasmids were cotransformed into a reporter strain by electroporation, and the protein-protein interaction assays were performed following the protocol supplied by the manufacturer (Stratagene). The interaction between two proteins is indicated by the expression level of a *lacZ* reporter gene. By testing the β-galactosidase activity of the reporter strains containing cloned genes on pBT and pTRG, the interaction between the two proteins can be tested.

Other Methods

Western blotting, ß-Galactosidase activity assays and statistical assays were done as described in Chapter 2. For twitching motility assays, bacteria were stabbed into a thin-layer LB plate and incubated overnight at 37°C. The LB plate was directly stained with Coomassie blue at room temperature for 5 min and destained with destaining solution.

Results

TTSS Is Repressed in a *prtR* Mutant

As described in Chapter 2, by screening a Tn insertion library consisting of 40,000 independent mutants, two *prtR* mutants were found to be defective in TTSS activity (Fig.

3-1). Complementation of the original *prtR*::Tn mutants with a *prtR* gene partially restored the TTSS activity (Fig. 3-1A). PrtR is a repressor of pyocin synthesis, which is a set of bacteriocins synthesized by *P. aeruginosa*. PrtR binds to the promoter region of the *prtN* gene and represses its expression. PrtN is also a DNA binding protein which recognizes a highly conserved sequence (P box) present upstream of pyocin synthesis genes and activates their expression (86). Based on this regulatory pathway, either the up-regulated PrtN is responsible for the TTSS repression or another gene under the control of PrtR mediates the TTSS repression. To test these possibilities, a *prtR prtN* double mutant was generated in the background of wild-type PAK. The resulting mutant, PAKΔ*prtNprtR*::Gm, had the same TTSS defect as the *prtR*::Tn5 mutant (Fig. 3-1A and B), and complementation by a *prtR* gene (pWW037) but not by a *prtN* gene (pWW031) restored the TTSS inducibility (Fig. 3-1A). Furthermore, introduction of a *prtN*-expressing clone in a high-copy-number plasmid (pWW031) in wild-type PAK had no effect on the TTSS activity (Fig. 3-1A and B). Thus, all of the above results indicated that PrtN is not involved in the TTSS repression. Therefore, it is likely that another gene(s) under the control of PrtR mediates the repression of the TTSS.

Identification of the PrtR-regulated Repressor of the TTSS

Since PrtR functions as a repressor, it might also repress the expression of a hypothetical TTSS repressor. With the mutation in *prtR*, this hypothetical repressor would be up regulated and therefore would repress the expression of the TTSS. Thus, upon inactivation of this repressor gene in the *prtR* mutant background, the TTSS activity should be restored to that of wild-type. To identify this hypothetical repressor, the Δ*prtNprtR*::Gm double mutant containing *exoT*::*lacZ* (pHW0006) was subjected to transposon mutagenesis. A plasmid containing a Tn5 transposon (pRL27) was

transferred from *E. coli* donor strain BW20767 into *P. aeruginosa* by conjugation. The double mutant strain Δ*prtNprtR*::Gm was chosen as a recipient, since it has an identical phenotype of a TTSS defect as the *prtR*::Tn mutant. More importantly, constitutive production of pyocin by a *prtR* mutant seems to have a detrimental effect on the *E. coli* donor strain which may lower the conjugation frequency.

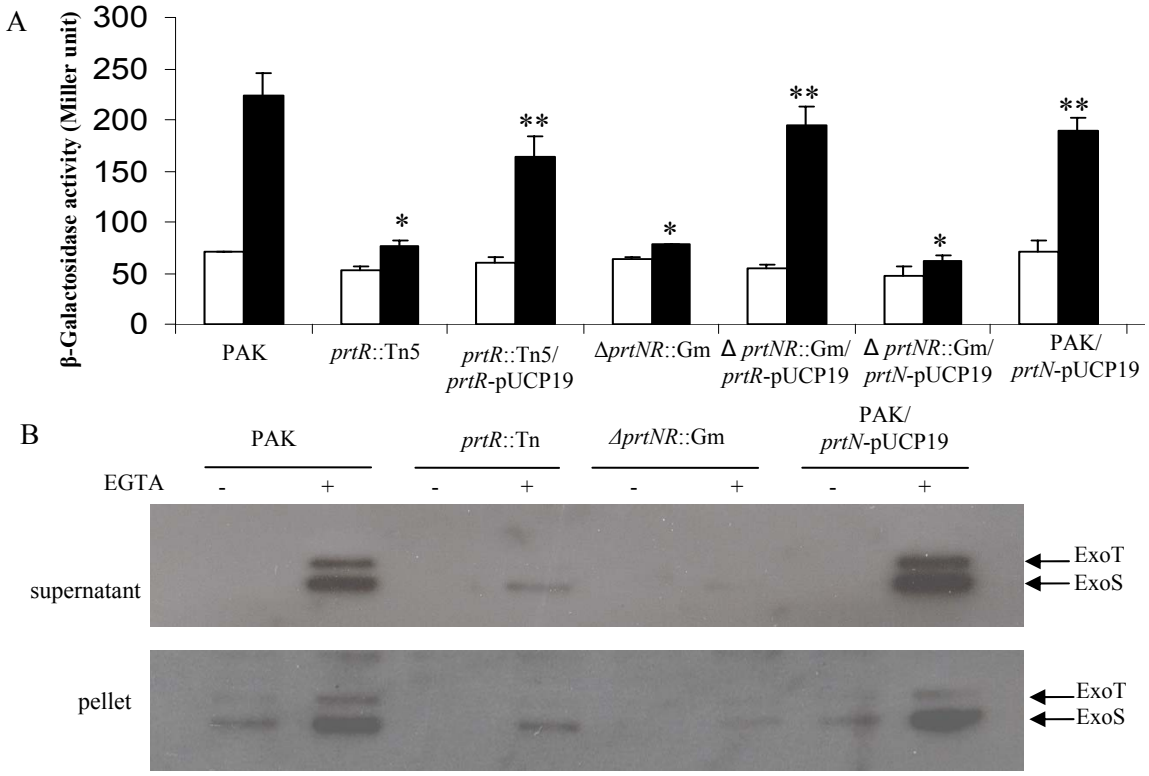

Figure 3-1. Expression and secretion of ExoS. (A) Expression of *exoS*::*lacZ* in the backgrounds of PAK, *prtR*::Tn5, *prtR*::Tn5 containing *prtR* expression plasmid pWW037 (*prtR*-pUCP19), Δ*prtNR*::Gm, Δ*prtNR*::Gm containing pWW037 (*prtR*-pUCP19) or *prtN* expression plasmid pWW031 (*prtN*-pUCP19), and PAK with pWW031 (*prtN*-pUCP19). Bacteria were grown to an OD_{600} of 1 to 2 in LB with (black bars) or without (white bars) EGTA before ß-galactosidase assays. (B) Cellular and secreted forms of ExoS in strains PAK, *prtR*::Tn5, Δ*prtNR*::Gm, and PAK containing pWW031 (*prtN*-pUCP19). Overnight bacterial cultures were diluted to 1% in LB or 3% in LB plus 5 mM EGTA and grown at 37°C for 3.5 h. Supernatants and pellets from equivalent bacterial cell numbers were loaded onto SDS-PAGE gels and immunoblotted with anti-ExoS antibody. Both ExoS and ExoT are indicated by arrows. Anti-ExoS polyclonal antibody also recognizes ExoT due to high homology between them. *, $P < 0.01$, compared to the values in PAK. **, $P < 0.05$, compared to the values in *prtR*::Tn5.

