

2005

An investigation into novel cellular roles of LasB elastase in *Pseudomonas aeruginosa*

Michelle M. Farrell Yingling
University of Dayton

Follow this and additional works at: https://ecommons.udayton.edu/graduate_theses

Recommended Citation

Farrell Yingling, Michelle M., "An investigation into novel cellular roles of LasB elastase in *Pseudomonas aeruginosa*" (2005). *Graduate Theses and Dissertations*. 2591.
https://ecommons.udayton.edu/graduate_theses/2591

This Thesis is brought to you for free and open access by the Theses and Dissertations at eCommons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of eCommons. For more information, please contact mschlangen1@udayton.edu, ecommons@udayton.edu.

An investigation into novel cellular roles of LasB elastase in *Pseudomonas aeruginosa*

THESIS

Submitted to

The College of Arts and Sciences of the

University of Dayton

In Partial Fulfillment of the Requirements for

The Degree

Master of Science in Biology

By

Michelle M. Farrell Yingling

UNIVERSITY OF DAYTON

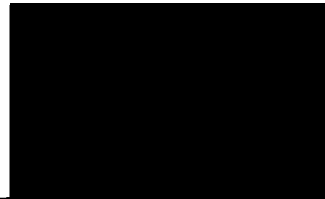
Dayton, Ohio

May, 2005

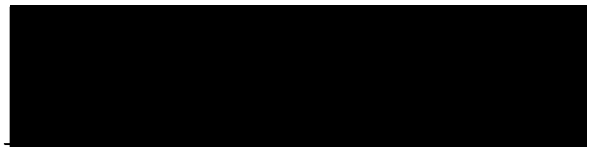
APPROVED BY:



Jayne Robinson, Ph.D.
Major Advisor



John Rowe, Ph.D.
Chairman, Department of Biology
Committee Member



Mark Nielsen, Ph.D.
Committee Member

ABSTRACT

An investigation into novel cellular roles of LasB elastase in *Pseudomonas aeruginosa*

Michelle M. Farrell Yingling
University of Dayton, 2005

Advisor: Jayne Robinson, Ph.D.

Biofilms have been described as “a community of microbes embedded in an organic polymer matrix, adhering to a surface.” The extracellular matrix (ECM) of microbial cells, which they produce, and in which they are embedded, is essential for allowing the cells to stick to biotic and abiotic surfaces. Microbial biofilms pose a serious medical threat, as they can form on medical devices and human tissues. *Pseudomonas aeruginosa* is a gram-negative bacterium that readily forms biofilms, and is a leading causative agent of nosocomial infections and death in cystic fibrosis patients. In addition to the ECM, two types of motility aid biofilm formation and virulence in *P. aeruginosa*: twitching motility and swimming motility. Also contributing to the virulence of *P. aeruginosa* is LasB elastase, an extracellular metalloprotease that is responsible for degrading human tissue. LasB elastase has recently been found to degrade a protein component of the ECM of *P. aeruginosa*. We investigated other possible cellular roles of LasB elastase in *P. aeruginosa* cells relating to biofilm formation, including motility and ECM production. We found that LasB elastase did not play a role in motility, but was essential for the normal protein composition of the ECM.

ACKNOWLEDGEMENTS

I owe gratitude and thanks to my advisor, Dr. Jayne Robinson, who provided me with guidance, creativity, and a research assistantship for this project. My special thanks to all of my committee members, Dr. Jayne Robinson, Dr. John Rowe, and Dr. Mark Nielsen. I would like to express my appreciation to them for their time, effort, and work in guiding my research and helping me throughout my thesis work. This could not have been completed without you. Also thanks to Dr. Mary Connolly, Tracy Collins, Paul DeLange, Amy Beumer, and Chris Moler. These intelligent, supportive, and caring previous and present coworkers in the lab made the past few years very enjoyable. They were also invaluable in providing sound advice and ideas. Thank you to the other faculty and staff in the biology department who have aided me along the way, especially Karen Bahr, Lynda Routley, and Kelly Bohrer. I am deeply grateful for having had the opportunity to know and work with all of you over the past years.

TABLE OF CONTENTS

Abstract.....	iii
Acknowledgements.....	iv
Table of Contents.....	v
List of Figures.....	vi
List of Tables.....	viii
Introduction.....	1
Literature Review.....	7
Methods.....	27
Results.....	40
Discussion.....	90
Appendix 1.....	102
References.....	103

LIST OF FIGURES

Figure 1. Biofilm formation by <i>Pseudomonas aeruginosa</i> (Dunne, 2002).....	4
Figure 2. Model of type IV pili assembly and retraction (Mattick, 2002).....	9
Figure 3. Chemotaxis genes in <i>P. aeruginosa</i> (Ferrandez <i>et al.</i> , 2002).....	11
Figure 4. Model of the main regulatory networks governing pilus biogenesis and twitching motility (Darzins, 1997).....	13
Figure 5. Population density-dependent gene regulation (Fuqua <i>et al.</i> , 2001).....	18
Figure 6. Model of the <i>P. aeruginosa</i> quorum-sensing hierarchy (McKnight <i>et al.</i> , 2000).....	21
Figure 7 . Elastase assay.....	55
Figure 8. Macroscopic stab assay for twitching motility.....	56
Figure 9. Macroscopic stab assay for swarming motility.....	57
Figure 10. Macroscopic stab assay for swimming motility.....	58
Figure 11. Rescue of mutant cells by wild-type cells.....	59
Figure 12. β -galactosidase assay for <i>lasB</i> expression.....	60
Figure 13. Western Blot analysis of surface pilin.....	61
Figure 14. Transmission electron micrographs of pili.....	62
Figure 15. Phage sensitivity assay.....	63
Figure 16. Transmission electron micrographs of pili-specific phage binding to <i>Pseudomonas aeruginosa</i> cells.....	64
Figure 17. Western Blot analysis of XcpT from isolated surface pilin.....	65
Figure 18. Western Blot analysis of XcpT from whole cell lysates.....	66
Figure 19. Western Blot analysis of XcpT from isolated surface appendages.....	67
Figure 20. Western Blot analysis of flagellin.....	68
Figure 21. Transmission electron micrographs of swarm cells and swim cells.....	69

Figure 22. Congo Red assay for ECM.....	70
Figure 23. Alcian Blue assay for ECM.....	71
Figure 24. Scanning electron micrographs of ECM.....	72
Figure 25. Macroscopic stab assay for swarming motility.....	73
Figure 26. Macroscopic stab assay for twitching motility.....	74
Figure 27. Rhamnolipid assay.....	75
Figure 28. AHL bioassays.....	76
Figure 29. Elastin plate assay.....	77
Figure 30. Elastase assay.....	78
Figure 31. PCR confirmation of 31878 (<i>lasB</i> transposon mutant).....	79
Figure 32. Macroscopic stab assay for twitching motility.....	80
Figure 33. Macroscopic stab assay for swarming motility.....	81
Figure 34. Western Blot analysis of surface pilin and XcpT.....	82
Figure 35. Rhamnolipid assay.....	83
Figure 36. AHL bioassay.....	84
Figure 37. 12% SDS-PAGE analysis of extracellular proteins.....	85
Figure 38. 7.5% SDS-PAGE analysis of extracellular proteins.....	86
Figure 39. 12% SDS-PAGE analysis of extracellular proteins.....	87
Figure 40. 15% SDS-PAGE analysis of extracellular proteins.....	88
Figure 41. 12% SDS-PAGE analysis of extracellular proteins.....	89
Figure 42. Colonization of <i>Candida albicans</i> by <i>Pseudomonas aeruginosa</i>	102

LIST OF TABLES

Table 1: Thermocycler settings.....	38
-------------------------------------	----

INTRODUCTION

Bacteria are known to live in two forms: attached to surfaces (sessile) and in suspension (planktonic). However, microorganisms have a stronger predisposition for being surface-bound. Early studies found that the majority of microbes in aquatic ecosystems are surface-bound rather than planktonic (ZoBell, 1943). This occurs in a wide range of ecosystems, and thus suggests that organisms attached to a surface may have advantages or greater survival over those in suspension (Costerton *et al.*, 1999; ZoBell, 1943).

When a community of microorganisms is attached to a surface the microbes form a biofilm. Biofilms have been described as “a community of microbes embedded in an organic polymer matrix, adhering to a surface” (Carpenter and Cerf, 1993). In nature biofilms are often composed of multiple species of microorganisms. The microbial cells stick to surfaces by means of an extracellular matrix (ECM), which they produce, and in which they are embedded (Deziel *et al.*, 2001). The ECM is composed of a variety of extracellular polysaccharides and proteins, and has often been referred to as “slime”. Once bacteria adhere to a surface they produce more ECM (Deziel *et al.*, 2001). Furthermore, bacterial cells can attach to surfaces that are either abiotic (inert materials) or biotic (living cells and tissues).

Microbial biofilms can be beneficial or detrimental to humans. Beneficial biofilms include those which are useful in nitrogen fixation, decomposition, and bioremediation of wastewater. However, even these can be damaging for many industries due to their corrosive nature. In addition, biofilms pose a serious medical threat, as they can form on medical devices, such as catheters, and thereby lead to infection of patients (Costerton *et al.*, 1999). Bacteria also form biofilms on or within human tissues, such as in the lungs of cystic fibrosis patients. Therefore, research into biofilm formation is necessary to gain a greater understanding of this mode of bacterial colonization and to combat the serious medical problems that stem from it.

Pseudomonas aeruginosa is a gram-negative bacterium that readily forms biofilms and gives rise to serious medical problems. It is a ubiquitous organism that is commonly found living in soil, water, and plants. As a pathogen, *P. aeruginosa* is opportunistic and a leading causative agent of nosocomial infections. It frequently infects immunocompromised patients, such as cancer and AIDS patients, and other individuals, such as those with burns or cystic fibrosis (Joklik *et al.*, 1992; Pollack, 1990; Smith *et al.*, 1996). *P. aeruginosa* forms copious biofilms in the lungs of cystic fibrosis patients and is often the ultimate cause of death in those individuals. At least one strain (PA14) of this bacterium has an unusually broad host range: causing infection and disease not only in humans but also mice, plants (*Arabidopsis*), nematodes (*C. elegans*), and insects (*Drosophila*) (Mahajan-Miklos *et al.*, 2000; Rahme *et al.*, 2000).

In addition to the ECM in *P. aeruginosa*, biofilm formation is aided by two types of cell surface appendages: Type IV pili (TFP) and flagella. These appendages are motorized structures found on the surface of a wide variety of bacteria. Flagella rotate

and propel cells through liquid environments (swimming motility), while pili extend and retract to pull cells along a solid surface (twitching motility). A third mode of motility, swarming, requires flagella, but is surface-associated (Kohler *et al.*, 2000; Rashid and Kornberg, 2000). Both flagella and pili are crucial for biofilm formation (O'Toole and Kolter, 1998). Biofilm formation involves a series of three steps as shown below in Figure 1 (Dunne, 2002). The first step in *P. aeruginosa* biofilm formation is adhesion whereby cells form a monolayer on the surface. The next step is aggregation, where cells migrate across the surface and congregate in microcolonies. The last step involves the increased production of exopolysaccharides by cells in the biofilm. Flagellar-dependent motility is necessary for the first step (primary adhesion) by propelling cells into close contact with the surface (Feldman *et al.*, 1998; Lawrence *et al.*, 1997; O'Toole and Kolter, 1998). Pili-mediated motility is necessary for the second step (aggregation). Cells migrate across the surface by twitching, which requires TFP and groups of cells (Semmler *et al.*, 1999). Pili are also important to biofilm formation because they act as adhesins promoting adherence of bacteria to both biotic and abiotic surfaces (Deziel *et al.*, 2001). Biofilm formation is reduced in *pilA* non-piliated mutants and enhanced in hyperpiliated, *pilT* mutants (Chiang and Burrows, 2003).

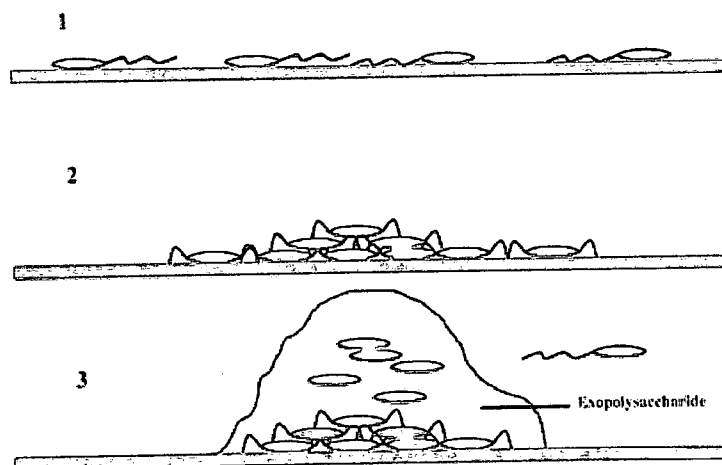


Figure 1. Biofilm formation by *Pseudomonas aeruginosa* (Dunne, 2002).

Recently, researchers discovered that twitching motility in *P. aeruginosa* is equivalent to social gliding motility (S-motility) in *Myxococcus xanthus* (Semmler *et al.*, 1999). As Type IV pili are required for twitching motility in *P. aeruginosa*, so is the case for S-motility in *M. xanthus* (Kaiser, 1979). In addition to Type IV pili, it has been shown that *M. xanthus* requires fibrils for S-motility (Yang *et al.*, 2000). Fibrils serve a role in coordinating movement during S-motility, although the mechanism is not known (Sun *et al.*, 1999). Fibrils are arrangements of the extracellular matrix, and thus are composed of extracellular polysaccharides and proteins (Behmlander and Dworkin, 1994). It has been shown that the polysaccharide component of the *M. xanthus* ECM mediates pilus retraction and S-motility (Li *et al.*, 2003). Besides their role in S-motility, fibrils also play a role in cell cohesion, linking neighboring cells to each other and to the surface they are gliding over (Yang *et al.*, 2000).

Besides twitching motility and TFP, *P. aeruginosa* and *M. xanthus* share several other similarities. Both bacteria produce an ECM that contains polysaccharides and extracellular metalloproteases, and both exhibit chemotaxis to dilauroyl phosphatidylethanolamine (Kearns *et al.*, 2001). FibA, a fibril-associated (ECM) protein in *M. xanthus*, shares a high level of sequence homology with LasB elastase in *P. aeruginosa* (Kearns *et al.*, 2002). Both proteins are metalloproteases that cleave a wide range of substrates.

Elastase, encoded by the *lasB* gene, contributes to virulence by degrading or inactivating tissue and immune system components. This important virulence factor is secreted via a type II secretion pathway analogous to the pathway used for type IV pilin secretion. Cells deficient in elastase production show reduced virulence (Cowell *et al.*, 2003).

LasB elastase is one of the major secreted proteins found in the ECM of *P. aeruginosa* (Moriwaka and Homma, 1985; Nicas and Iglewski, 1985; Tommassen *et al.*, 1992; Nouwens *et al.*, 2002; Nouwens *et al.*, 2003). Another major component of the ECM is CbpD, chitin-binding protein, with a high level of homology to polysaccharide-binding proteins (Foldes *et al.*, 2000). LasB has been found to act on the mature 43 kDa CbpD protein (CbpD-43) in the ECM, forming two degradation products (Foldes *et al.*, 2000).

We hypothesized that LasB elastase, with its similarities to FibA in *M. xanthus* and major presence in the ECM, may also play a role in motility, thus contributing to biofilm formation. Motility affects three important aspects of a bacterium's survival: acquisition of nutrients, avoidance of predation/stress, and dispersal of populations.