The Tn insertion mutants were spread on LB agar plates containing 20 μg/ml X-Gal, 2.5 mM EGTA, and proper antibiotics. Blue colonies were looked for in which the TTSS repressor under the control of PrtR should have been knocked out. About 100,000 Tn insertion mutants were screened. Thirty blue colonies were picked and cultured in liquid LB for ß-galactosidase assay. Sixteen mutants showed restored TTSS activity compared to the parent strain. Sequence analysis of the Tn insertion sites showed that 14 mutants had Tn insertions at a single locus (PA0612) at nine different positions. PA0612 encodes a hypothetical protein with a consensus prokaryotic DksA/TraR C4-type zinc-finger motif. The *dksA* gene product suppresses the temperature-sensitive growth and filamentation of a *dnaK* deletion mutant of *E. coli* (66), while TraR is involved in plasmid conjugation (30). These proteins contain a C-terminal region thought to fold into a four-cysteine zinc finger (30). Its homologues also exist in other gram-negative bacteria, such as *Pseudomonas syringae*, *Pseudomonas putida*, *E. coli*, *Salmonella enterica* serovar *Typhimurium*, and *Shigella flexneri*. However, the functions of these gene homologues have not been studied. The remaining two mutants contained a Tn insertion in the genes PA2265 and PA5021, respectively. PA2265 encodes a putative gluconate dehydrogenase. Promoter analysis (http://www.fruitfly.org/seq_tools/promoter.html) indicates it is in the same operon with an upstream gene, PA2264, as well as a downstream gene, PA2266. PA2264 is an unknown gene, while PA2266 encodes a putative cytochrome c precursor. PA5021 encodes a probable sodium:hydrogen antiporter. Promoter analysis indicated that two downstream genes, PA5022 and PA5023, are in the same operon with PA5021, where

PA5022 and PA5023 encode two unknown proteins. We further pursued the regulation and function of PA0612 in this study.

PA0612 and PA0613 Form an Operon Which Is Under the Control of PrtR

Promoter analysis predicted that PA0612 and PA0613 may form an operon, while the pyocin synthesis gene PA0614 has its own promoter. The downstream gene (PA0613) encodes an unknown protein. On the chromosome of PAO1, PA0612 is located next to the *prtR* gene in the opposite direction. In the promoter region of PA0612, a 14-base sequence was observed that was also present as a direct repeat in the predicted *prtN* promoter region, which might be the PrtR recognition site (Fig. 3-2) (86). Therefore, it is highly likely that the expression of PA0612 is under the control of PrtR and mediates the repression of TTSS.

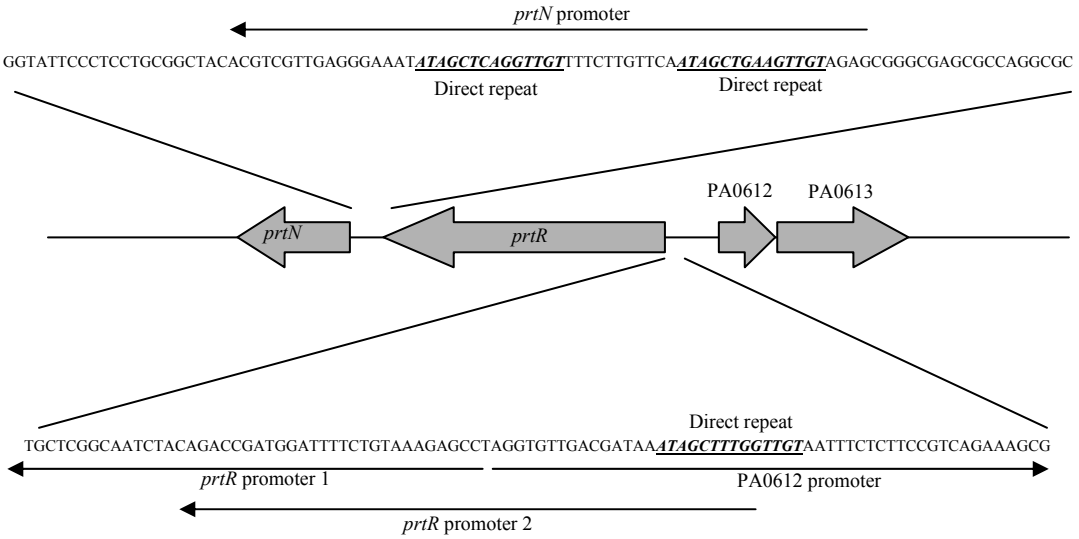

Figure 3-2. Genetic organization and putative promoter regions of *prtN*, *prtR*, PA0612-3. Computer-predicted promoters of *prtN*, *prtR*, PA0612-613, and PA0614 are indicated with arrows. Two promoters are predicted for the prtR gene and are designated promoters 1 and 2. The potential PrtR binding sequences are underlined. The arrow of each open reading frame represents the transcriptional direction.

To confirm the prediction that PA0612 and PA0613 are in the same operon, a pair of primers annealing to the 5' end of PA0612 (612GS1) and 3' end of PA0613 (613BT2)

was designed for RT-PCR analysis (Table 3-2). A 649-bp PCR product was amplified using total RNA isolated from *prtR*::Tn or Δ*prtNprtR*::Gm (Fig. 3-3A), and the size was the same as that when PAK genomic DNA was used as template (data not shown). However, when total RNA from PAK or PAK/pWW031 (*prtN* overexpresser) was used as template, a faint PCR product could be seen (Fig. 3-3A), indicating low abundance of this transcript. These results suggested that PA0612 and PA0613 are in the same operon, which is under the negative control of PrtR. Transcription of PA0612 was investigated further by real-time PCR. Expression of PA0612 mRNA in *prtR*::Tn and Δ*prtNR*::Gm was 30- and 38-fold greater than that in PAK, respectively, while overexpression of the *prtN* gene had little effect on the transcript level of PA0612 (Fig. 3-3B). To further confirm this, the promoter of PA0612 was fused with a promoterless *lacZ* gene on plasmid pDN19lacZ, and the resulting fusion construct (pWW048-1) was introduced into various strain backgrounds for the ß-galactosidase assay. As shown in Fig. 3-4, the expression of PA0612 was up regulated in both *prtR*::Tn and Δ*prtNprtR*::Gm mutant backgrounds compared to that in PAK or PAK overexpressing *prtN* (PAK/pWW031), further proving that the expression of PA0612 and PA0613 is repressed by PrtR. The above results also reaffirmed our earlier conclusion that *prtN* has no effect on the expression of PA0612 and PA0613.

PA0612 Is Required for the Repression of the TTSS In the *prtR* Mutant

Since PA0612 and PA0613 are in the same operon, insertion of a Tn in PA0612 will have a polar effect on the expression of PA0613. To test which of the two genes is required for the TTSS repression in the *prtR* mutant, deletion mutants of PA0612 and PA0613 and the PA0612 PA0613 double mutant were generated in the background of the Δ*prtNprtR*::Gm mutant. The production and secretion of ExoS, as judged by Western

blotting, were restored in the PA0612 and PA0612-013 mutants but not in the PA0613 mutant (Fig. 3-5A). The reporter plasmid of *exoT*::*lacZ* (pHW0006) was further transformed into these mutants and subjected to a ß-galactosidase assay. As the results show in Fig. 3-5B, transcription of the *exoT* gene was partially restored in the backgrounds of Δ*prtNprtR*::GmΔPA0612-013 and Δ*prtNprtR*::GmΔPA0612 mutants, while they remained repressed in the background of the Δ*prtNprtR*::GmΔPA0613 mutant, indicating that PA0612 is required for repression of the TTSS in the *prtR* mutant background.