Recent evidence has led to a greater appreciation that motility, and the flagella and pili that are the agents of motility, play a central role in biofilm formation by *P. aeruginosa*. The formation and composition of these biofilm communities depend on the individual and collective abilities of their members to adhere, move, metabolize, replicate and finally detach and relocate. We also investigated the contribution of LasB elastase to the extracellular matrix, which is an essential component of biofilm formation. The results of this research could lead to a greater understanding of how LasB, an important virulence factor that is crucial in host tissue exploitation, also contributes to motility and extracellular matrix structure and function in *P. aeruginosa*. Such understanding may result in the following significant outcomes: a greater understanding of how *P. aeruginosa* controls motility and biofilm formation, and the development of rational mechanisms to control biofilm formation, and hence the pathogenicity of this bacterium.

LITERATURE REVIEW

Twitching motility and type IV pili (TFP)

Twitching motility is a means of rapid colonization of surfaces by bacteria and requires TFP (Kaiser, 1979; Semmler *et al.*, 1999). This type of motility is essential for colonial behavior, which includes the formation of biofilms and fruiting bodies, and colonization of hosts during infection (O'Toole and Kolter, 1998; Shi, *et al.*, 1996; Ward and Zusman, 1997; Watson *et al.*, 1996). The term "twitching" was first used to describe motility in *Acinetobacter calcoaceticus* (Lautrop, 1961). It is now known to occur in a wide range of microorganisms, including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Vibrio cholera*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*. *Myxococcus xanthus* also exhibits twitching motility, but in this organism it is referred to as social gliding motility, or S-motility (Semmler *et al.*, 1999).

Pseudomonas aeruginosa is the primary model for twitching motility studies. This microorganism is an opportunistic pathogen that is one of the common agents of infection in immunocompromised individuals (Bodey, 1983). Its hosts include animals and plants such as fruit flies, mice, and mustard plants. Important for infection is the ability of *P. aeruginosa* to form biofilms, and biofilm formation is dependent upon

twitching motility and type IV pili (Costerton, 1999; O'Toole and Kolter, 1998; Potera, 1999).

TFP are multifunctional structures known to mediate: 1) adhesion to host cells and inanimate surfaces 2) twitching motility 3) DNA uptake and 4) phage infection. TFP are considered virulence determinants: in mutants with pili that are rendered nonfunctional, infectivity of hosts is reduced (Comolli *et al.*, 1999; Hazlett *et al.*, 1991). Nearly 40 genes located in several clusters have been identified to be involved in the complex biogenesis and function of TFP of *P. aeruginosa* (Hobbs and Mattick, 1993).

Type IV pili, composed of polymerized PilA (or pilin) monomers, are 5-7 nm in diameter and several micrometers in length. The pilin subunits are arranged in a helical conformation with 5 subunits per turn. A short leader sequence with a positive charge exists on the pilin subunit as well as a hydrophobic amino terminus that forms the core of the pilus fiber. However, the leader sequence is proteolytically removed from the pilin, and the newly exposed N-terminal phenylalanine is methylated by PilD, a bifunctional enzyme that acts as both a peptidase and a methyltransferase (Nunn and Lory, 1991; Strom *et al.*, 1993). The general structure of TFP is conserved among bacteria that possess these structures (Hazes *et al.*, 2000; Keizer *et al.*, 2001). However, pilin undergoes posttranslational modifications in some species and strains, including *Pseudomonas aeruginosa* strain 1244 in which pilin is glycosylated (Castric *et al.*, 2001).

The assembly of type IV pili requires several proteins: pilin itself, PilD (peptidase and methyl transferase enzyme), minor pilin-like proteins, a nucleotide binding protein, an inner membrane protein, and an outer membrane protein (Alm and Mattick, 1997; Hobbs *et al.*, 1993; Lauer *et al.*, 1993; Lory and Strom, 1997; Russel, 1998; Strom *et al.*,

1993). Figure 2 depicts the model for type IV pili assembly (Mattick, 2002). PilD, which is necessary for pili assembly, cleaves the leader sequence and *N*-methylates the pilin as discussed earlier (Nunn *et al.*, 1990). Assembly of the processed pilin (PilA) then occurs on a base of the minor pilin proteins PilE, PilV, PilW, PilX, and FimU. (Alm *et al.*, 1996; Alm and Mattick, 1995; Alm and Mattick, 1996; Russell and Darzins, 1994). This assembly requires PilB, a traffic ATPase, and PilC, an inner membrane protein (Nunn *et al.*, 1990). The pilus is finally extruded through PilQ, a multimeric outer membrane protein that forms a gated pore in the outer membrane (Martin *et al.*, 1993).

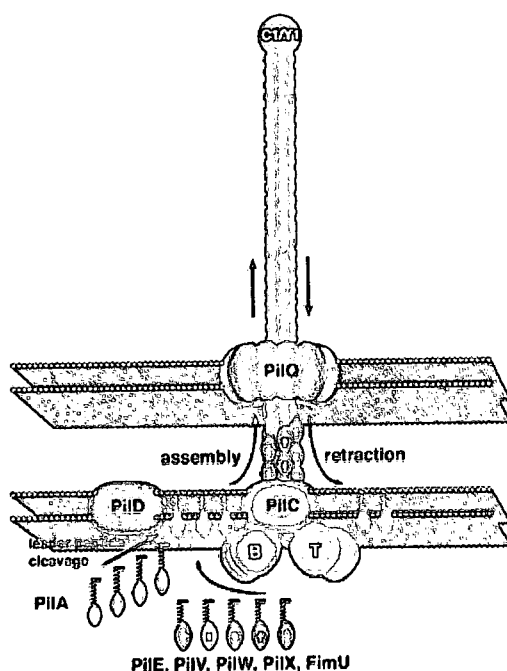


Figure 2. Model of type IV pili assembly and retraction (Mattick, 2002).

The mechanism of twitching motility is the retraction of type IV pili, first concluded by Bradley (1980), and recently confirmed by Merz *et al.* (2000) and Skerker and Berg (2001). The pili extend, attach at their distal tips, exert force (at least 10pN), and retract (Skerker and Berg, 2001). The rate of pili extension and retraction is approximately 0.5 $\mu\text{m}/\text{sec}$. Besides motility, pili retraction is also necessary for pili-specific bacteriophage infection (Bradley, 1980). Pili retraction is believed to occur through pilus filament disassembly mediated by PilT, an ATPase (Maier *et al.*, 2002; Merz *et al.*, 2000; Okamoto and Ohmori, 2002). PilT mutants are hyperpilated and deficient in twitching motility due to an inability to retract their pili (Bradley, 1980; Whitchurch *et al.*, 1991; Wolfgang *et al.*, 2000). Pilin degradation also relies on PilT (Wolfgang *et al.*, 2000).

Many genes have been implicated in the transcriptional regulation and chemosensory pathways that control TFP expression and twitching motility in *Pseudomonas aeruginosa*. PilS-PilR, a two-component sensor-regulator pair, controls transcription of *PilA*, and thus expression of pilin and twitching motility (Hobbs *et al.*, 1993). PilS is a sensory protein that is located at the pole of the cell in the inner membrane (Boyd and Lory, 1996; Boyd, 2000). It is believed that when PilS encounters an environmental signal, it autophosphorylates and transfers the phosphate to PilR, the response regulator (Figure 2, Darzins and Russell, 1997). Then PilR interacts with RpoN and RNA polymerase to activate transcription of *pilA*. The signal that PilS responds to is presently unknown.

Twitching motility in *Pseudomonas aeruginosa* is controlled by a chemosensory signal transduction system named *chp*, and also referred to as *pil*. *P. aeruginosa* possesses five chemosensory systems (Ferrandez et al., 2002; Stover et al., 2000; Croft et al., 2000) as depicted in Figure 3 (Ferrandez et al., 2002). The Cluster IV chemosensory system controls twitching motility and contains the *pilGHIJKchpABCDE* genes (Ferrandez et al., 2002). Two chemosensory systems control flagella rotation in swimming motility (*che*) (Kato, 1999; Masduki, 1995), and the other two have unknown functions. In the *che* system, methyl-accepting chemotaxis proteins (MCPs) induce autophosphorylation of CheA, a histidine kinase. CheA then phosphorylates CheY, a response regulator. CheY reverses the rotation direction of flagella. Some other proteins, such as CheW, CheR, CheB, and CheZ, modulate the methylation state of the MCPs, dephosphorylation of CheY, or act as adaptors between CheA and the MCPs. The *chp* chemosensory system is similar to the *che* system but is more complex. In the *chp* system there are three CheY-like response receiver domains, products encoded by the genes *pilG* and *pilH*, and the C-terminus of the protein encoded by *chpA* (Darzins 1993; Darzins, 1994). PilJ is the MCP, ChpA is the CheA homolog, and PilG and PilH are CheY homologs. *pilJ*, *chpA*, and *pilG* mutants are defective in twitching motility, while *pilH* mutants exhibit aberrant twitching motility (Darzins 1993; Darzins, 1994). The model for how the *pilGHIJK* gene cluster and *chpA* (also called *pilL*) controls twitching motility is shown in Figure 4 (Darzins, 1997). In this model, PilJ (an MCP in the inner membrane) senses an unknown environmental signal, which is followed by the activation of PilL (CheA) and the subsequent transfer of a phosphate from PilL to PilG and/or PilH.

Since PilG and PilH are CheY homologs and CheY directly controls flagellar rotation, it is thought that PilG and PilH may play similar roles in pili production and twitching motility.

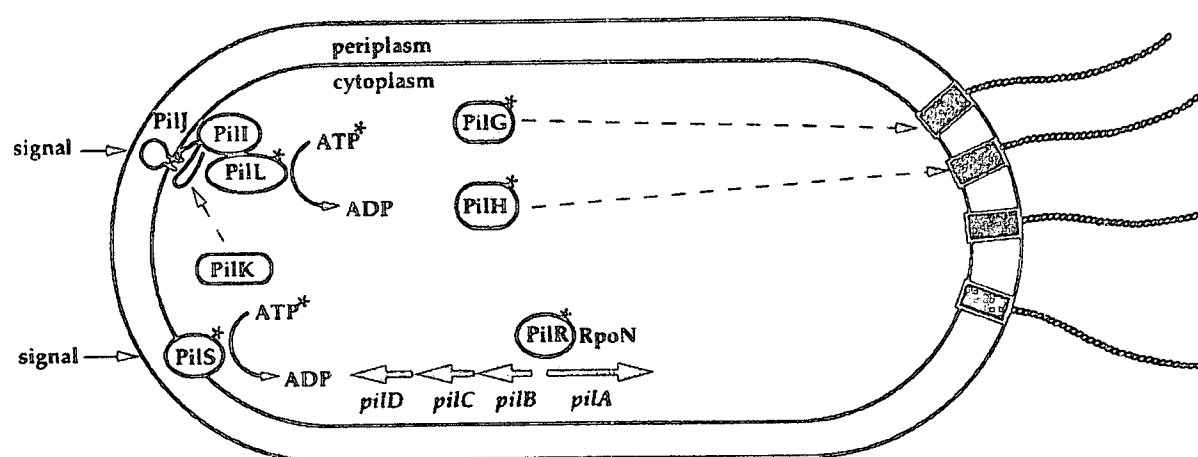


Figure 4. Model of the main regulatory networks governing pilus biogenesis and twitching motility (Darzins, 1997).

Quorum-sensing systems in *P. aeruginosa* play a role in twitching motility. It has been demonstrated that normal twitching motility is dependent upon the *las* and *rhl* quorum-sensing systems (Glessner *et al.*, 1999). Quorum-sensing controls the transcription of several virulence factors, and it is associated with high cell densities and cell-cell communication. Each system consists of an autoinducer synthase (LasI or RhII)

and a transcriptional activator (LasR or RhIR). The diffusible signaling molecule, the autoinducer, and the activator molecule bind to each other and activate transcription of the genes under control of the systems. Glessner *et al.* (1999) showed that *lasI* and *rhII* mutants that are unable to synthesize the autoinducers are defective in twitching motility.

Swimming motility, flagella, and chemotaxis

Swimming motility in *Pseudomonas aeruginosa* is a flagellum-dependent mode of motility for moving through liquid environments or a (less than 0.4%) agar medium. A single polar flagellum is accountable for this mode of motility. The flagellum operates by rotating clockwise or counterclockwise to propel cells through the aqueous environment and to move in specific directions. The direction of movement is instructed by chemotaxis to chemical stimuli (Masduki, 1995).

Chemotaxis is the movement of an organism toward chemical attractants and away from chemical repellants (Adler, 1966). The *che* genes are responsible for swimming chemotactic responses to chemical stimuli, as described above and depicted in Figure 2. A MCP functions as a receptor and binds a chemical attractant, triggering sensory signal transduction. CheA becomes phosphorylated and transfers a phosphate to CheY. CheY-P then interacts with the rotational “switch” protein, FliM, in the flagella motors and the flagella change direction and rotate clockwise. The cell “tumbles.” Without an attractant bound, flagella rotate counterclockwise to propel the cell in one

direction. The cell “runs.” When cells swim up a concentration gradient of an attractant, they run more than they tumble (chemotaxis).

Running and tumbling depends on the phosphorylation/dephosphorylation of CheY. CheA is responsible for phosphorylating CheY, while CheZ is responsible for dephosphorylating CheY. CheY also dephosphorylates itself. The system is reset by the MCPs’ methylation state. Even though the attractant is still present, MCP methylation will reset the signaling activity of the receptors and counterbalance the effect of the binding of the attractant. This guarantees continued movement of the cell up a concentration gradient of an attractant. A methyltransferase (CheR) adds methyl groups to MCPs, and a methylesterase (CheB) removes the methyl groups.

Swarming motility and flagella

It has recently been shown that flagella mediate swarming motility in addition to swimming motility in *Pseudomonas aeruginosa* (Rashid and Kornberg, 2000; Kohler *et al.*, 2000). However, swarmer cells are elongated and may contain two polar flagella (Rashid and Kornberg, 2000). Kohler *et al.* (2000) report that type IV pili are also necessary for swarming motility in *Pseudomonas aeruginosa* due to their *pilA* mutant being deficient in swarming motility. However, Rashid and Kornberg (2000) report that their *pilA* mutant was not defective in swarming motility, indicating that pili may not be essential for swarming motility.

Recent reports indicate factors that may play a role in swarming motility. It has been indicated that swarming motility is regulated by nitrogen availability, and is dependent on the *las* and *rhl* quorum-sensing systems (Kohler *et al.*, 2000). Rhamnolipids, synthesized by *rhlAB*, have been shown to be essential for swarming (Kohler *et al.*, 2000). Several species of bacteria secrete a surfactant that is associated with swarming, including *Proteus mirabilis* and *Serratia liquefaciens* (Gygi *et al.*, 1995; Lindum *et al.*, 1998). Quorum-sensing has also been implicated in controlling swarming motility in *Serratia liquefaciens* (Eberl *et al.*, 1996). More recently, a study in which autoinducers were degraded in *P. aeruginosa* showed that both swarming motility and virulence gene expression were reduced, signifying the importance of quorum-sensing in swarming motility by *P. aeruginosa*. (Reimmann *et al.*, 2002).

Quorum-sensing

Quorum-sensing (QS) in bacteria involves the regulation of genes and behaviors in response to changes in the local number and proximity of siblings and allows cooperation among members of a community (Miller and Bassler, 2001; Whitehead *et al.*, 2001). QS is dependent on the synthesis, exchange and perception of small signal molecules between bacteria. These QS signals typically activate specific receptors that function as transcriptional regulators. In gram-negative bacteria, which are responsible for most plant and animal diseases, the most common QS signals are *N*-acyl homoserine lactones (AHLs) (Whitehead *et al.*, 2001). Figure 5 depicts the QS paradigm for control

of gene expression (Fuqua *et al.*, 2001). Random mutagenesis revealed that over 250 different genes were affected by the AHL-mediated QS in *Pseudomonas aeruginosa* (Rahme *et al.*, 2000; Whiteley *et al.*, 1999). Recent transcriptome analyses indicate that 6 to 10% of the *P. aeruginosa* genome is affected by QS (Schuster *et al.*, 2003; Wagner *et al.*, 2003). Thus, AHL signaling is both common among relevant bacteria and can affect many aspects of their growth, survival and interactions with other species of bacteria. Most bacteria in the environment however coexist with other species of bacteria, and there is also evidence for interspecies communication (McKenney *et al.*, 1995).

One of the first organisms discovered to use quorum-sensing was *Vibrio fischeri*, a marine bacterium that produces light. Initial research in quorum-sensing focused on this microorganism. *V. fischeri* produces an autoinducer that regulates luciferase, a light producing enzyme (Nealson, 1970). Acyl-HSL quorum sensing in *V. Fischeri* relies on the synthesis of the autoinducer by the LuxI protein, an acyl-HSL synthase. The acyl-HSLs produced leave the cell by diffusion down their concentration gradient and accumulate in the environment. The concentration of acyl-HSLs in the environment increases during growth of bacterial populations (see Figure 5, Fuqua, 2001). Therefore, the concentration of acyl-HSLs reflects cell density. Once the concentration of acyl-HSLs reaches a critical threshold, the acyl-HSLs subsequently interact with transcription regulators, LuxR proteins, and the complex activates transcription of genes that are regulated by quorum-sensing. One of the target genes is LuxI, which creates a positive feedback circuit. Another target is the gene encoding luciferase, thus the bacteria only synthesize luciferase and produce light when cell density is high.

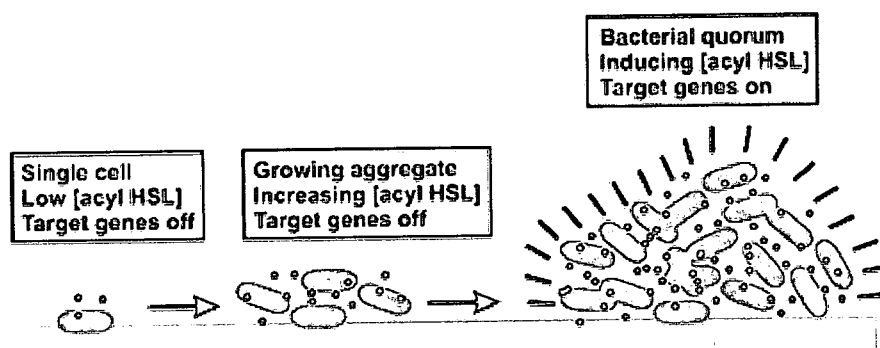


Figure 5. Population density-dependent gene regulation (Fuqua *et al.*, 2001).

Quorum-sensing plays a role in the formation of biofilms in *Pseudomonas aeruginosa* (Davies *et al.*, 1998). Virulence factors controlled by QS include LasB (elastase), LasA (protease), pyocyanin, hydrogen cyanide, alkaline protease and exotoxin A (Parsek and Greenberg *et al.*, 2000). A *rhlR* mutant displays reduced virulence (Tang *et al.*, 1996). In pathogenic microorganisms such as *P. aeruginosa*, quorum sensing may provide early protection in the host because it delays the host defense response by delaying the production of quorum-sensing controlled virulence factors until high cell densities are reached (Costerton *et al.*, 1999). QS also influences the ability of bacteria to form surface-associated biofilms (Davies *et al.*, 1998). There are two quorum-sensing systems in *P. aeruginosa*, the *las* and *rhl* systems (Figure 6). The AHL synthases, or LuxI homologues, are LasI and RhII, and the corresponding regulatory proteins that the

autoinducers bind to, or LuxR homologues, are LasR and RhlR, respectively (Gambello and Iglewski, 1991; Latifi *et al.*, 1995; Ochsner and Reiser, 1995; Passador *et al.*, 1993). LasI is responsible for the synthesis of the autoinducer, *N*-(3-oxododecanoyl)-L-homoserine lactone, or 3-oxo-C12-HSL (Passador *et al.*, 1993; Pearson *et al.*, 1994). RhlI is responsible for the synthesis of the autoinducer, *N*-(butanoyl)-L-homoserine lactone, or C4-HSL (Pearson *et al.*, 1995; Winson *et al.*, 1995). *P. aeruginosa* virulence can be attenuated by the inhibition of QS (Hentzer *et al.*, 2003).

The two quorum-sensing systems, *las* and *rhl*, form a regulatory hierarchy (Figure 5). LasR and the LasI autoinducer (3-oxo-C12-HSL) activate *rhlR* transcription (Latifi *et al.*, 1996; Pesci *et al.*, 1997). LasR and 3-oxo-C12-HSL also regulate the synthesis of *Pseudomonas* quinolone signal (PQS) (Pesci, 1999; McKnight *et al.*, 2000). PQS (2-heptyl-3-hydroxy-4-quinolone) is a second type of signaling molecule. It is involved in regulating the transcription of *rhlR*, *rhlI*, and *lasB* genes (Pesci, 1999; McKnight *et al.*, 2000). However, PQS is not considered to be involved in quorum-sensing because it is not dependent on cell density, as its concentration peaks during late stationary phase (McKnight *et al.*, 2000). Later studies identified the PQS structural genes, transcriptional regulator, and response effector (Gallagher *et al.*, 2002). The PQS structural gene *pqsH* is regulated by the *las* QS system. Thus, the regulation of *lasB* is complex requiring both an intact *las* QS system as well as PQS (Calfee *et al.*, 2001; McKnight *et al.*, 2000).

Several studies on the global regulation of quorum-sensing have been performed. It has been found that a CRP homologue, Vfr, controls *lasR* expression (Albus *et al.*, 1997). GacA, a response regulator, is also essential for *lasR* expression, as well as for the full synthesis of C4-HSL (Reimann, 1997). C4-HSL production is negatively regulated

by RpoS, a stationary-phase sigma factor (Whiteley *et al.*, 2000). A second sigma factor, RpoN, is also a regulator of *rhII* transcription (Thompson *et al.*, 2003; Heurlier *et al.*, 2003). Another negative regulator of quorum sensing is RsaL, which represses *lasI* transcription (DeKievit *et al.*, 1999). The LuxR homologue QscR regulates the expression of several quorum-sensing genes including *lasI* and *rhII* (Chugani *et al.*, 2001). The synthesis of QscR in turn is regulated by GacA (Ledgham *et al.*, 2003). The expression of *lasI* and *rhII* is also regulated postranscriptionally by the global posttranscriptional regulators RsmA and DksA (Pesci *et al.*, 2001; Kohler *et al.*, 2003). In addition, MvaT, is a global regulator of virulence gene expression, as well as AlgR2, which negatively modulates *lasR* and *rhIR* expression (Diggle *et al.*, 2002; Ledgham *et al.*, 2003).

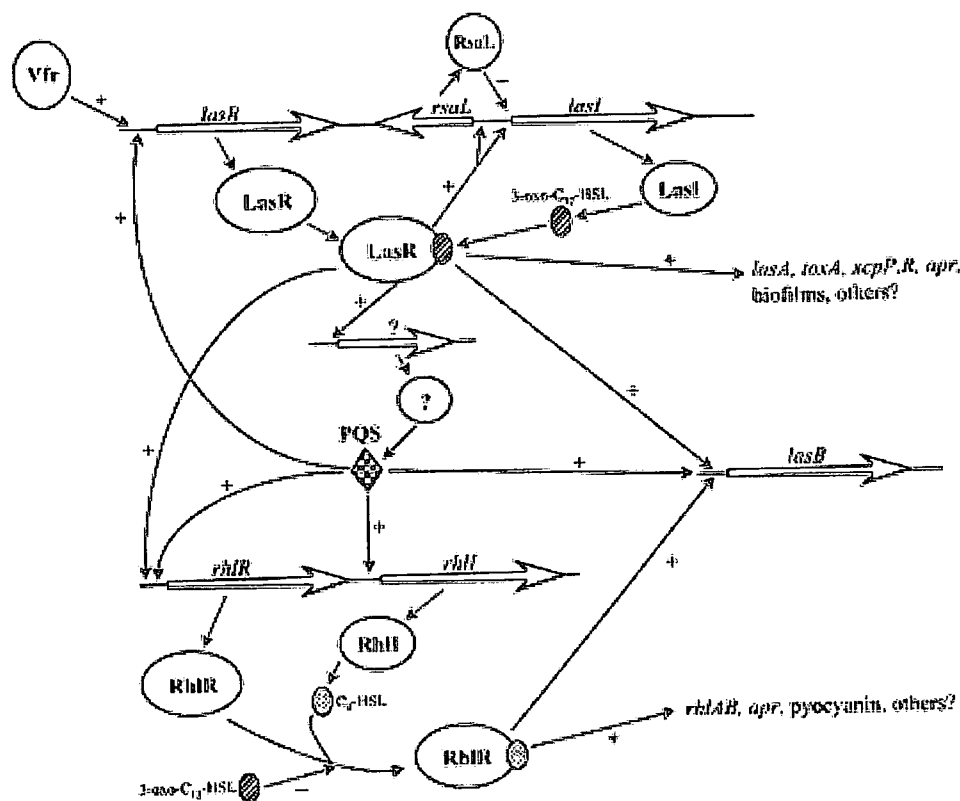


Figure 6. Model of the *P. aeruginosa* quorum-sensing hierarchy (McKnight *et al.*, 2000).

LasB elastase and Type II Secretion System (TTSS)

Elastase, encoded by the *lasB* gene, is a virulence factor that is regulated by QS and PQS as mentioned above. *P. aeruginosa* cells deficient in LasB elastase production show decreased virulence (Cowell *et al.*, 2003). A high throughput screening of a collection of 7968 mutants of *P. aeruginosa* in a rat model of chronic respiratory

infection showed that a *lasB* mutant failed to cause infection and could not survive in the lung (Potvin *et al.*, 2003).

Elastase is synthesized when cells are in late logarithmic phase of growth or when cell density is high. Elastase contributes to the virulence of *P. aeruginosa* due to its function as a metalloprotease and its ability to degrade or inactivate tissue and immune system components. The tissue components that elastase degrades include elastin, collagen, and fibrin (Moriyama, 1964; Wremland and Wadstrom, 1977; Heck *et al.*, 1986). The immune system components that elastase inactivates are immunoglobulin, serum complement factors, gamma interferon and tumor necrosis factor cytokines (Doring *et al.*, 1981; Heck *et al.*, 1990; Hong and Ghebrehewet, 1992; Schultz and Miller, 1974; Parmely *et al.*, 1990).

Elastase is synthesized as a preproenzyme, with the “pre-” part acting as a signal peptide (Bever and Iglewski, 1988). It is an extracellular protein, so it must be translocated across both the inner and outer membrane. The N-terminal signal peptide mediates translocation across the inner membrane into the periplasmic space. After entering the periplasm, the protein proceeds to fold with the aid of its propeptide which functions as an intramolecular chaperone (Braun *et al.*, 1996; McIver *et al.*, 1995). It is then processed by autoproteolytic cleavage before being secreted (McIver *et al.*, 1991). LasB is retained in the periplasm in an active oligomeric form in mucoid cells, and to a lesser extent in nonmucoid cells (Filloux *et al.*, 1998). Elastase is translocated across the outer membrane via the type II general secretion system (TTSS). This secretion pathway is similar to and overlaps with the system that operates pilin secretion.

In *P. aeruginosa* there are two type II systems (TTSS): Xcp and Hxc. Proteins encoded by the *xcp* genes mediate the secretion of LasB elastase across the outer membrane (Filloux *et al.*, 1990; Gustin *et al.*, 1996). Both the propeptide and mature elastase are secreted, and the propeptide is extracellularly degraded (Braun *et al.*, 1998). Besides elastase, alkaline phosphatase, exotoxin A, lipases, and phospholipase C are secreted via the Xcp transport system (Filloux *et al.*, 1998). Alkaline phosphatase LapA is secreted via the Hxc transport system (Ball *et al.*, 2002).

The TFP assembly machinery is similar to the Xcp transport machinery. In fact, these two systems share a common component: PilD, also called XcpA (Bally *et al.*, 1992; Nunn and Lory, 1993). This protein has two catalytic functions: as a peptidase and transmethylase that processes pilin (PilA) by cleaving and methylating the pilins before they are assembled into a pilus (Nunn and Lory, 1991). Other similar components between pilus assembly and type II secretion include the secretins, PilQ and XcpQ, and ATPases, PilT and XcpR (Bitter and Tommassen, 1999; Planet *et al.*, 2001). Also, the pilin subunit, PilA, shares homology with the Xcp pseudopilins, XcpT, XcpU, XcpV, XcpW, and XcpX (Bally *et al.*, 1992; Bleves *et al.*, 1998; Nunn and Lory, 1993). Besides homology among components of the TFP assembly pathway and the Xcp secretion pathway, it has been found that the two systems have some interaction. Hong-Mei *et al.* (1997) discovered that PilA is required for efficient secretion of exoenzymes by the Xcp secretion system, and that XcpT can form heterodimers with PilA. Recently it was shown that when overexpressed, XcpT pseudopilins are assembled into a pilus-like structure that is called the type II pseudopilus (Durand *et al.*, 2003). The pseudopilus is not made of a single filament, rather it is a bundle of fibrils with a diameter similar in size

to pili (7nm and 5.2nm, respectively) and has similar adhesive capabilities (Durand *et al.*, 2003).

Myxococcus xanthus S-motility and the Extracellular Matrix (ECM)

Twitching motility in *P. aeruginosa* is equivalent to social gliding motility (S-motility) in *Myxococcus xanthus* (Darzins, 1994; Semmler *et al.*, 1999). TFP are required for both twitching motility in *P. aeruginosa* and S-motility in *M. xanthus* (Kaiser, 1979; Kaiser and Crosby, 1983; Rosenbluh and Eisenbach, 1992; Wu and Kaiser, 1995). In addition to TFP, *M. xanthus* requires fibrils for S-motility (Yang *et al.*, 2000). Fibrils are arrangements of the extracellular matrix (ECM), and thus are composed of extracellular polysaccharides and proteins (Behmlander and Dworkin, 1994). Besides their role in S-motility, fibrils also play a role in cell cohesion, linking neighboring cells to each other and to the surface they are gliding over (Yang *et al.*, 2000).

Fibril-deficient mutants of *M. xanthus* are hyperpiliated and defective in S-motility (Sun *et al.*, 1999). Recently, it was shown that it is the polysaccharide, and not the protein, component of the *M. xanthus* ECM that mediates pilus retraction, and that S-motility and normal piliation is restored upon the addition of chitin, a homopolymer of *N*-acetylglucosamine (Li *et al.*, 2003). Interestingly, S-motility in wild-type cells was inhibited by *N*-acetylglucosamine monomers. Li *et al.* (2003) suggests that specific

amino-substituted polysaccharides in the ECM are bound by specific receptors on TFP and form the basis for pilus retraction, and therefore S-motility in *M. xanthus*.