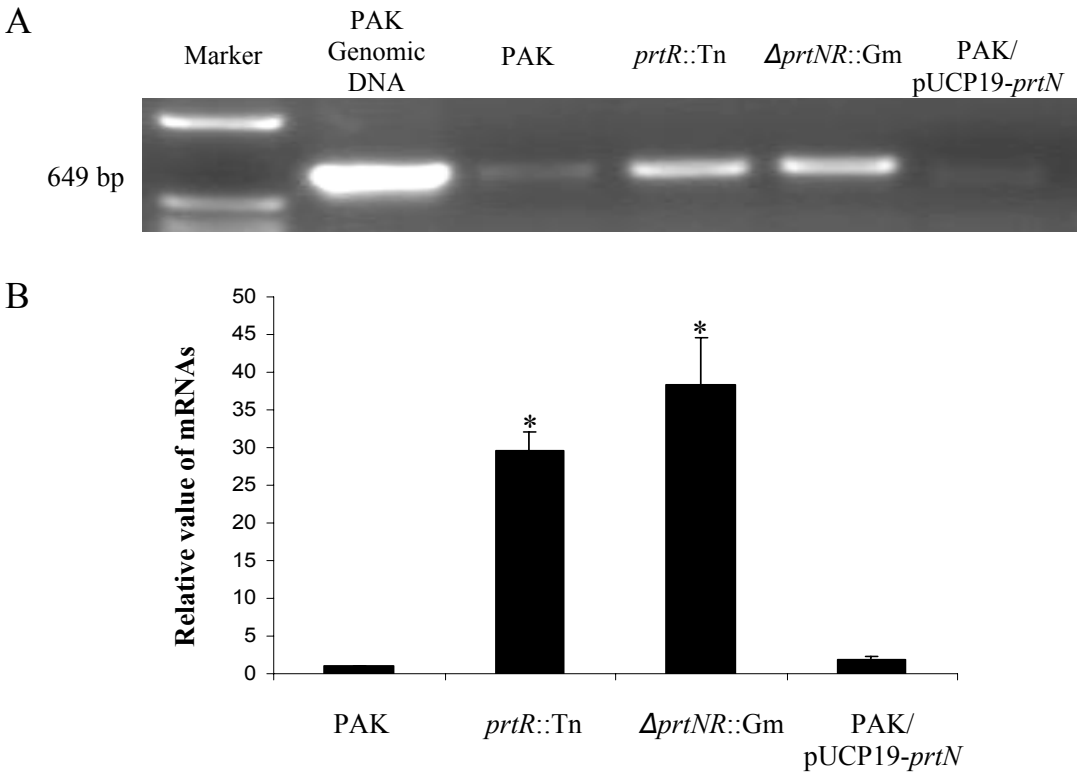

Figure 3-3. Expression of PA0612 is repressed by *prtR*. (A) RT-PCR of the PA0612-0613 operon. Total RNA was isolated from PAK, *prtR*::Tn5, Δ*prtNR*::Gm, and PAK/pWW031. One microgram of RNA from each sample was used to synthesize cDNA, and the cDNA was diluted 100-fold for subsequent PCR amplification. The primers used in the PCR anneal to the 5' end of PA0612 and the 3' end of PA0613. (B) Quantitation of PA0612 gene expression by real-time PCR. Data are expressed relative to the quantity of PA0612 mRNA in PAK. *, $P < 0.01$, compared to the values in PAK.

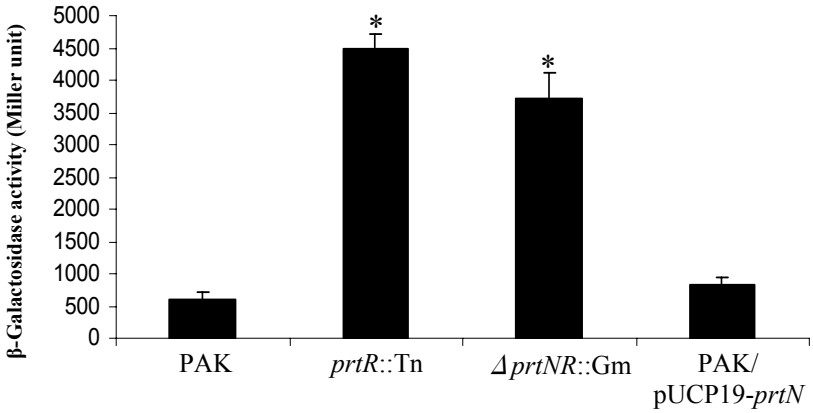

Figure 3-4. Expression of PA0612::*lacZ* (pWW048-1) in PAK, *prtR*::Tn5, Δ*prtNR*::Gm, and PAK/pWW031. Bacteria were grown in LB for 10 h before ß-galactosidase assays. *, $P < 0.01$, compared to the values in PAK.

The TTSS of PAK can directly deliver ExoS, ExoT, and ExoY into the host cell, resulting in cell rounding and lifting (46, 125, 131). HeLa cells were infected with wild-type PAK and *prtR* mutants at a MOI of 20. Upon infection by PAK, almost all of the HeLa cells were rounded after 2.5 h. Under the same conditions, the PAK*exsA*::Ω mutant, a TTSS-defective mutant, had no effect on HeLa cell rounding; similar to the PAK*exsA*::Ω mutant, low cytotoxicity was seen with mutant strains *prtR*::Tn, Δ*prtNprtR*::Gm, and Δ*prtNprtR*::GmΔPA0613. However, Δ*prtNprtR*::GmΔPA0612-013 and Δ*prtNprtR*::GmΔPA0612 caused comparable levels of HeLa cell lifting as that seen with PAK. Quantitative assay of the cell lifting was further performed by crystal violet staining of the adhered cells after 4 h of infection. As shown in Fig. 3-5C, mutant strains Δ*prtNprtR*::GmΔPA0612-013 and Δ*prtNprtR*::GmΔPA0612 showed similar cytotoxicity as wild-type PAK. However, *PrtR*::Tn, Δ*prtNprtR*::Gm, and Δ*prtNprtR*::GmΔPA0613 showed much-reduced cytotoxicity. The above observations clearly indicated that PA0612, but not PA0613, is required for the TTSS repression in the *prtR* mutant background. We designate this newly identified repressor gene as pseudomonas type III repressor gene B or, *ptrB*.