P. aeruginosa and *M. xanthus* share many similarities in addition to twitching motility and TFP. Both bacteria produce an ECM that contains polysaccharides and extracellular metalloproteases and both exhibit chemotaxis to dilauroyl phosphatidylethanolamine (PE), a component of all cell membranes (Kearns *et al.*, 2001). In *M. xanthus*, FibA, a fibril-associated ECM protein, is required for chemotaxis to PE (Kearns *et al.*, 2002). FibA shares a high level of sequence similarity (50%) with LasB elastase in *P. aeruginosa*, and both of these metalloproteases cleave a wide range of substrates.

P. aeruginosa ECM and LasB elastase

The ECM or EPS (extracellular polymeric substance) of *Pseudomonas aeruginosa* has not been fully characterized. Alginate and other polysaccharides are thought to be major components of the ECM, yet a recent report shows no substantial differences in biofilm architecture between wild-type and *algD* mutants of *P. aeruginosa* strains PAO1 and PA14 (Wozniak *et al.*, 2003). A chemical analysis of the EPS of *P. aeruginosa* biofilms revealed the primary carbohydrates present in strains PAO1 and PA14 to be glucose, rhamnose and mannose, along with *N*-acetyl sugars and KDO (Wozniak *et al.*, 2003). In addition to polysaccharides the ECM of *P. aeruginosa*

contains excreted and secreted cellular products, including a variety of proteins as well as nucleic acids (Sutherland, 2001).

LasB elastase is one of the major secreted proteins found in the ECM of *P. aeruginosa* (Moriyama and Homma, 1985; Nicas and Iglewski, 1985; Tommassen *et al.*, 1992; Nouwens *et al.*, 2002; Nouwens *et al.*, 2003). Another major component of the ECM is CbpD, chitin-binding protein, with a high level of homology to polysaccharide-binding proteins (Folders *et al.*, 2000). The *lasB* and *cbpD* genes are both regulated by QS, and both proteins are secreted through the Xcp system. LasB has been found to act on the mature 43 kDa CbpD protein (CbpD-43) in the ECM, forming a major product of 30 kDa (CbpD-30), and a minor product of 23 kDa (CbpD-23) (Folders *et al.*, 2000). The mature form of CbpD was found to bind chitin, and the formation of this enzyme-substrate complex protects CbpD from action by LasB. CbpD-30 and CbpD-23, however, are unable to bind chitin and are proposed to be degradation products. In the absence of chitin, the major forms of CbpD found in the ECM are the 30 kDa and 23 kDa forms. Interestingly, CbpD was found in the ECM of clinical isolates, but not soil isolates, of *P. aeruginosa* (Folders *et al.*, 2000).

If *P. aeruginosa* requires a specific polysaccharide in the ECM for pilus anchoring and retraction like *M. xanthus*, any alterations in the structure and composition of the ECM can be expected to affect twitching motility. It seems probable that the absence of an important protein such as LasB, which is known to have multiple substrates in the ECM, would have significant effects on the ECM and therefore affect twitching motility.

METHODS

Elastolysis Assay

An assay of elastase activity was performed as described previously (Bjorn *et al.*, 1979) with modifications (Pearson *et al.*, 1997). *P. aeruginosa* cells were grown overnight in PTSB (5% peptone, 0.25% tryptic soy, pH 7.0) with shaking at 37°C. Cells were subcultured (1% inoculum) and grown to mid-log phase in PTSB ($OD_{600nm} = 0.9-1.0$), washed and resuspended in PTSB to OD_{600nm} of 0.05. Cell suspensions were incubated with shaking at 37°C, and culture supernatants were filtered (0.2µm pore size). Triplicate 50ul samples of culture filtrates were added to tubes containing 20mg Elastin-Congo Red (ECR) and 1ml Tris buffer (0.1M Tris pH 7.2, 1mM $CaCl_2$). Tubes were incubated for 18 hours at 37°C with rotation, then 0.1ml of 0.12M EDTA solution was added, and tubes were placed on ice. Insoluble ECR was removed by centrifugation at 10,000 x g for 10 minutes. Supernatants were used to measure the OD at 495nm. A red colored supernatant indicated elastase activity.

Elastin plate assay

LB (1.5% agar) plates were overlaid with 5ml of 0.5% elastin, 0.8% Nutrient Agar mixtures and solidified at room temperature overnight (Toder *et al.*, 1991). The following day, elastin agar plates were streaked with *P. aeruginosa* wild-type and mutant cultures. Clearing zones surrounding bacterial growth indicate elastase activity.

Complementation of *lasB* mutant strains

The *lasB* complementing plasmid, pRB1804SF, described by Toder *et al.* (1991) was isolated from *E.coli* TB1 using QIAprep Spin Miniprep Kit (Qiagen), and confirmed by agarose gel electrophoresis. *P. aeruginosa* mutant cells grown overnight, subcultured the following day, and grown to early log phase were prepared for transformation by centrifuging cells at 10,000 x g and resuspending in 300mM sucrose 3 times. A 40µl volume of mutant cells was transformed by electroporation with 5ul DNA (isolated pRB1804SF) solution. Cells were incubated at 37°C in 3ml LB broth for 2 hours before plating on selective media (LB plates containing 200 µg/ml carbenicillin) to select for *lasB* mutant cells that had been complemented with pRB1804SF, the *lasB* complementing plasmid containing a carbenicillin resistance marker.

Motility Assays

LB (1.0% agar) plates were stab inoculated with *P. aeruginosa* wild-type or mutant cells and incubated at 37°C for 24 hours to determine twitching motility. Agar (0.5%) plates containing 8g/L Nutrient Broth and 5g/L glucose were stab inoculated with *P. aeruginosa* cells and incubated at 37°C for 24 hours to determine swarming motility. LB (0.3% agar) plates were stab inoculated with *P. aeruginosa* cells and incubated at 37°C for 24 hours to determine swimming motility. Twitching motility was also visualized at the microscopic level. Plate grown *Pseudomonas aeruginosa* cells were point inoculated on a block of LB agar (1.0%) on microscope slides, covered with a coverslip, and incubated at 37°C for 4-5 hours in a petri dish as previously described (Glessner *et al.*, 1999). Cells were visualized using brightfield microscopy (60x objective lens).

Twitching motility rescue assay

P. aeruginosa cells (wild-type, mutant, and wild-type expressing GFP) were grown overnight in LB broth and diluted the following day to an OD_{600nm} of 0.3. Equal numbers of mutant cells and wild-type expressing GFP cells were mixed, as well as wild-type and wild-type expressing GFP cells for a control. A 1μl volume of the cell mixtures was placed on LB (1% agar) squares positioned on microscope slides. The cell mixtures were covered with coverslips and incubated at 37°C for 5 hours, and the twitch zone edges were viewed using fluorescence and brightfield microscopy (60x objective lens).

β -galactosidase assay for *lasB* expression

Wild-type cells containing the *lasB::lacZ* fusion were grown overnight in LB broth. Cells were subcultured the following day and grown to an optical density (OD_{600nm}) of 0.6. Triplicate tubes were prepared with Z Buffer, SDS, and chloroform, and were vortexed for 10 seconds before the addition of samples. Tubes were equilibrated at 28°C for 5 minutes before adding ONPG to each tube. Na_2CO_3 was added to the first tube of each set to stop the reaction when it turned pale yellow in color, and the time was recorded. The other 2 tubes of each set were allowed to react for twice that amount of time. Tubes were centrifuged for 5 minutes at maximum speed, and the OD_{420nm} was determined and converted to β -galactosidase (Miller) units.

Pili Isolation and Purification

P. aeruginosa wild-type and mutant cells were grown on LB plates overnight at 37°C, resuspended in water to an OD_{600nm} of 5.0, and vortexed vigorously for 45 seconds to release pili. Cells were removed by centrifugation twice at 2000 x g for 10 minutes. Supernatants were diluted with 1 X PBS to a volume of 20 ml and centrifuged at 12,000 x g for 20 minutes, then the supernatant was filtered (0.2 μ m pore size), equilibrated to pH 4.5 with 0.1M NaOAc (pH 3.9), and incubated for 2 hours on ice. Pili were recovered by centrifugation at 12,000 x g for 20 minutes. Pellets were resuspended in 0.1 ml of 1 X PBS, and analyzed on 12% SDS polyacrylamide gels (BioRad).

Western Blot analysis of pilin

Following SDS-PAGE, purified pili samples were transferred to a nitrocellulose membrane by electroblotting at 100V for 15 minutes. Blots were washed twice with water and incubated in Blocking Solution (1% BSA in TBST) for 30 minutes. A 1/4000 dilution of primary antibody (rabbit anti-pilin antibody) was added to the blocking solution, and the membrane was incubated for 60 minutes, then washed three times in TBST (0.05% Tween in TBS). The membrane was incubated with a 1/5000 dilution of secondary antibody (AP-conjugated anti-rabbit antibody) in blocking solution for 30 minutes, washed three times in TBST, and rinsed in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl). Bands indicating pilin were revealed by using Western Blue substrate (Promega).

Phage Sensitivity Assay

Pseudomonas aeruginosa cells were grown overnight in LB broth. 100µl of cells were added to melted LB (0.7% agar) and overlayed onto LB (1.5% agar) plates. The plates were dried for 20-30 minutes, and then 4µl of phage were spotted onto the overlay containing bacterial cells and dried for 10 minutes. The plates were incubated at 37°C overnight, and the presence of clear plaques the following day indicate infection and lysis of bacterial cells.

Microscopic observation of fluorescent-labeled phage

Phage (10^{10} - 10^{11} p.f.u./ml) were labeled with YO-PRO-1 iodide fluorescent dye (Molecular Probes, Inc.) by mixing 20 μ l of phage with 1 μ l of dye (1mM in DMSO) and incubating for 2 days in the dark at 4°C. *Pseudomonas aeruginosa* cells were grown overnight in LB broth or swarm plates and then suspended in LB to OD_{600nm}=0.5. Phage were diluted 1:50, and 5 μ l of phage were mixed with 5 μ l of cells on a glass microscope slide. Cells and phage were visualized using fluorescence microscopy (60x objective lens).

Surface appendage isolation

Wild-type and mutant cells were grown overnight on LB (1% agar) plates. Cells were harvested and suspended in CTX + 10mM MgCl₂. The suspension was passed through an 18-gauge needle of a syringe to shear off the surface appendages. The suspension was centrifuged twice at 15,000rpm for 15 minutes to remove bacterial cells from the surface-appendage enriched supernatant.

Whole cell lysate preparation

Wild-type and mutant cells were grown overnight on LB (1% agar) plates. Cells were harvested and suspended in CTX + 10mM MgCl₂. Cell suspensions were centrifuged at 10,000 x g for 5 minutes, and cells were washed 3 times in CTX + 10mM MgCl₂. Cells were resuspended in CTX + 10mM MgCl₂ and boiled for 10 minutes.

Western Blot analysis of XcpT

Following SDS-PAGE, purified pili samples, surface appendage samples, or whole cell lysate samples were transferred to a nitrocellulose membrane by electroblotting at 100V for 15 minutes. Blots were washed twice with water and incubated in Blocking Solution (1% BSA in TBST) for 30 minutes. A 1:5000 dilution of primary antibody (rabbit anti-XcpT antibody) was added to the blocking solution, and the membrane was incubated for 60 minutes, then washed three times in TBST (0.05% Tween in TBS). The membrane was incubated with a 1:5000 dilution of secondary antibody (AP-conjugated anti-rabbit antibody) in blocking solution for 30 minutes, washed three times in TBST, and rinsed in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl). Bands indicating XcpT were revealed by using Western Blue substrate (Promega).

Western Blot analysis of flagellin

Pseudomonas aeruginosa cells were grown overnight on LB (1.5% agar) plates and resuspended in CTX+0.005M MgCl₂. Cells were collected by centrifugation at 10,000 x g for 5 minutes and washed 3 times in CTX+0.005M MgCl₂. Washed cells were solubilized in Laemmli buffer and boiled for 10 minutes, then separated on 12% SDS polyacrylamide gels (Biorad). Proteins were transferred to a nitrocellulose membrane by electroblotting at 100V for 50 minutes. That membrane was washed twice with water and incubated in Blocking Solution (1% BSA in TBST) for 30 minutes. A 1/50,000 dilution of primary antibody (rabbit anti-flagellinB antibody) was added to the blocking solution, the membrane was incubated for 60 minutes, then washed three times in TBST (0.05% Tween in TBS). The membrane was incubated with a 1/5000 dilution of secondary antibody (AP-conjugated anti-rabbit antibody) in blocking solution for 30 minutes, washed three times in TBST, and rinsed in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl). Bands indicating flagellin were revealed by using Western Blue substrate (Promega).

Transmission Electron Microscopy

To observe flagella, *Pseudomonas aeruginosa* cells were grown overnight on swarm plates or in LB broth. EM grids were dipped into cells, washed three times with water, and stained with 0.5% phosphotungstic acid for 50 seconds. The grids were examined in

the TEM. To observe pili, blocks of 1.0% agar were stab inoculated with *Pseudomonas aeruginosa* cells, covered with a coverslip, and incubated at 37°C for 4 hours. EM grids were prepared same as above. To observe PO4 pili-specific phage binding, *Pseudomonas aeruginosa* cells were grown overnight on agar (1.5%) plates and suspended in LB to $OD_{600nm}=0.5$. Phage were diluted 1:50 and equivalent amounts of phage and cells were mixed. EM grids were prepared the same as above.

Congo Red Assay

LB (1.0% agar) plates containing 10 µg/ml Congo Red were stab inoculated with *Pseudomonas aeruginosa* cells and incubated at 37°C for 48 hours. The following day, coloration of bacterial colonies was observed. Red coloration indicates binding of Congo Red by cells.

Alcian Blue Assay

LB (1.0% agar) plates containing 0.2g/L Alcian Blue were stab inoculated with *Pseudomonas aeruginosa* cells and incubated at 37°C for 48 hours. The following day, coloration of bacterial colonies was observed. Blue coloration indicates binding of Alcian Blue by cells.

Scanning Electron Microscopy

Pseudomonas aeruginosa cells were grown on LB plates overnight and resuspended in LB to an OD_{600nm} of 0.6 - 0.8. The cells were washed in 10 mM MOPS buffer and resuspended in agglutination buffer (10 mM MOPS pH 6.8, 10 mM MgCl₂, 1 mM CaCl₂). Cell suspensions were collected by vacuum filtration onto a Nuclepore polycarbonate membrane (48mm diameter, 0.2µm pore size). The membrane was saturated in primary fixative (3% glutaraldehyde in 0.1M sodium cacodylate, 500ppm Ruthenium Red) for 1 hour, rinsed 4 times in 0.1M sodium cacodylate for 2 minutes each rinse, and saturated in post fix (2% osmium tetroxide in 0.1M sodium cacodylate) for 90 minutes. The cells were dehydrated with sequential washes in 30, 50, 70, 90, and 100% ethanol, dried with hexamethyldisilazane:ethanol (50:50) for 15 minutes, and then 100% HMDS for 20 minutes. The membrane was coated with gold (15 seconds, 45milliamps) and examined in the SEM

Rhamnolipid assay

Plates for detecting rhamnolipids were prepared as described by Kohler *et al.* (2000). The medium was prepared by supplementing M8 salts with 0.2% glucose, 2 mM MgSO₄, trace elements, 0.0005% methylene blue, 0.02% cetyltrimethylammonium bromide, and 0.05% glutamate. 1.6% agar was used to solidify the medium. Plates were spotted with *P. aeruginosa* cells grown overnight on LB (1.5% agar) plates, and were incubated at

37°C for 24 hours, followed by incubation at room temperature for 2-5 days. The appearance of a blue halo surrounding the inoculation site indicates the production of rhamnolipids.