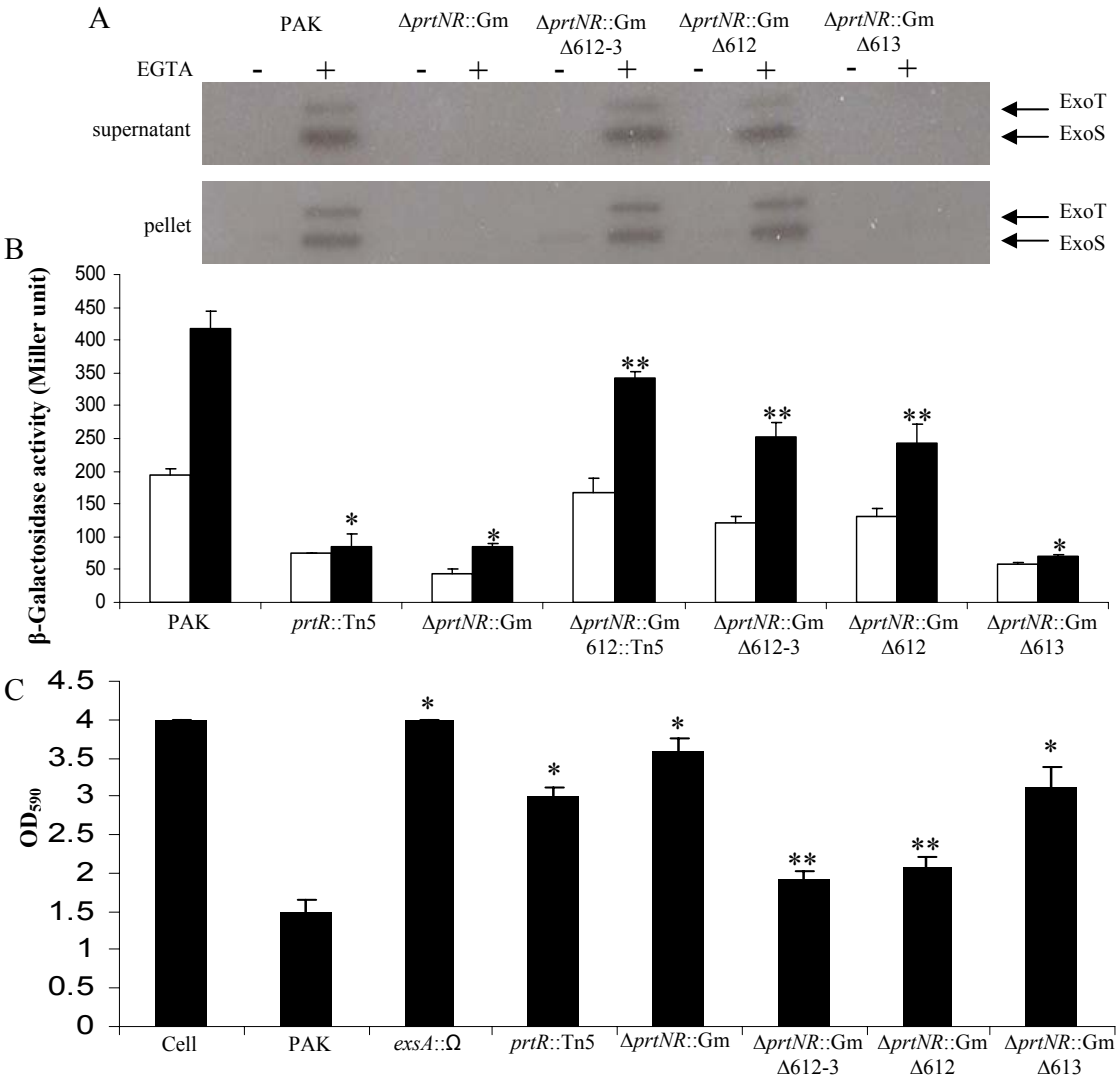

Figure 3-5. Characterization of ExoS expression and cytotoxicity. (A) Cellular and secreted forms of the ExoS in strains PAK, Δ*prtNR*::Gm, Δ*prtNR*::GmΔPA0612-0613, Δ*prtNR*::GmΔPA0612, and Δ*prtNR*::GmΔPA0613. Overnight bacteria cultures were diluted to 1% in LB or 3% in LB plus 5 mM EGTA and grown at 37°C for 3.5 h. Supernatants and pellets from equivalent bacterial cell numbers were loaded onto SDS-PAGE gels and immunoblotted with anti-ExoS antibody. Both ExoS and ExoT are indicated by arrows. (B) Expression of *exoT*::*lacZ*(pHW0005) in the backgrounds of PAK, Δ*prtNR*::Gm, Δ*prtNR*::GmΔPA0612-0613, Δ*prtNR*::GmΔPA0612, and Δ*prtNR*::GmΔPA0613. Bacteria were grown to an OD_{600} of 1 to 2 in LB with (black bars) or without (white bars) EGTA before ß-galactosidase assays. (C) Cell lifting assay. HeLa cells were infected with PAK, *prtR*::Tn5, Δ*prtNR*::Gm, Δ*prtNR*::GmΔPA0612-0613, Δ*prtNR*::GmΔPA0612, and Δ*prtNR*::GmΔPA0613 at an MOI of 20. After a 4-hour infection, cell lifting was measured with crystal violet staining (see Materials and Methods for details). *, $P < 0.01$, compared to the values in PAK; **, $P < 0.01$, compared to the values in Δ*prtNR*::Gm.

The Expression of *exsA* Is Repressed by PtrB in *prtR* mutants

The master activator of TTSS genes is ExsA. It is the last gene in the *exsCEBA* operon (144). The great reduction of ExoS and ExoT in *prtR* mutants may occur through the repression of *exsA* expression. To test the transcription of *exsA*, an *exsC*::*lacZ* reporter plasmid was introduced into the *prtR* mutants. As shown in Fig. 3-6, the expression of the *exsCEBA* operon was greatly reduced in *prtR* and Δ*prtNR* mutants. Deletion of PA0612-3 and *ptrB*, but not PA0613, partially restored the promoter activity of *exsC*. Since ExsA is also the activator of its own operon (60), the repression may be on the transcriptional, translational or protein level. So I further tested the interaction between ExsA and PtrB.

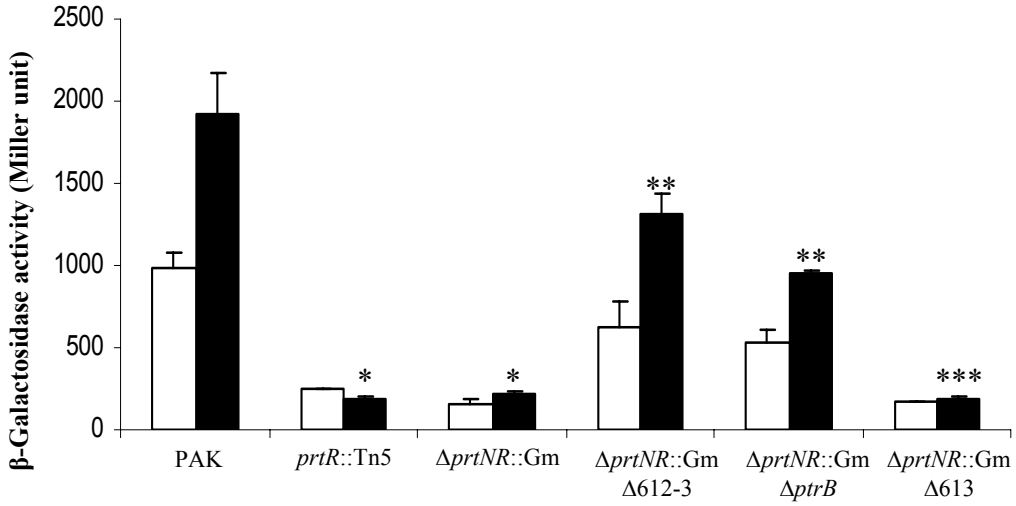

Figure 3-6. Expression of *exsA* operon in *prtR* mutants. Bacteria were grown to an OD_{600} of 1 to 2 in LB with (black bars) or without (white bars) EGTA before ß-galactosidase assays. *, $P < 0.01$, compared to the values in PAK; **, $P < 0.01$, compared to the values in Δ*prtNR*::Gm; ***, $P < 0.001$, compared to the values in Δ*prtNR*::GmΔ*ptrB*.

PtrB Might Not Directly Interact with ExsA

In earlier reports, it has been shown that ExsA activity can be repressed by interaction with ExsD or PtrA (47, 89). We wanted to test if the TTSS repressor function of PtrB is achieved through a direct interaction with the master regulator, ExsA. A

bacterial two-hybrid system (Stratagene) was used to test the interaction between the two components. *ptrB* and *exsA* were each cloned into bait (pBT) or prey (pTRG) plasmids, fused with λCI and RNA polymerase (RNAP) α-subunit at C terminus, respectively. Interaction between the two tested proteins can stable λCI and RNAP in the promoter region of a *lacZ* gene and activates its expression. Thus, the interaction of two proteins was indicated by the expression of *lacZ* in the reporter strain. ß-Galactosidase assay results, however, did not suggest a direct interaction between PtrB and ExsA, although strong interaction was observed between the positive controls provided (Fig. 3-7). Therefore, the mechanism of TTSS repression in the *prtR* mutant might not involve a direct binding of PtrB to ExsA. Negative results were also obtained in similar tests between PtrB and PA0613, indicating no direct interaction of the two small proteins encoded in the same operon.