Acyl-homoserine lactone (AHL) extraction

P. aeruginosa cells were grown overnight, subcultured the following day, and grown to an OD_{590nm} of 0.9. To stop growth, 10ml acidified ethyl acetate was added to each tube. Ethyl acetate AHL-containing supernatants were extracted and extracted 2 more times with 2x volumes of acidified ethyl acetate. Any water in the samples was precipitated with sodium sulfate. The ethyl acetate containing AHLs was evaporated completely with argon gas and stored at -20°C.

Detection of AHLs using bioreporters

The dried AHL extracts were dissolved in 200µl ethyl acetate and spotted on thin-layer chromatography (TLC) plates. TLC plates were run in 70% methanol/30% water solvent to separate the AHLs, and the plates were dried for 10 minutes. TLC plates were overlayed with agar-bioreporter mixtures and incubated overnight at 30°C. *Chromobacterium violaceum*, *Agrobacterium tumefaciens*, and *Aeromonas hydrophila* 536 cultures were used as bioreporters. AHLs are indicated by the production of colored

pigments (*Chromobacterium violaceum* and *Agrobacterium tumefaciens*) or bioluminescence (*Aeromonas hydrophila* 536) in the presence of specific AHLs. *Aeromonas hydrophila* overlays were exposed to film for 15 seconds to detect bioluminescence.

Polymerase Chain Reaction confirmation of *lasB* transposon mutant

The forward (genomic) primer was designed to the *lasB* gene sequence (5'-tttctacgcttgacctgtgttcg-3'), and the reverse (transposon) primer was designed to the *phoA* transposon sequence (5'-cgggtgcagtaatatcgccct-3'). Tubes were prepared with forward and reverse primers (1.5ul), dNTPs (1.5ul), dH₂O (83ul), and Taq buffer (10ul), and were heated to 95°C in thermocycler. A colony of 31878 (*lasB* transposon mutant) was added to each tube, except the negative control tube. 1ul Taq polymerase was added to each tube, and PCR was performed as described in Table 1. PCR products were analyzed by gel electrophoresis (0.8% agarose gel, 70V) and detected with ethidium bromide and a UV transilluminator.

Table 1: Thermocycler settings.

Step	Temperature	Time	Notes
1	94 degrees	10'	initial denaturing
2	94 degrees	30"	denaturing
3	64 degrees	30"	annealing
4	72 degrees	3'	extension
5	go to step 2		30 cycles then step 6
6	72 degrees	7'	final extension
7	4 degrees	hold	

Isolation and concentration of extracellular matrix proteins

P. aeruginosa cells were grown overnight on LB plates or in 10 ml LB broth at 37°C. Plate-grown cells were collected in CTX + 10mM MgCl₂. Plate-grown and broth-grown cells were passed through an 18-gauge needle of a syringe, then centrifuged at 15,000 rpm for 15 minutes to remove cells. Supernatants were collected and centrifuged again for 15 minutes. The proteins of the supernatant were concentrated in a 10% trichloroacetic acid (TCA) solution for 1 hour at 4°C. Proteins were recovered by centrifugation at 4°C for 30 minutes. Pellets were washed twice in cold 90% acetone and resuspended in 40ul CTX + 10mM MgCl₂. Samples were boiled for 10 min.

SDS-PAGE detection of extracellular protein differences

Concentrated extracellular proteins were separated and analyzed on 12%, 15%, and 7.5% SDS polyacrylamide gels (Biorad). Following SDS-PAGE, proteins were visualized by silver staining (SilverSnap Stain Kit, Pierce).

RESULTS

Evaluation of $\Delta lasB$ mutant phenotypes

Elastase activity. To verify that *lasB* is disrupted in PAO-B1 ($\Delta lasB$ mutant) cells, we investigated the ability of PAO-B1 cells to produce elastase. Elastase acts on its substrate, elastin, by cleaving it. The production of elastase in PAO1 wild-type cells, mutant PAO-B1 cells, and complemented PAO-B1 cells was measured using Elastin-Congo Red (ECR) as described previously (Bjorn *et al.*, 1979) with modifications (Pearson *et al.*, 1997). PAO-B1 mutant cells did not exhibit any elastase activity compared to PAO1 wild-type cells (Figure 7). The complemented PAO-B1 mutant (containing the *lasB* complementing plasmid, pRB1804SF) was restored in elastase activity.

Twitching, swarming, and swimming motility. *Pseudomonas aeruginosa* cells are capable of three forms of motility: swimming, swarming, and twitching motility. Twitching motility is a flagella-independent form of surface motility that is mediated by Type IV pili (Bradley, 1980) and is essential for biofilm formation (O'Toole and Kolter, 1998). Swarming motility enables cells to move across the surface of a semi-solid

medium, and may require Type IV pili as well as flagella (Kohler *et al.*, 2000). In contrast, swimming motility takes place in liquids and requires flagella only. In order to determine whether *lasB* is involved in any of the three forms of *P. aeruginosa* motility, macroscopic 'stab' assays were performed. Twitching motility was assayed using LB agar (1%) plates that were stab inoculated with *P. aeruginosa* cells as described previously (Glessner *et al.*, 1999). The diffuse zone represents cells moving by twitching motility at the bottom of the petri dish. PAO-B1 mutant cells displayed substantially reduced twitching motility compared with wild-type cells (Figure 8). Complemented mutant cells displayed twitch zones larger than the mutant. To determine the involvement of *lasB* in swarming motility, agar (0.5%) plates containing 8g/L Nutrient Broth and 5g/L glucose were stab inoculated. The branching white zone represents cells moving by swarming motility on the agar surface. Swarming motility was substantially reduced in PAO-B1 mutant cells (Figure 9). In contrast, swimming motility was unaffected in PAO-B1 mutant cells (Figure 10).

Interestingly, wild-type cells could 'rescue' the twitch-defective PAO-B1 mutant cells. To demonstrate this, equal numbers of GFP-labeled PAO1 cells and PAO-B1 cells were inoculated together on the surface of a twitch plate. As a control, equal numbers of wild-type and GFP-labeled wild-type cells were mixed. After 5 hours of incubation at 37°C the edge of the twitch zone was viewed. It was determined that the pattern and number of cells expressing GFP (wild-type cells) at the edge of the twitch zone were equivalent whether the co-inoculated partner was unmarked wild-type cells or $\Delta lasB$ mutant cells (Figure 11).

Expression of *lasB*. Since PAO-B1 cells were reduced in twitching motility and swarming motility, but not swimming motility, we hypothesized that the expression of *lasB* could differ with respect to surface or liquid environments. The expression of *lasB* under the three motility conditions (twitch, swarm, and swim) was investigated by performing a β -galactosidase assay using a *lasB::lacZ* fusion in PAO1 wild-type cells. There were no significant differences in *lasB* expression between *P. aeruginosa* cells grown in broth with those grown on standard agar (1.5%), twitch agar (1%) or swarm agar (0.5%) plates (Figure 12).

Level of piliation. *lasB* was necessary for normal twitching motility, which requires type IV pili. The reduced ability of the PAO-B1 mutant cells to twitch could be due to the lack of pili, or reduced number of pili. To determine whether *lasB* is essential for the production or assembly of pilin monomers into pili on the cell surface, we performed a Western Blot analysis of total surface pilin recovered from wild-type and PAO-B1 mutant cells. Pili were isolated from the cell surface by vigorous vortexing of cells, and recovered by isoelectric point precipitation as previously described (Glessner *et al.*, 1999). Recovered surface pili samples were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with rabbit anti-pilin primary antibody, followed by anti-rabbit AP conjugated secondary antibody. As shown in Figure 13A, PAO-B1 mutant cells contained surface pili. Thus, *lasB* does not affect twitching motility by inhibiting the assembly of surface pili in *P. aeruginosa*. Western blot analysis for surface pili was also performed on wild-type and PAO-B1 mutant cells grown on twitch plates. It was discovered that PAO-B1 cells possessed

more surface pilin than wild-type cells under this condition (Figure 13B). Transmission electron microscopy confirmed the presence of pili on the surface of PAO-B1 cells, and showed that they were in fact hyperpiliated (Figure 14). Not only did PAO-B1 mutant cells have more pili coming out of the poles of the cells, but also from all around the cell. The pili were peritrichous. It was confirmed that PAO-B1 cells possessed pili, thus the reduced twitching motility of PAO-B1 mutant cells was not due to the lack of surface piliation. This led us to investigate whether the pili were functional.

Phage sensitivity. *Pseudomonas aeruginosa* is sensitive to type IV pili-specific phage. The phage bind to receptors on the pili, and when the pili retract the phage are brought into the cell. This leads to cell infection and subsequent lysis, resulting in a clear plaque on agar plates. A loss of pili function would therefore result in reduced sensitivity to pili-specific phage infection. Pili-specific phage (D3112 and PO4) and virulent non-pili-specific phage (UT1) were spotted onto agar plates containing an overlay with *Pseudomonas aeruginosa* cells. Wild-type cells were susceptible to infection by the pili-specific phage, producing clear plaques, but PAO-B1 cells were much more resistant to infection (Figure 15). In contrast, both wild-type and PAO-B1 mutant cells were susceptible to infection by UT1, a virulent non-pili-specific phage.

Visualization of pili-specific phage. Pili are multi-functional structures that function in twitching motility, adherence, and binding of phage. To test if pili-specific phage could bind to pili on PAO-B1 cells, we labeled D3112 and PO4 phage with YO-PRO-1 fluorescent dye to observe binding of phage to *P. aeruginosa* cells with

fluorescence microscopy. We found that PAO-B1 cells were able to bind the fluorescent-labeled phage. Therefore, the increased resistance of PAO-B1 cells to pili-specific phage infection may be due to defective retraction of pili into the cell. The pili may not be functional in that they do not retract, thus accounting for decreased susceptibility to pili-specific phage and decreased twitching motility in $\Delta lasB$ mutant cells. To determine if the pili were indeed defective in retraction, we used transmission electron microscopy to observe PO4 pili-specific phage bound to pili on PAO1 and PAO-B1 cells in order to see pili in different stages of retraction. Short pili covered with phage were visible at the poles of PAO1 cells (Figure 16). However, on PAO-B1 cells phage were observed attached to pili, but the pili remained long and unretracted (Figure 16).

Composition of Type IV pili. Since PAO-B1 cells were found to be hyperpiliated (Figure 16), we sought to determine the subunit composition of these pili. Pseudopili, which are composed of XcpT, are produced when *xcpT* is overexpressed (Durand *et al.*, 2003). Hong-Mei *et al.* (1997) found that XcpT can form heterodimers with PilA. It is possible that the hyperpiliation seen on PAO-B1 mutant cells is partially due to an excess of PilA, as well as the presence of XcpT. Perhaps XcpT and PilA are complexed. We performed a Western Blot analysis of isolated surface pili using anti-PilA and anti-XcpT antibodies. Wild-type and mutant cells were grown on twitch plates, pili were removed by shearing, and the pilin was subsequently recovered by isoelectric point precipitation using the pI of PilA (4.3). Under these conditions there was no detectable XcpT in isoelectric point precipitated surface pilin of either PAO1 or PAO-B1 mutant cells (Figure 17), whereas both reacted with anti-PilA antibodies (Figure 13). We also

analyzed the levels of XcpT in whole cell lysates and found them to be equivalent in wild-type and $\Delta lasB$ mutant cells (Figure 18). However, we found XcpT to be present in surface appendages recovered from wild-type and mutant cells grown under twitch conditions when all surface appendages, not just pili, were isolated using the method described by Durand *et al.*, (2003) (Figure 19). This method does not rely on isoelectric point precipitation and would thus be expected to precipitate both PilA and XcpT (with a theoretical pI of 6.2).

Level of flagellation. *lasB* was necessary for normal swarming motility, which requires flagella. However, *lasB* was not necessary for swimming motility, which also requires flagella. Rashid and Kornberg (2000) recently reported that swarming *P. aeruginosa* cells possessed multiple polar flagella. Therefore, the reduced ability of PAO-B1 mutant cells to swarm could be due to a reduced number of flagella. To determine whether *lasB* is involved in the production of flagella, we performed a Western Blot analysis of total flagellin from PAO1 and PAO-B1 mutant cells. Samples were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with rabbit anti-flagellin B primary antibody, followed by anti-rabbit AP conjugated secondary antibody. As shown in Figure 20, PAO-B1 mutant cells showed levels of flagellin equivalent to wild-type cells by Western Blot analysis.

Since flagella are necessary for swarming and swimming motility, but PAO-B1 cells that have flagella were deficient in swarming but not swimming, possibly the flagella used for each type of motility are different from each other. Transmission electron microscopy confirmed the presence of flagella on the surface of PAO-B1 mutant

cells, and was used to examine flagella on cells grown in LB broth (swim conditions) and on semi-solid agar (0.5%) plates (swarm conditions). Similar to the findings of Rashid and Kornberg (2000), many wild-type cells observed from swarm plates possessed multiple polar flagella (Figure 21A). Usually, two polar flagella per cell were observed. However, PAO-B1 mutant cells from swarm plates possessed only a single polar flagellum in most cases (Figure 21B). In general, flagella were more abundant on wild-type cells than mutant cells. Also, the flagella on wild-type cells appeared thick and strong, and were sigmoidal in shape. In contrast, flagella on PAO-B1 cells appeared thinner and were often straight or bent. PAO1 and PAO-B1 cells grown in broth were both shown to possess a single polar flagellum that was long and sigmoidal in shape (Figure 21C,D).

Extracellular matrix determination. *Pseudomonas aeruginosa* produces extracellular polysaccharides that enable it to form biofilms. *Myxococcus xanthus* also has an extracellular matrix (Arnold and Shimkets, 1988.), exhibits gliding motility which is similar to twitching (Semmler *et al.*, 1999), forms extensive biofilms, and contains an extracellular protein (FibA) that is similar to elastase (Kearns *et al.*, 2002). Because of these similarities between *Myxococcus xanthus* and *Pseudomonas aeruginosa*, we sought to determine if *P. aeruginosa* also has its extracellular matrix arranged as fibrils as *M. xanthus* does, and whether *lasB* is necessary for the normal production of the extracellular matrix (ECM). To assay for the presence of an extracellular matrix, we incorporated Congo Red dye into agar plates. Colonies of *Myxococcus xanthus* cells that have an ECM assimilate the dye and appear red in color (Arnold and Shimkets, 1988.).

The Congo Red dye binds to polysaccharides in the ECM. *M. xanthus* matrix-deficient *dsp* mutants do not assimilate the dye and remain colorless (Arnold and Shimkets, 1988). LB agar (1%) plates containing 10 ug/ml Congo Red were stab inoculated with *Pseudomonas aeruginosa* cells. We found that PAO1 cells assimilated the dye and turned red; however, PAO-B1 mutant cells did not (Figure 22).

Alcian Blue is a dye that binds to acidic polysaccharides in the ECM of *Pseudomonas aeruginosa* cells (Sauer *et al.*, 2001). Cells possessing the acidic polysaccharides in the matrix assimilate the dye and turn blue in color. LB agar (1%) plates containing 0.2 g/L Alcian Blue were stab inoculated with *Pseudomonas aeruginosa* cells. We found that PAO1 cells assimilated the dye and turned blue; however, PAO-B1 mutant cells appeared to be a darker blue, indicating that more Alcian Blue was bound to the ECM of PAO-B1 mutant cells than wild-type cells (Figure 23).