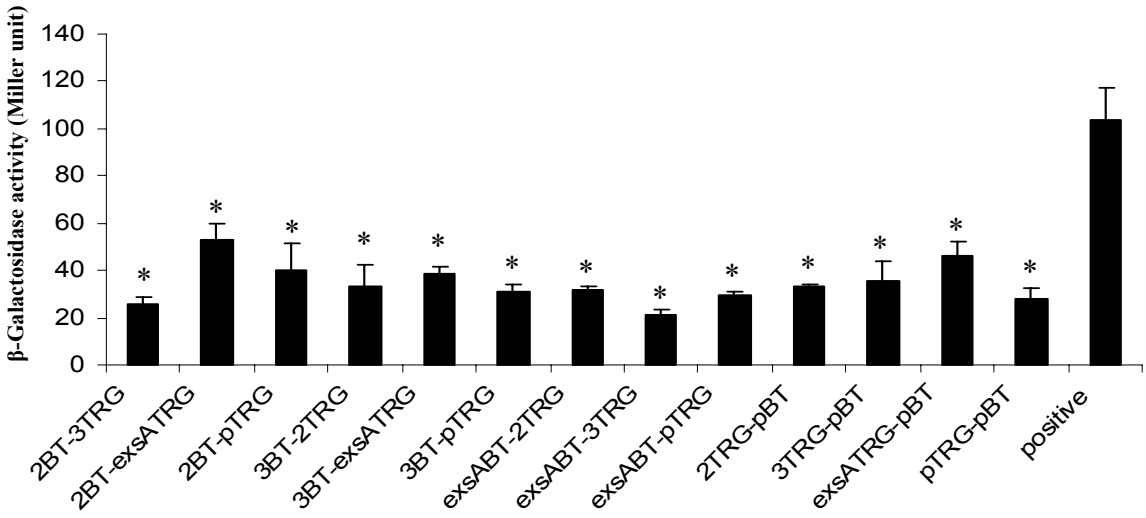

Figure 3-7. Monitoring of protein-protein interactions by the BacterioMatch two-hybrid system. pBT, bait vector; pTRG, target vector; 2BT, *ptrB* cloned into bait vector; 2TRG, *ptrB* cloned into target vector; 3BT, PA0613 cloned into bait vector; 3TRG, PA0613 cloned into target vector; *exsA*BT, *exsA* cloned into bait vector; *exsA*TRG, *exsA* cloned into target vector; positive, positive control provided by the manufacturer. *, $P < 0.01$, compared to the values in the positive control.

Mitomycin C-mediated Suppression of the TTSS Genes Requires PtrB

Pyocin production can be triggered by mutagenic agents, such as mitomycin C. In response to the DNA damage, RecA is activated and cleaves PrtR, similar to LexA cleavage by RecA in *E. coli* during the SOS response (90). In the absence of PrtR, the expression of *prtN* is derepressed, resulting in up regulation of the pyocin synthesis genes. Under this circumstance, the *ptrB* gene should also be up regulated, resulting in TTSS repression. To test this prediction, wild-type PAK was treated with mitomycin C under TTSS inducing and noninducing conditions and the expression of ExoS was monitored by Western blot analysis. In previous reports, 1 μg/ml of mitomycin C was shown to be able to induce pyocin synthesis (90). After treatment with 1 μg/ml of mitomycin C for 1.5 h, the OD_{600} of PAK began to decrease with or without EGTA due to the toxic effect of the mitomycin C (Fig. 3-8A); therefore, we collected the samples 1.5 h after mitomycin C treatment. Two culture methods were used. One was to grow PAK with mitomycin C for 30 min and then EGTA was added to induce TTSS for 1 hour. The other was to add mitomycin C and EGTA at the same time and induce for 1 hour. Experimental results showed that when wild-type PAK was treated with mitomycin C and EGTA at the same time, normal TTSS activation was observed. However, when cells were treated with mitomycin C 30 min before the addition of EGTA, a clear repression of the TTSS was observed (Fig. 3-8B). To test whether the *ptrB* gene mediates the repression of the TTSS by mitomycin C, a deletion mutant of *ptrB* was further generated in the background of wild-type PAK. Deletion of *ptrB* in PAK had no effect on the expression of the TTSS (Fig. 3-8B and C). Interestingly, even with the 30-min pretreatment of mitomycin C (1 μg/ml), production of ExoS in the PAKΔ*ptrB* mutant was activated by EGTA, even higher than that without mitomycin C treatment

(Fig. 3-7B). Clearly, mitomycin C-mediated suppression of the TTSS requires the *ptrB* gene.

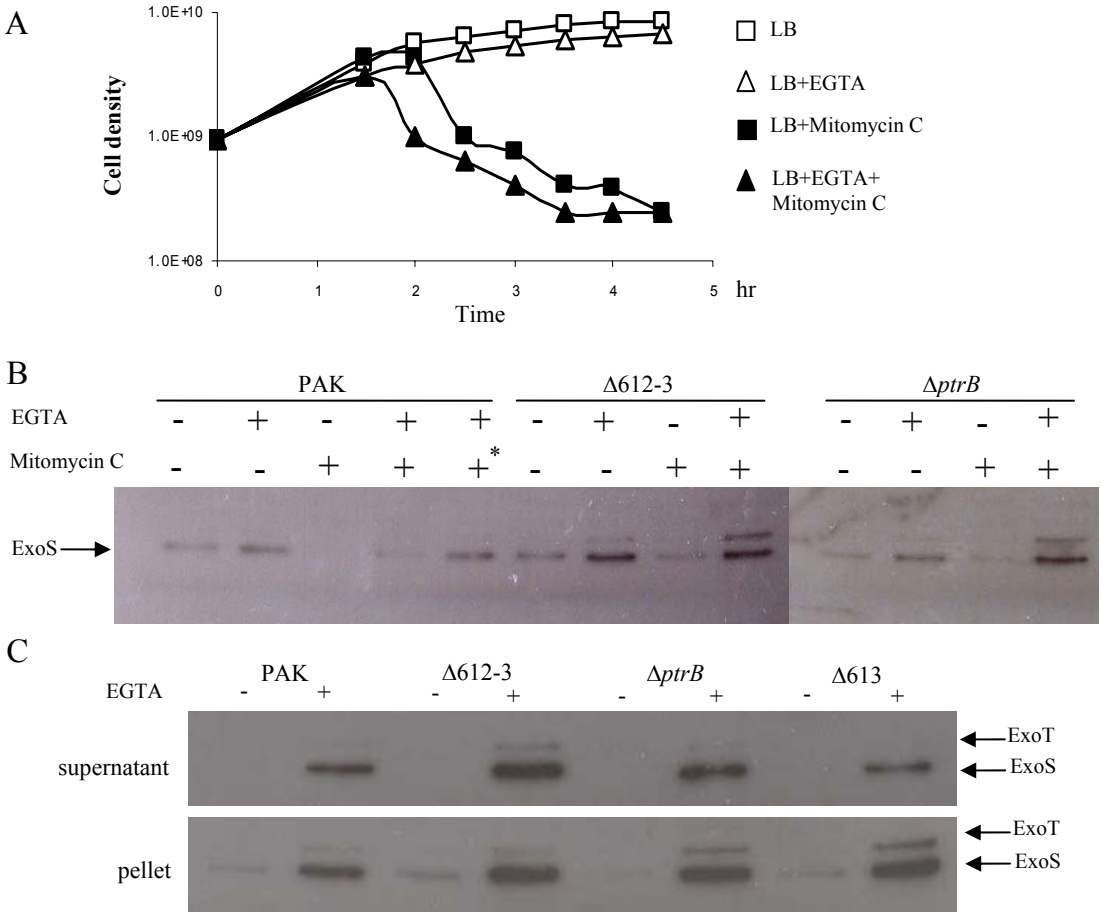

Figure 3-8. Effect of mitomycin C on bacteria growth and TTSS activity. (A) An overnight culture of PAK was diluted to an OD_{600} of 0.8 in LB, LB plus 1 μg/ml mitomycin C, LB plus 5 mM EGTA, or LB plus 1 μg/ml mitomycin C plus 5 mM EGTA. The OD_{600} of each sample was measured at 30-min intervals. The cell densities were calculated based on the OD_{600}. (B) Overnight cultures of PAK, PA0612-0613, and Δ*ptrB* were diluted to an OD_{600} of 0.5 with LB or LB plus 1 μg/ml mitomycin C. After 30 min, EGTA was added to the culture medium at a final concentration of 5 mM. One hour later, each culture was mixed with protein loading buffer. Samples derived from equivalent bacterial cell numbers were loaded onto SDS-PAGE gels and immunoblotted with anti-ExoS antibody. *, PAK was grown in LB for 30 min, and then both mitomycin C and EGTA were added at the same time. (C) Overnight cultures of PAK, PA0612-0613, and Δ*ptrB* strains were diluted to 1% in LB or 3% in LB plus 5 mM EGTA and grown at 37°C for 3.5 h. Supernatants and pellets from equivalent bacterial cell numbers were loaded onto SDS-PAGE gels and immunoblotted with anti-ExoS antibody.

Twitching Motility Was Not Affected by the *prtR* mutation

The TTSS genes have been shown to be affected by Vfr and CyaA/B, homologues of CRP and cyclic AMP synthase (140). Vfr is well known for its involvement in the regulation of twitching motility (8), flagellum synthesis (26), type II secretion (140), and quorum sensing (3). Recently, FimL was found to regulate both the TTSS and twitching motility through Vfr (137). To test whether mutation of *prtR* affects twitching motility, strains with *prtR* and *ptrB* mutations were subjected to a stab assay. Mutation in the *prtR* or *ptrB* gene had no effect on twitching motility (Fig. 3-9), indicating that the repression of the TTSS in the *prtR* mutant does not go through the Vfr pathway.

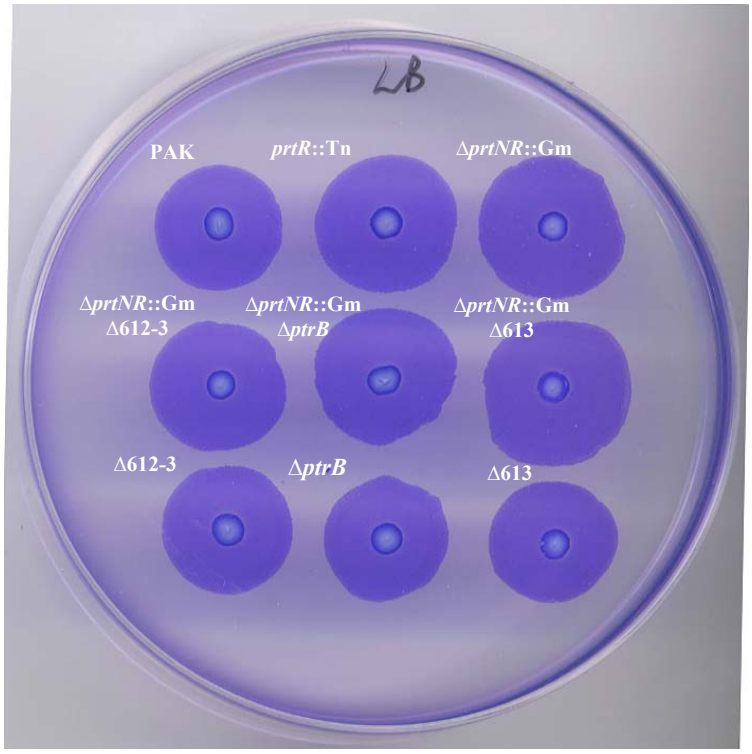

Figure 3-9. Twitching motility of *prtR*, *ptrB* and PA0613 mutants. The bacteria of each strain were stabbed into a thin-layer LB agar. The plate was incubated at 37°C over night. The whole plate was directly stained with Coomassie blue at room temperature for 5 min and destained with destain solution.

Discussion

During early infection of cystic fibrosis patients, *P. aeruginosa* produces S-type pyocins (9); however, the exact physiological role played by pyocins is unclear. Pyocins might ensure the predominance of a given strain in a bacterial niche against other bacteria of the same species. The pyocin production starts when adverse conditions provoke DNA damage. Under these conditions, the effect of pyocins is likely to preserve the initial predominance of pyocinogenic bacteria against pyocin-sensitive cells (90). Upon activation by DNA-damaging agents, RecA mediates the cleavage of PrtR, derepressing the expression of *prtN*, resulting in active synthesis of pyocins. Thus, the pyocin synthesis is dependent on the SOS response, resembling those responses of temperate bacteriophages in *E. coli* (16, 90). Indeed, DNA-damaging agents, such as UV irradiation and mitomycin C, induce the synthesis of pyocins in a *recA*-dependent manner (90). Apparently, in response to the DNA damage stress signal, *P. aeruginosa* not only turns on the SOS response system for DNA repair and pyocin synthesis but also actively represses the energy expensive type III secretion system, an example of coordinated gene regulation for survival.

Along the regulatory pathway, mutation of the *prtR* gene results in the up regulation of *prtN* (86). We found that PrtN is not responsible for the repression of the TTSS; rather, *ptrB* next to and under the control of *prtR* is required for the TTSS repression. We also found that the downstream gene PA0613 was in the same operon with PA0612. Homologues of these genes are also found in *Pseudomonas putida* (PP3039 and PP3037) and *Pseudomonas syringae* (PSPT03417 and PSPT03419), where they seem to also form operon structures, although with one additional gene between them (PP3038 or PSPR03418). The promoter of *ptrB* contains a 14-base sequence that

was also found in the *prtN* promoter (86), which may be a binding site for PrtR. Considering that PrtR is the ortholog of λCI, which functions as a homodimer (16), PrtR may also form a dimer. Whether PrtR recognizes these potential binding sites is not known. Interestingly, the PtrB protein contains a prokaryotic DksA/TraR C4-type zinc-finger motif (www.pseudomonas.com). The *dksA* gene product suppresses the temperature-sensitive growth and filamentation of a *dnaK* deletion mutant of *E. coli* (66), while TraR is involved in plasmid conjugation (30). These proteins contain a C-terminal region thought to fold into a four-cysteine zinc-finger (30). *Yersinia sp*. also encodes a small-sized protein, YmoA (8 kDa), which negatively regulates the type III secretion system (79). YmoA resembles the histone-like protein HU and *E. coli* integration host factor; thus, it is likely to repress type III genes through its influence on DNA conformation. Whether PtrB exerts its repressor function through interaction with another regulator or through binding to specific DNA sequences present in the TTSS operons or their upstream regulator genes is not known. It would also be interesting to investigate on what other genes of the *P. aeruginosa* genome PtrB effects on.

It is not surprising that *P. aeruginosa* has multiple regulatory networks, since 8% of its genome codes for regulatory genes, indicating that *P. aeruginosa* has dynamic and complicated regulatory mechanisms responding to various environmental signals (108, 124). Also, due to the requirement of a large number of genes, construction of the type III secretion apparatus is an energy-expensive process. Thus, *P. aeruginosa* might have evolved multiple signaling pathways to fine-tune the regulation of the type III secretion system in response to the environmental changes. Similarly, *Yersinia* has been reported to have several regulators, such as an activator, VirF, and repressor molecules, LcrQ,

YscM1, YscM2, and YmoA, that are involved in the control of *yop* gene transcription (20, 139, 142). Current efforts are focused on the elucidation of the molecular mechanism by which PtrB mediates suppression of the TTSS. Also, the relevance of the two additional genes, PA2265 and PA5021, to the regulation of the TTSS needs more investigation.

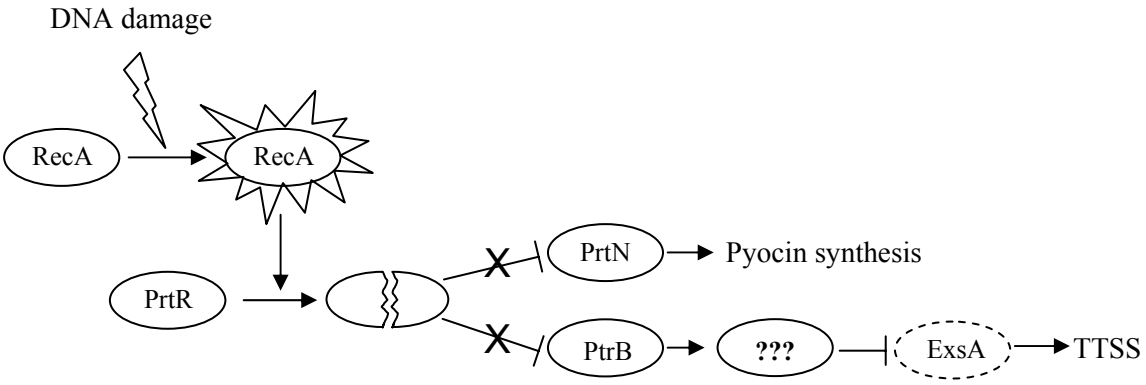

Figure 3-10. Proposed model of PtrB-mediated TTSS repression. In wild-type PAK, PrtR represses the expression of *prtN* and *ptrB*. In response to DNA damage, RecA is activated and cleaves PrtR, resulting in increased expression of *prtN* and *ptrB*. PrtN activates the expression of pyocin synthesis genes, while PtrB represses the type III secretion genes directly or through additional downstream genes.