LasB elastase, an ECM protein, acts as a protease and degrades the mature 43 kDa CbpD protein in the ECM, forming two degradation products (Folders *et al.*, 2000). Due to its presence in the ECM and its function as a protease that has been shown to act on at least one other ECM protein, we speculated that LasB elastase is likely to affect the structure of the ECM. We investigated this further using scanning electron microscopy to observe the extracellular matrix on the surface of wild-type and mutant cells. We found that the ECM of PAO1 cells was organized into long, thin fibrils that extended to other surrounding cells, similar to *M. xanthus* (Figure 24). However, the structure of the ECM of PAO-B1 mutant cells was different. Mutant cells did not possess the long fibrils. Instead, the ECM appeared to be in clumps on the surface of the cells (Figure 24).

Level of rhamnolipids. Recent reports indicate factors that may play a role in swarming motility. Rhamnolipids, synthesized by *rhlAB*, have been shown to be essential for swarming motility (Kohler *et al.*, 2000), but not twitching motility (Figure 25,26). We have found that PAO-B1 cells exhibit reduced swarming motility, and this may be in part due to our findings that they lack multiple polar flagella under swarm conditions. But since rhamnolipids are also essential for swarming motility, we analyzed the level of rhamnolipids produced by wild-type and PAO-B1 mutant cells using rhamnolipid detection plates. PAO-B1 cells were severely reduced in rhamnolipid levels (Figure 27). Complemented PAO-B1 mutant cells, which were fully restored in elastase activity, remained severely reduced in rhamnolipid production.

AHL levels. We found that LasB elastase is necessary for normal swarming motility and rhamnolipid production. The synthesis of rhamnolipids is under the control of the *rhl* quorum-sensing system, which is turned on by the *las* quorum-sensing system. This led us to investigate the possibility that elastase affects rhamnolipid synthesis via production of short-chain AHLs (quorum-sensing autoinducers). To determine whether LasB affects AHL levels in *P. aeruginosa*, AHLs were extracted from PAO1 and PAO-B1 cells, separated using thin-layer chromatography, and detected using bioreporters to assay for the presence of AHLs. Broth-grown cells were assayed for OdDHL, OHHL, C4-HSL, C6-HSL, and C8-HSL. PAO1 cells possessed all of these AHLs, however PAO-B1 mutant cells lacked C4-HSL, the *rhl* system autoinducer (Figure 28). Complemented PAO-B1 mutant cells did not produce C4-HSL either.

Discovery of second-site mutation. Complemented PAO-B1 cells were fully restored in elastase activity, partially restored in twitching and swarming motility, but not restored in rhamnolipid and AHL production. The lack of rhamnolipids and C4-HSL in complemented PAO-B1 cells led us to believe that PAO-B1 mutant cells had a second-site mutation that was responsible for the phenotypes seen in PAO-B1 cells that could not be restored by complementation with the *lasB* containing plasmid, pRB1804SF. We contacted Barbara Iglewski who had provided us with the PAO-B1 cells constructed in her lab. She informed us that the mutant cells were constructed in a wild-type background that was later determined to have an overexpression of the MexEF efflux pump. An overexpression of *mexEF* has been found to affect the production of virulence factors (including LasB elastase), levels of rhamnolipids, and levels of C4-HSL (Kohler *et al.*, 2001). In fact, *mexEF* overexpression led to a decrease in transcription of *rhlAB* and *rhlI*, the genes responsible for rhamnolipid and C4-HSL synthesis. This would explain the decreased levels of both of these molecules in PAO-B1 cells that overexpress *mexEF*. However, if our experiments had been comparing PAO-B1 cells to their parent cells, like we thought, we would not have seen any differences in the levels of rhamnolipids and C4-HSL.

Barbara Iglewski also informed us that she had many different types of wild-type PAO1. Some had the *mexEF* overexpression and some did not. The *mexEF* overexpression leads to a large decrease in rhamnolipid production. If the wild-type we had been working with was the parent of PAO-B1 and contained the *mexEF* overexpression, we would have found very little rhamnolipid production. However, the wild-type PAO1 we were working with produced high levels of rhamnolipids (Figure

27). The results of the rhamnolipid and AHL assays, together with the information provided to us by B. Iglewski about the PAO-B1 and wild-type strains, led us to believe that we were not working with the parent of PAO-B1. Instead, we had a wild-type PAO1 that differed in respect to not possessing the *mexEF* overexpression. Therefore, wild-type cells (no *mexEF* overexpression) would show high levels of rhamnolipids and C4-HSL, but PAO-B1 mutant cells (*mexEF* overexpression) would show low levels of rhamnolipids and C4-HSL. This is what our results have shown (Figures 27, 28).

To determine if any of the phenotypic differences we had observed in PAO-B1 mutant cells were due to the *lasB* mutation and not overexpression of *mexEF*, we obtained different $\Delta lasB$ mutants from a mutant library at the University of Washington. These mutants were constructed by the insertion of a transposon into the genome (Jacobs *et al.*, 2003). We were provided with several probable $\Delta lasB$ mutant strains that needed to be verified, and the parental wild-type strain (MPAO1) in which the mutants were constructed.

Re-evaluation of $\Delta lasB$ mutant phenotypes

Elastase activity. The transposon mutants and wild-type (MPAO1) cultures received from the University of Washington were first screened for elastase activity on plates overlayed with elastin. If functional elastase was produced, the elastase would cleave the elastin and produce clearing zones on the plates. Most transposon mutants

produced a clearing zone similar to wild-type MPAO1, however, the $\Delta lasB$ mutant 31878 displayed a reduced clearing zone and thus elastase activity (Figure 30). An elastolysis assay was performed to provide further verification that *lasB* is disrupted in mutant 31878 cells. We determined the amount of elastase activity on Elastin-Congo Red (ECR) as described previously (Bjorn *et al.*, 1979) with modifications (Pearson *et al.*, 1997). 31878 mutant cells did not exhibit elastase activity as MPAO1 cells did (Figure 29). The complemented 31878 mutant (containing the *lasB* complementing plasmid, pRB1804SF) was restored in elastase activity.

Verification of transposon insertion in *lasB*. The location of the transposon insertion in the *lasB* gene was verified by polymerase chain reaction (PCR). Primers were designed to the *lasB* gene sequence and the *phoA* transposon, and PCR was performed according to the specifications provided by the University of Washington for mutant verification. PCR products were detected by ethidium bromide staining of DNA products separated by gel electrophoresis. The genomic primer started at base pair 11 in the *lasB* gene, while the transposon should have been inserted at base pair 520 in the *lasB* gene. The *phoA* transposon was 138 base pairs in length. Therefore, the expected PCR product was 647 base pairs (509 base pairs of *lasB* gene sequence plus 138 base pairs of transposon sequence). The product from our PCR of 31878 mutant cells corresponded to the expected size product for an insertion of the *phoA* transposon in the *lasB* gene (Figure 31).

Twitching and swarming motility. In order to determine whether *lasB* was involved in *P. aeruginosa* twitching or swarming motility, macroscopic 'stab' assays

were performed. Twitching motility was assayed using LB agar (1%) plates that were stab inoculated with *P. aeruginosa* cells as described previously (Glessner *et al.*, 1999). The diffuse zone represents cells moving by twitching motility at the bottom of the petri dish. MPAO1 and 31878 mutant cells were equivalent in twitching motility (Figure 32). Swarming motility was assayed using agar (0.5%) plates containing 8g/L Nutrient Broth and 5g/L glucose that were stab inoculated with *P. aeruginosa* cells. The branching white zone represents cells moving by swarming motility on the agar surface. MPAO1 and 31878 mutant cells were equivalent in swarming motility (Figure 33).

Level of piliation. Since 31878 $\Delta lasB$ mutant cells exhibited normal twitching motility, we suspected that they were not altered in type IV pili production and assembly as had been found previously with PAO-B1 cells. We performed a Western Blot analysis of surface appendages recovered from wild-type and $\Delta lasB$ mutant cells grown under twitch conditions. Surface appendages were sheared off cells and isolated as described by Durand *et al.* (2003). Recovered surface appendages were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with either rabbit anti-pilin or anti-XcpT primary antibody, followed by anti-rabbit AP conjugated secondary antibody. As shown in Figure 34, there were no differences in surface pilin and XcpT levels between MPAO1 and 31878 $\Delta lasB$ mutant cells.

Level of rhamnolipids and AHLs. Since 31878 mutant cells exhibited normal swarming motility, we suspected that they were not altered in rhamnolipid or AHL production as had been found previously with PAO-B1 cells. Both MPAO1 and 31878

cells exhibited equivalent levels of rhamnolipids, which were barely detectable levels compared to PAO1 cells used previously in comparison with PAO-B1 cells (Figure 35). AHL levels were also found to be equivalent between MPAO1 and 31878 mutant cells (Figure 36).

Extracellular matrix determination. LasB elastase has been shown to degrade Chitin binding protein D (CbpD) in the ECM, forming two degradation products (Foldes *et al.*, 2000). Elastase is a metalloprotease present in the ECM, and it may have other substrates in the matrix that have gone undiscovered thus far. To investigate the possibility of other substrates of elastase in the ECM, we isolated extracellular proteins from wild-type and mutant cells to determine if any differences exist in their extracellular protein profiles. MPAO1 and 31878 cells grown in LB broth and on LB (1.5% agar) plates were harvested, passed through a 18-gauge needle of a syringe, and centrifuged. The cell pellets were discarded and the extracellular protein-containing supernatants were collected and concentrated by 10% TCA precipitation. Proteins were separated by 12%, 15%, and 7.5% SDS-PAGE and visualized by silver staining. Wild-type and $\Delta lasB$ mutant samples collected from plates exhibited differences in their extracellular protein profiles. 12% SDS-PAGE analysis showed the presence of a protein just above 53,500Da in 31878 cells but not MPAO1 cells, and differences in several proteins between 36 and 53kDa (Figure 37). 7.5% gels were used to obtain optimal resolution of high molecular weight proteins, and it was discovered that 31878 cells differed from MPAO1 in the presence/absence of a protein just under 71kDa (Figure 38).

Besides investigating differences between wild-type and mutant cells, we also discovered differences in the extracellular proteins between broth-grown and plate-grown cells. Figure 39 reveals a dark, sharp band at 36,100Da in plate-grown cells that is barely visible in broth-grown cells separated by 12%SDS-PAGE. In addition, broth-grown cells display a distinct dark band at 29,500Da that is much lighter in plate-grown cells and slightly below the broth-grown cells' band (Figure 39). Figure 40 shows a difference in a low molecular weight protein resolved by 15% SDS-PAGE gels. In plate-grown cells there is a protein band just below 21,300Da. In broth-grown cells there is a band that is further below the plate-grown cells' band.

SyproRuby staining, which is compatible with mass spectrometry analysis (unlike silver staining) was also used to visualize protein differences, although with less success (Figure 41). Banding pattern differences could not be discerned.

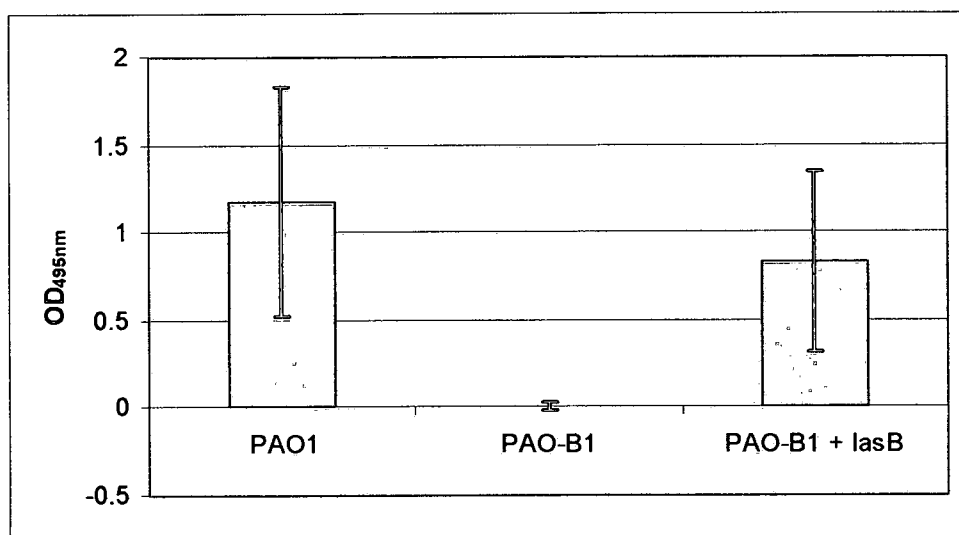


Figure 7. Elastolysis assay. Elastase production was measured using Elastin-Congo Red (ECR) substrate.

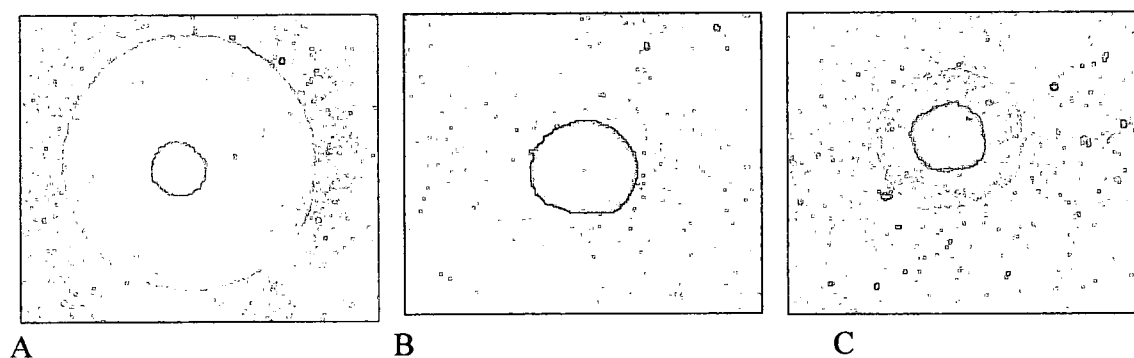


Figure 8. Macroscopic stab assay for twitching motility. LB (1% agar) plates were stab inoculated to the bottom of the plate and incubated at 37°C for 24 hours. The twitch zone (diffuse zone), at the petri dish/agar interface, is a measure of twitching motility. The center, dense zone is surface colony growth. (A) PAO1 (wild-type), (B) PAO-B1 ($\Delta lasB$ mutant), (C) PAO-B1 + pRB1804SF (complemented PAO-B1 mutant).