Based on our results, we propose a model for the repression of the TTSS induced by DNA damage (treatment with mitomycin C) (Fig. 3-10). DNA damage induces the SOS response, in which RecA is activated. RecA cleaves PrtR, resulting in the up regulation of *prtN* and *ptrB*. PrtN activates the expression of pyocin synthesis genes, while PtrB represses the TTSS genes. How PtrB represses the TTSS is not known. In the bacteria two-hybrid system, I failed to detect the interaction between PtrB and ExsA. However, PtrB is a small protein (~6.7 kDa), and when fused with either λCI or RNAP α-subunit, its interaction with ExsA might be affected due to conformational change or

steric hindrance. Further experiments are needed to study the interaction between PtrB and ExsA.

CHAPTER 4
DISCUSSION AND FUTURE DIRECTIONS

The TTSS and Environmental Stresses

Repression of the TTSS under Environmental Stresses

The TTSS of *P. aeruginosa* is under the control of a complicated regulatory network. ExsA, an AraC-type protein, is the master activator of the TTSS. Two proteins, ExsD and PtrA, have been found to directly interact with ExsA. ExsD is an anti-activator, inhibiting the activity of ExsA (89). PtrA is an *in vivo* inducible protein and represses the activity of ExsA through direct binding. *In vitro*, the expression of PtrA is inducible by high copper stress signal through a CopR/S two-component regulatory system (47). Over expression of multi-drug efflux systems MexCD-OprJ and MexEF-OprN leads to repression of the TTSS (75). The expression of multi-drug efflux systems are usually triggered by antibiotics which is a detrimental stress. We also found that mutation in the *mucA* gene not only results in overproduction of alginate but also causes repression of the TTSS (Chapter 2). MucA-regulated alginate production is induced by environmental stresses, such as high osmolarity, reactive oxygen intermediates, and anaerobic environment (45, 84). Metabolic imbalance was also shown to cause repression of the TTSS, which represents a nutritional stress (23, 107). In Chapter 3 we reported that mutation in the *prtR* gene resulted in repression of the TTSS. PrtR is a repressor whose activity is regulated by DNA damage (90), yet another stress signal. Mitomycin C, a mutagenic agent, can indeed repress the activity of the TTSS. My preliminary data showed that heat shock could also cause repression of the TTSS. These

discoveries indicate that the TTSS is effectively turned off under various environmental stresses, which might be an important survival strategy for this microorganism. Since mounting an effective resistance against stress requires a full devotion of energy, turning off other energy-expensive processes, such as the TTSS, will be beneficial to the bacterium.

Indication for the Control of *P. aeruginosa* Infection

Mutation in TTSS renders *P. aeruginosa* avirulence in a burned mouse model (59). In a mouse model of *P. aeruginosa* pneumonia and a rabbit model of septic shock, antibodies against PcrV (required for effectors translocation) are able to decrease lung injury and ensure survival of the infected animals (37, 113, 120). These results indicated that inactivation of the TTSS is a prospective therapeutic strategy. Since environmental stresses can lead to the repression of the TTSS, drugs can be designed towards components in the stress response signaling pathways, such as DNA damage, heat shock, metabolism imbalance, copper stress, etc. The more we know about the regulatory pathways, the more candidate targets we will have. This strategy might be extended to the control of other virulence mechanisms, such as biofilm formation. During the chronic infection in CF lungs, *P. aeruginosa* grows under a low oxygen environment in the form of a biofilm. Quorum sensing mutants (*lasR* or *rhlR*) are unable to survive in the anaerobic condition, due to the metabolic intoxication by nitric oxide (145). Therefore, drugs targeting the quorum sensing system might facilitate the eradication of *P. aeruginosa* biofilm (51). Indeed, some non-native AHLs (autoinducers of the *las* and *rhl* quorum sensing systems) have been found to disrupt *P. aeruginosa* biofilm formation (40).

Regulation of the TTSS under Environmental Stresses

Among all the environmental stresses that induce TTSS repression, only one regulatory pathway (PtrA) is well understood (47). Based on the experimental data for PtrA and PtrB, it is possible that each environmental stress involves a specific TTSS repressor, such as PtrA for copper stress and PtrB for DNA damage stress (Chapter 3). Some of these repressors may even have common regulators. For example, cAMP and Vfr are required for the TTSS. Any environmental signals affecting cAMP level or Vfr activity will affect the TTSS. It will be interesting to measure the expression level of Vfr as well as cAMP level under various environmental stresses.

The ExsA activity is under the direct control of a regulatory cascade, consisting of ExsE, ExsC and ExsD (Fig.1-1) (27, 89, 106, 130). Each of the components can also be the target of regulation under stress conditions. Activation of ExsA depends on the secretion of ExsE through the TTSS machinery. Any environmental stresses that block the ExsE secretion will result in inhibition of the ExsA function (106, 130). Furthermore, expression of *exsA* may also be affected by stress responses.

Expression of ExsA

exsA is the last gene in the *exsCEBA* operon as shown in Fig. 4-1, which is activated by ExsA itself (144). It is not known which sigma factor recognizes this operon promoter. Interestingly, the predicted *exsB* open reading frame (ORF) seems not translated in either *P. aeruginosa* or *E.coli* (43). Neither point mutation of the *exsB* start cordon nor over expression of *exsB* had any effect on the TTSS activity (43, 106). However, deletion of the *exsE* and *exsB* region (StuI sites, Fig 4-1) resulted in a drastic reduction of the TTSS activity. Since ExsE is a TTSS repressor, mutation in *exsE* should lead to derepression of the TTSS (106). These results indicate that the *exsB* region (DNA

or RNA) might affect the transcription or translation of ExsA. It will be interesting to delete only the *exsB* region and test the effect on the expression of *exsA*.

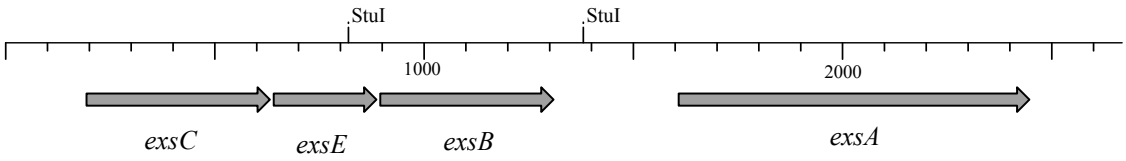

Figure 4-1. Structure of the *exsCEBA* operon. The ORFs and transcription directions are indicated as arrows.

Transcriptional control

The *exsB* DNA fragment might control the transcription of *exsA* gene through the formation of a secondary structure. This type of regulation usually happens at the promoter region, where RNA polymerase or regulators bind to (16). Since *exsA* does not have its own promoter immediate upstream of its coding sequence (144), it is unlikely that *exsB* DNA has this type of function.

Post-transcriptional control

Microarray analysis and *lacZ* transcriptional fusion experiments indicate that the mRNA level of *exsCEBA* operon does not change much under TTSS inducing vs. non-inducing conditions (72, 140). A real-time PCR experiment is needed to precisely determine the mRNA levels of each ORF and the region between *exsB* and *exsA*. Despite the minimal increase at the transcriptional level, the ExsA protein level increased significantly under TTSS inducing conditions as judged by Western blot analysis (27), suggesting that the expression of ExsA is under post-transcriptional control. Well known mechanisms of the post-transcriptional controls include mRNA stability or formation of secondary structures which affect translation efficiency (16, 74). In prokaryotes, untranslated mRNA tends to be degraded quickly by endoribonucleases or exonucleases (70). The translation of an mRNA can be affected by secondary structures formed by

endogenous sequences or with an antisense RNA. A likely hair-pin structure has indeed been found in the *exsB*-*exsA* junction (Fig. 4-2) which may blocks the access of the ribosome to its binding site for the translation for *exsA*. Experimental tests, deletion as well as site-directed mutagenesis, are needed to confirm this possibility.

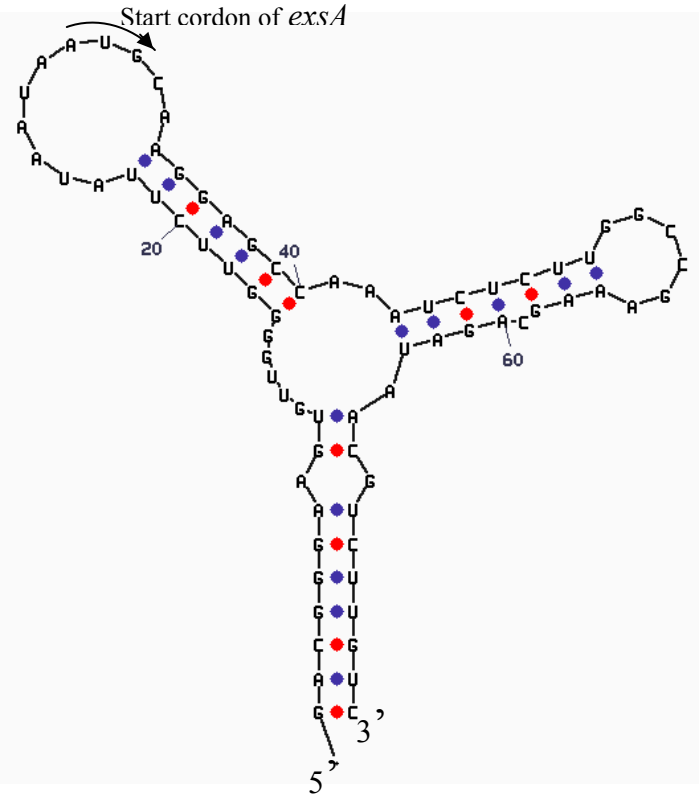

Figure 4-2. The secondary structure of *exsA* mRNA 5' terminus. The sequence was analyzed by mfold (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/).

The mRNA stability and secondary structure can also be controlled by small RNAs (sRNAs). sRNAs, with length range from 50 to 200 nucleotides, are used by bacteria to rapidly tune gene expression in responding to changing environments (83, 123). sRNAs usually anneal to 5' untranslated region (5' UTR) of target mRNAs. The effects of sRNA binding include increase or decrease of mRNA stability, exposure or blockage of ribosome binding site. Most interactions between sRNA and target mRNA require a small protein called Hfq. Mutation of Hfq in *P. aeruginosa* resulted in impaired

twitching motility and attenuation of virulence when injected intraperitoneally into mice (122). It will be interesting to test the TTSS activity in the *hfq* mutant, which may give us a clue whether sRNAs are involved in the TTSS regulation.

In summary, the transcriptional and translational control of *exsA* is not clear at present time. Understanding the regulatory mechanism of *exsA* may help us to clarify the relationship between TTSS and many other genes that affect its activity. Also, it will help us to develop strategies to control *P. aeruginosa* infections.

Transposon Mutagenesis

My project started from the construction and screening of Tn insertional mutant libraries. This strategy is a powerful tool in searching genes related to certain phenotype. The success of this method relies on the high efficiency of transposition, special characteristics of the Tn and sensitive screening methods.

Mutagenesis Efficiency

Usually, the Tn is on a suicide plasmid and transferred into the recipient through conjugation or sometimes by electroporation. In my experiments, the growth phase of *E.coli* donor strain was important, with the highest efficiency achieved by using cells grown to OD_{600}=0.6-1.0. The growth phase of *P. aeruginosa* recipient strain seems less important. The optimum donor to recipient ratio ranged between 3:1 and 8:1, with about $5X10^8$ recipient cells in each conjugation mixture.

During the growth of the conjugation mixtures (121), *P. aeruginosa* seems to kill *E.coli*, resulting in low conjugation efficiency. This killing can be repressed by performing the conjugation on nutrient agar. Probably, *P. aeruginosa* produces fewer bactericidal factors when grown on nutrient agar medium compared to the L-agar.

Another factor limiting the conjugation efficiency is the DNA modification and restriction system of the recipient, which mediates the degradation of foreign DNA. Growth of the recipient at 42ºC for at least 2 hours before conjugation can greatly increase the mutagenesis efficiency, presumably due to the repression of the DNA modification and restriction system.

In most of my experiments, 1-3x10^4 Tn insertion mutants can readily be obtained from each conjugation. *P. aeruginosa* has about 5600 genes; thus theoretically, 3x10^4 mutants should provide about 5-fold coverage of these genes (63).

Characteristics of the Tn

Most Tn insertional mutagenesis do not ensure every target gene being hit by the Tn, although statistically the number of the mutants should saturate the whole genome. Tns seem preferentially to insert in certain regions while avoiding other regions, so called hot and cold spots, respectively. The Tn used in my research is a derivative of Tn5 (71). In my Tn5 mutagenesis experiments, no insertion was found in the TTSS region, suggesting it is a cold spot for the Tn5. In agreement with my experience, a Tn5 insertion library constructed in strain PAO1 by Jacobs et al. (University of Washington Genome Center, Seattle) has also concluded that the coding region of the TTSS apparatus is a clod spot (63). Testing of different transposons might identify ones that can readily transpose into the TTSS region.

Screen Sensitivity

The success of Tn mutagenesis experiments also depends on the screening strategy. Two types of screening methods are widely used. One is to individually test for phenotypes of interests, which provides a high accuracy. However, it takes a lot of manpower and is cumbersome. The other one is to do large scale screening on the whole

library. By this method, a large number of mutants can be screened quickly, although the accuracy is compromised. Usually, this method requires a reporter gene, either encoded on the Tn or harbored by the recipient strain. In my experiments, an *exoT*::*lacZ* fusion on plasmid was used as the reporter. On plates containing X-gal, the density of blue color of each colony represents the *exoT* promoter activity. With this method, 100,000 mutants can be screened in less than one hour. The shortcoming of this method is that the color density is judged by eyes; thus many mutants with interesting phenotypes might have been missed. Indeed, although I successfully identified two genes, *mucA* and *prtR*, which are required for the TTSS activity, no other genes known to regulate the TTSS were identified. Possibly either I missed those colonies with minor changes in blue color or the Tn insertion libraries were not saturated. Other Tn with more sensitive screening methods might be needed to identify additional TTSS related genes.

In summary, I developed a screening system for the identification of the TTSS related genes. From the Tn insertion libraries constructed in wild type PAK containing *exoT*::*lacZ* reporter plasmid, two genes, *mucA* and *prtR*, were found to be related to the TTSS. I further studied the regulatory relationship between MucA and the TTSS as well as PrtR and the TTSS. In the *mucA* mutant, AlgU and AlgR are required for the repression of the TTSS. In the *prtR* mutant, a newly identified gene, *ptrB* is up regulated and responsible for the repression of the TTSS. Wild type *P. aeruginosa* strain will turn into mucoid phenotype in response to some environmental stresses, such as anaerobic environment, high osmolarity and reactive oxygen intermediates. PrtR is a regulator of pyocin synthesis, it responses to DNA damage. All my results suggest that TTSS will be

repressed under environmental stresses (10), which may provide a potential strategy for the control of the TTSS activity and improve the treatment of *P. aeruginosa* infection.

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BIOGRAPHICAL SKETCH

Weihui Wu was born in Tianjin, People's Republic of China, in June, 1976. From 1988 to 1994, he attended Tianjin No.2 middle school and high school. In 1994, he received admission from Nankai University, where he started his study in microbiology. After obtaining a Bachelor of Science degree in the summer of 1998, Weihui continued to study microbiology as a graduate student. He spent the next three years in studying *Bacillus thuringiensis* and received a Master of Science degree in 2001. After that, he decided to continue his study in microbiology. In August, 2001, Weihui came to America as a graduate student in the Interdisciplinary Program in Biomedical Sciences at the University of Florida. One year later, he joined Dr. Shouguang Jin's laboratory. In the next four years, he studied the regulation of the type III secretion system in *Pseudomonas aeruginosa* under the supervision of Dr. Shouguang Jin. After obtaining a Ph.D. degree in microbiology and immunology, Weihui plans to continue to pursue his research career in the biomedical field.