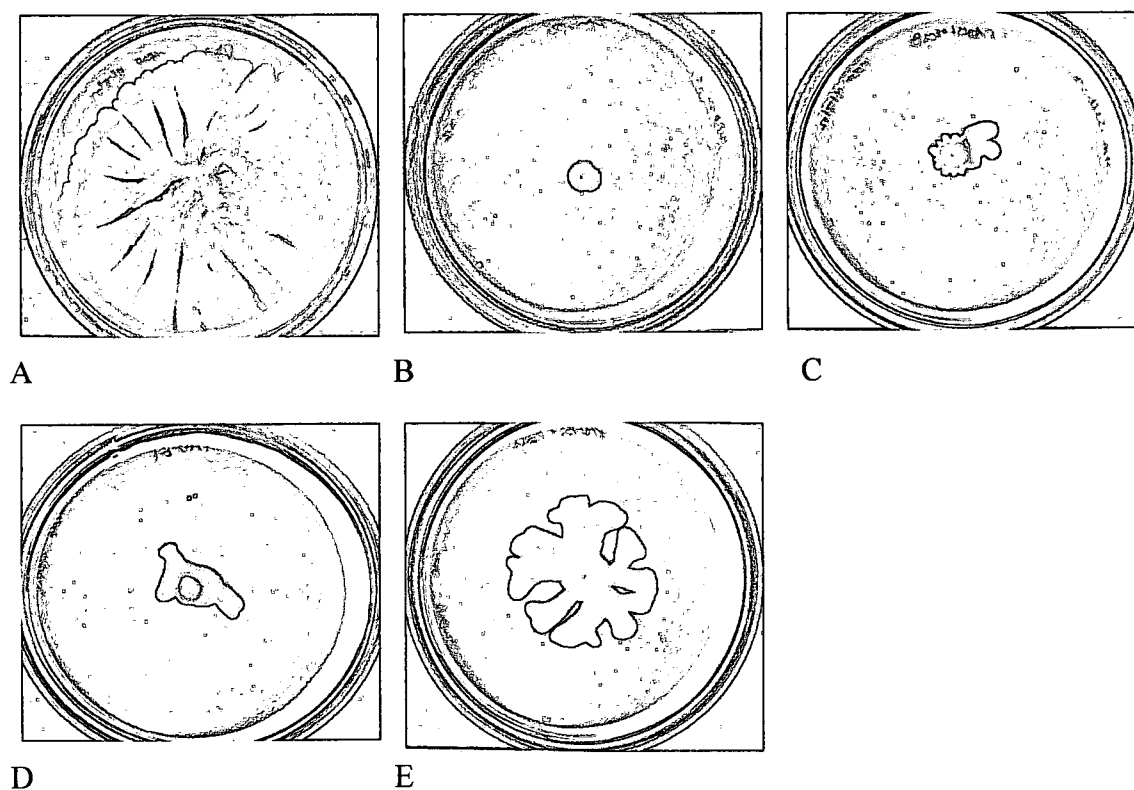


Figure 9. Macroscopic stab assay for swarming motility. Agar (0.5%) plates containing 8g/L Nutrient Broth and 5g/L glucose were stab inoculated with *P. aeruginosa* cells and incubated at 37°C. (A) PAO1, (B) PAO-B1, and (C) complemented PAO-B1 after 24 hours incubation. (D) PAO-B1 and (E) complemented PAO-B1 after 48 hours incubation.

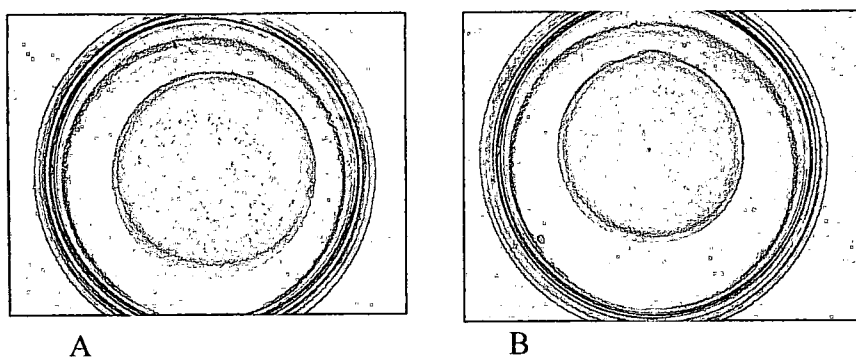


Figure 10. Macroscopic stab assay for swimming motility. LB (0.3% agar) plates were stab inoculated to the bottom of the plate and incubated at 37°C for 24 hours. (A) PAO1, (B) PAO-B1.

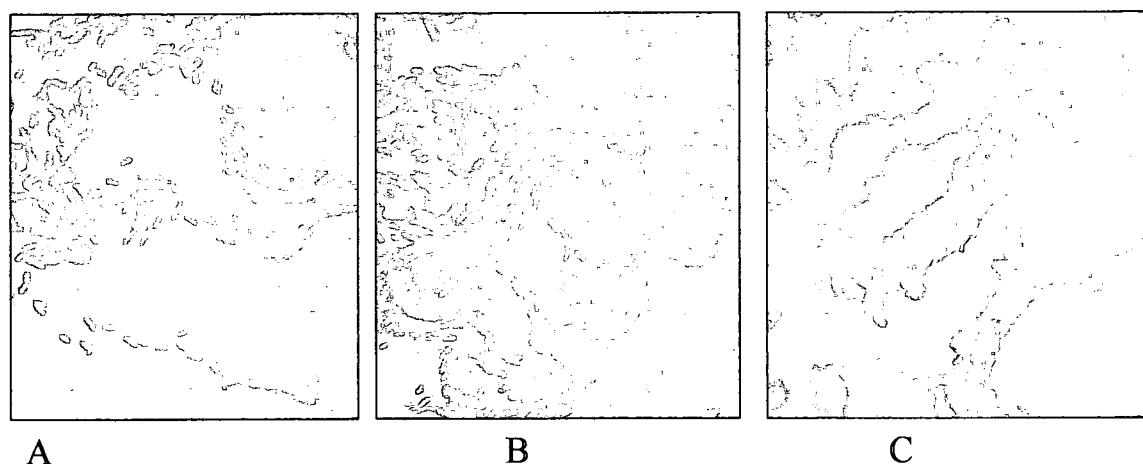


Figure 11. Rescue of mutant cells by wild-type cells. Equal numbers of GFP-containing wild-type cells and either wild-type or *lasB* mutant cells were mixed, placed on twitch plates, and observed using fluorescence and brightfield microscopy. (A) GFP-containing PAO1 cells mixed with PAO1 cells, (B) GFP-containing PAO1 cells mixed with PAO-B1 cells, (C) GFP-containing PAO1 cells mixed with $\Delta pilJ$ mutant cells. Dark areas between GFP marked cells in A and B represent cells not expressing GFP which were visible in bright field.

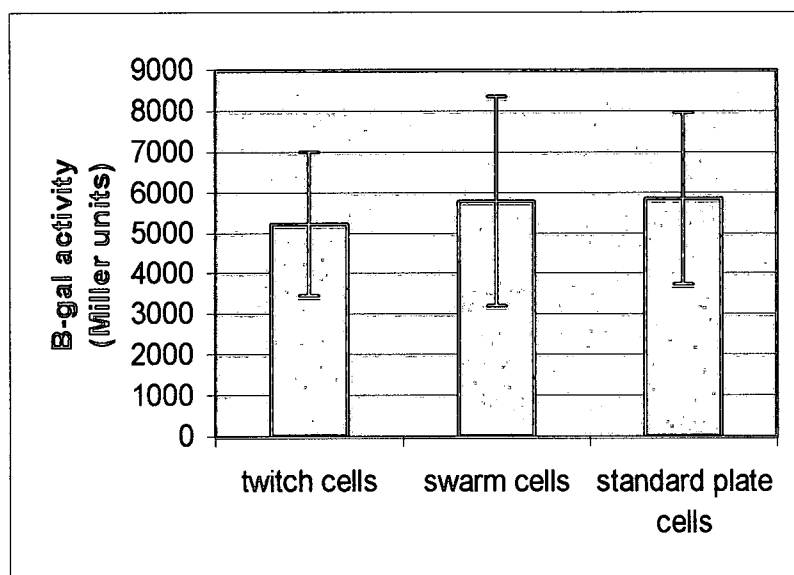


Figure 12. β -galactosidase assay for *lasB* expression. PAO1 cells containing *lasB::lacZ* were grown on twitch plates, swarm plates, and standard LB (1.5% agar) plates and assayed for β -galactosidase activity.

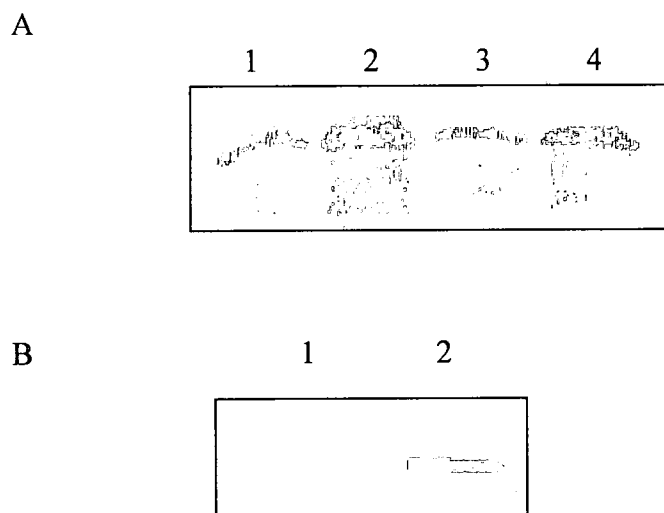


Figure 13. Western Blot analysis of surface pili. Surface pili were sheared off cells by vortexing, precipitated by isoelectric point precipitation, and recovered by centrifugation. Proteins were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with rabbit anti-pilin primary antibody followed by AP-conjugated anti-rabbit secondary antibody. (A) Cells grown on LB 1.5% agar plates. Lane 1, PAO1 pilin; lane 2, PAO-B1 pilin; lane 3, overexpression of *lasB* in PAO1 pilin; lane 4, complemented PAO-B1 pilin. (B) Cells grown on LB 1% agar twitch plates. Lane 1, PAO1 pilin; lane 2, PAO-B1 pilin.

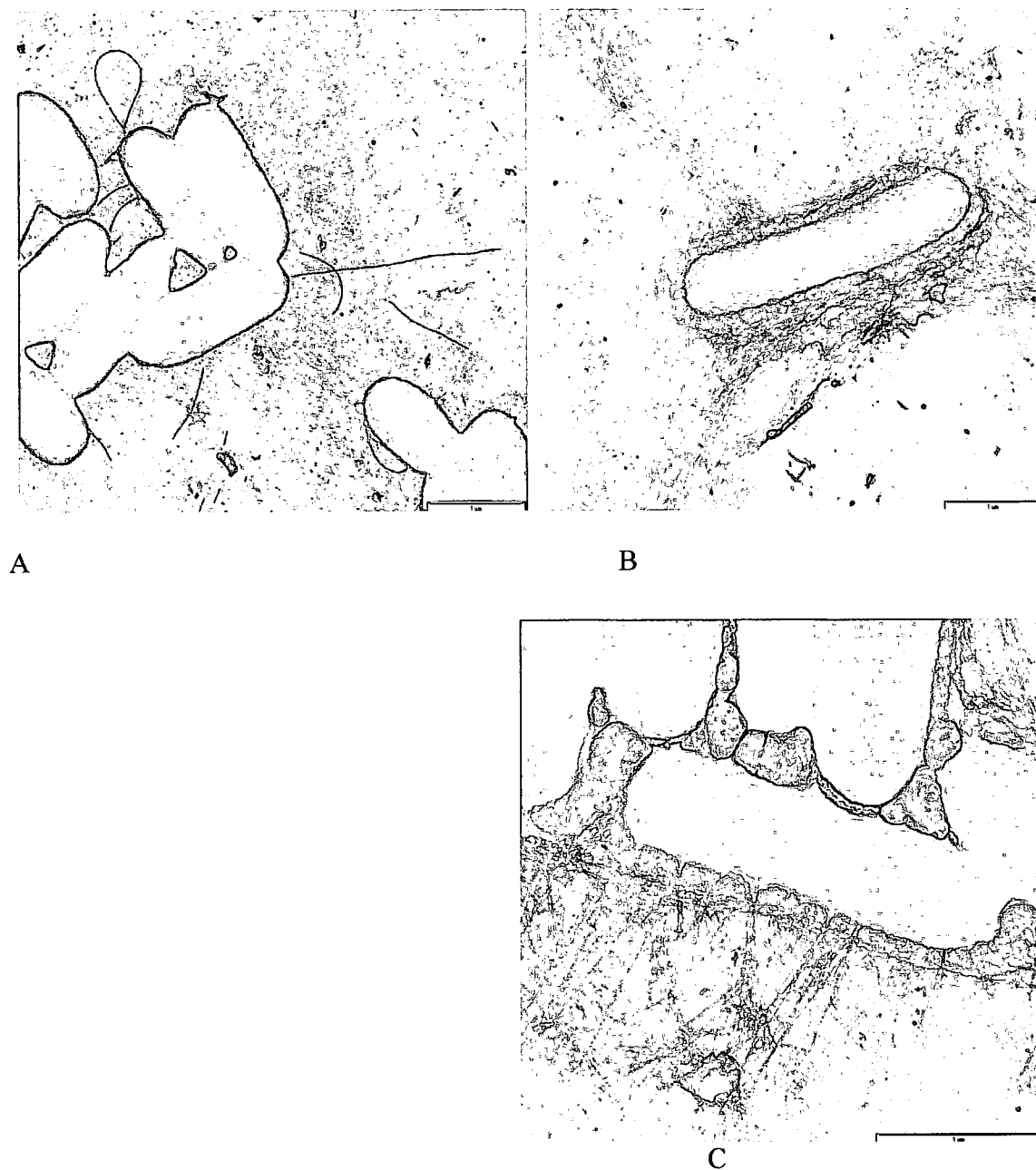


Figure 14. Transmission electron micrographs of pili. Blocks of LB 1.0% agar plates were stab inoculated with wild-type and *lasB* mutant cells, covered with a coverslip, and incubated for 4 hours. Cells from the agar/coverslip interface were blotted onto EM grids, washed three times with water, and stained with 0.5% phosphotungstic acid for 50 sec. The grids were examined by TEM. (A) PAO1, (B, C) PAO-B1.

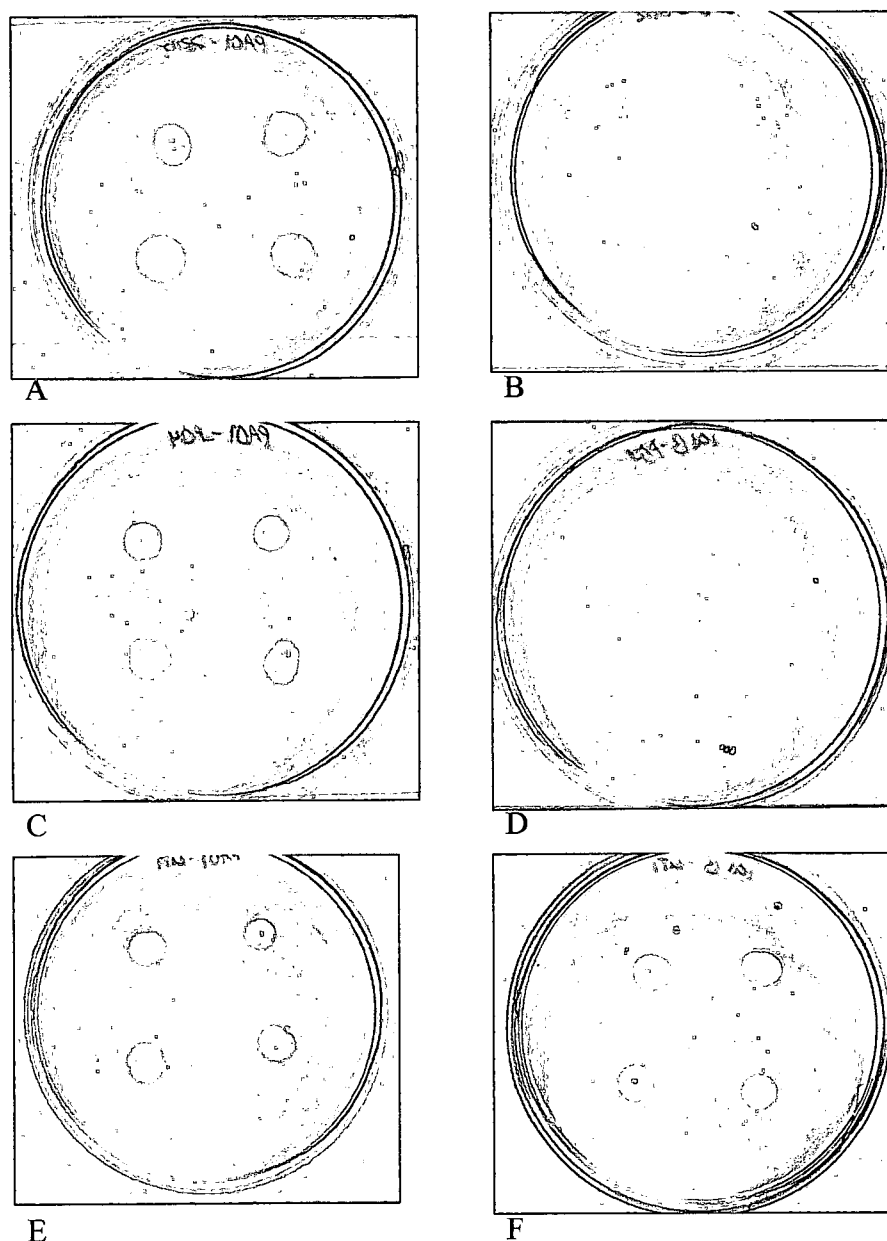
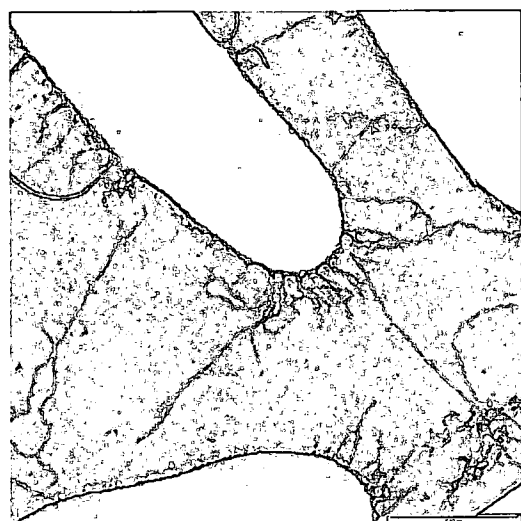


Figure 15. Phage sensitivity assay. D3112 or PO4 pili-specific phage were spotted onto LB (1.5% agar) plates containing *Pseudomonas aeruginosa* cells. D3112 infected cells: (A) PAO1, (B) PAO-B1. PO4 infected cells: (C) PAO1, (D) PAO-B1. UT1 non pili-specific phage were spotted onto agar plates containing *Pseudomonas aeruginosa* cells: (E) PAO1, (F) PAO-B1.



A



B



C

Figure 16. Transmission electron micrographs of pili-specific phage binding to *Pseudomonas aeruginosa* cells. Cells were grown overnight on LB agar (1.5%) plates, resuspended in LB broth and mixed with PO4 pili-specific phage. EM grids were placed in the mixture of cells and phage for 1 min., washed three times with water, and stained with 0.5% phosphotungstic acid for 50 sec. The grids were examined by TEM. (A, B) PAO1, (C) PAO-B1.

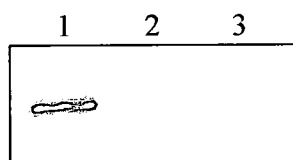


Figure 17. Western Blot analysis of XcpT from isolated surface pilin. Surface pili were sheared off cells (grown overnight on LB 1% agar twitch plates) by vortexing, precipitated by isoelectric point precipitation, and recovered by centrifugation. Proteins were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with rabbit anti-XcpT primary antibody followed by AP-conjugated anti-rabbit secondary antibody. Lane 1, isolated PAO1 whole cell protein containing XcpT; lane 2, PAO1 pilin; lane 3, PAO-B1 pilin.

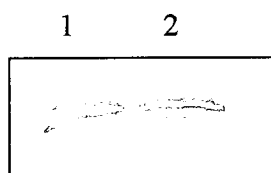


Figure 18. Western Blot analysis of XcpT from whole cell lysates. Cells grown overnight on LB 1% agar twitch plates were suspended in Laemmli Sample Buffer and boiled for 10 minutes. Proteins were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with rabbit anti-XcpT primary antibody followed by AP-conjugated anti-rabbit secondary antibody. Lane 1, PAO1; lane 2, PAO-B1.

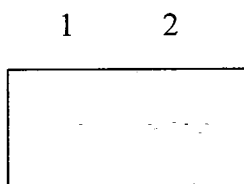


Figure 19. Western Blot analysis of XcpT from isolated surface appendages. Surface appendages were sheared off cells (grown overnight on LB 1% agar twitch plates) by passing through a 19-gauge needle of a syringe, and bacterial cells were removed by centrifugation (Durand *et al.*, 2003). Proteins were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with rabbit anti-XcpT primary antibody followed by AP-conjugated anti-rabbit secondary antibody. Lane 1, PAO1; lane 2, PAO-B1.

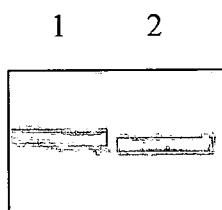


Figure 20. Western Blot analysis of flagellin. Cells were grown overnight on LB (1.5% agar) plates, suspended in CTX buffer supplemented with 0.005M MgCl_2 , and solubilized in Laemmli sample buffer. Proteins were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with rabbit anti-flagellinB primary antibody followed by AP-conjugated anti-rabbit secondary antibody. Lane 1, PAO1 flagellin; lane 2, PAO-B1 flagellin.

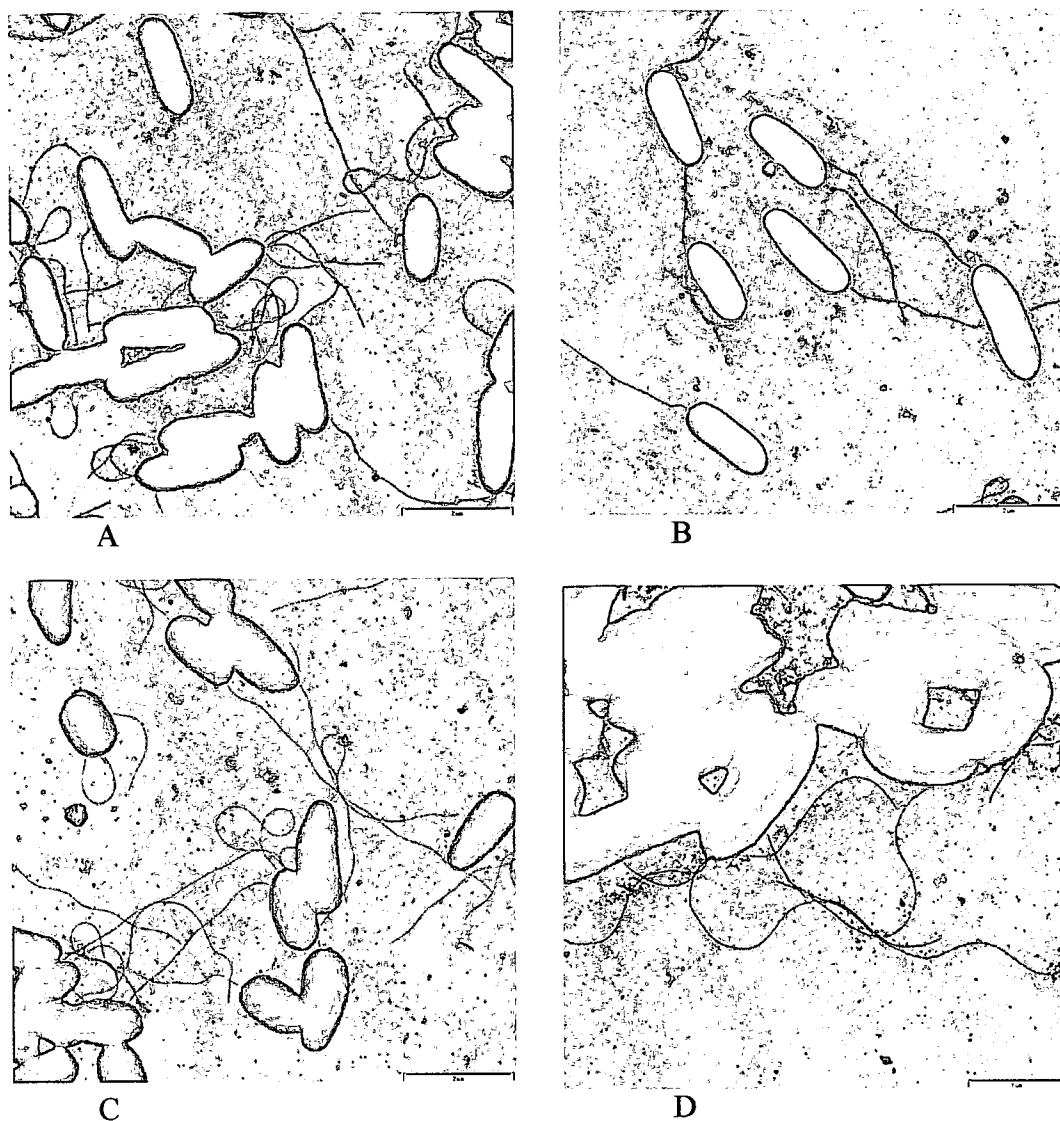
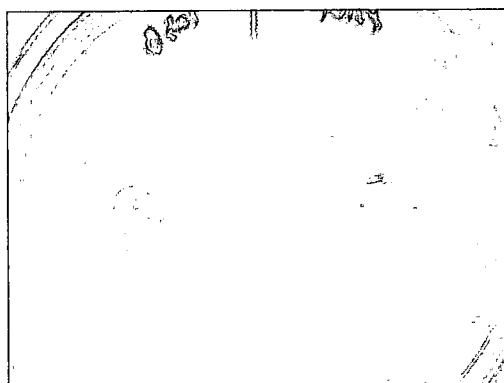


Figure 21. Transmission electron micrographs of swarm cells and swim cells. Cells were blotted onto EM grids, washed three times with water, and stained with 0.5% phosphotungstic acid for 50 sec. The grids were examined by TEM. (A) PAO1 cells from a swarm plate, (B) PAO-B1 cells from a swarm plate, (C) PAO1 cells from LB broth, (D) PAO-B1 cells from LB broth.



A

B

Figure 22. Congo Red assay for ECM. LB (1.0% agar) plates containing 10 $\mu\text{g/ml}$ Congo Red were stab inoculated with cells and incubated at 37°C for 48 hours. (A) PAO-B1, (B) PAO1.

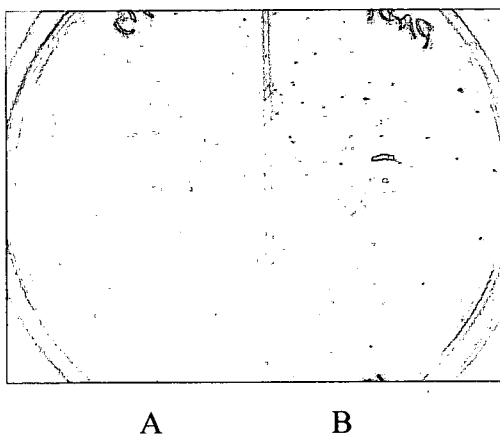
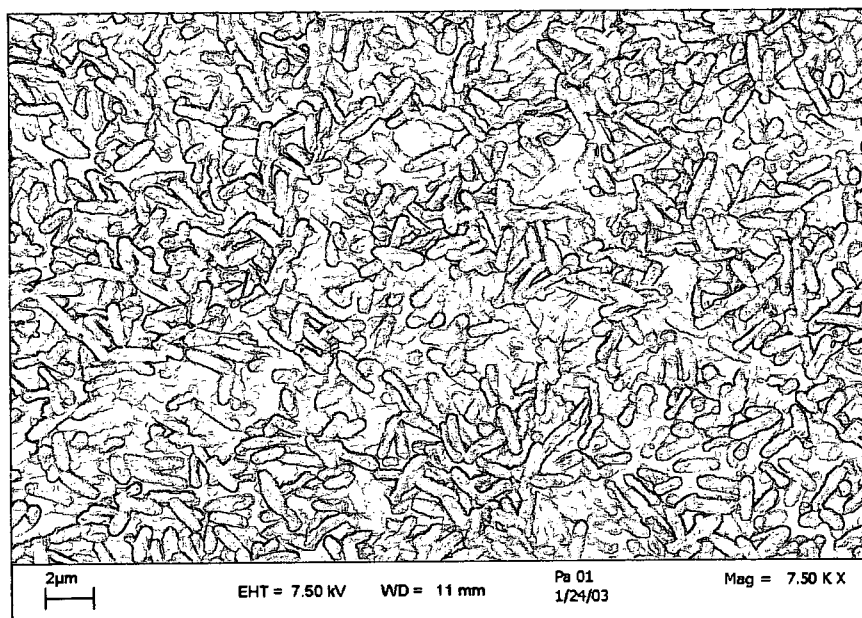
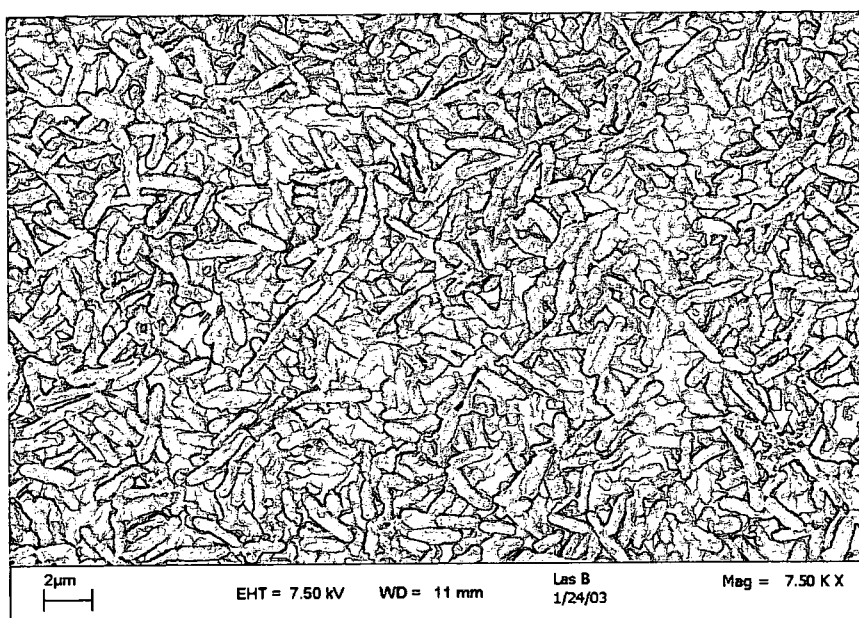


Figure 23. Alcian Blue assay for ECM. LB (1.0% agar) plates containing 0.2g/L Alcian Blue were stab inoculated with cells and incubated at 37°C for 48 hours. (A) PAO-B1, (B) PAO1.



A



B

Figure 24. Scanning electron micrographs of ECM. Cells were collected by filtration on a Nuclepore polycarbonate membrane (48mm diameter, 0.2μm pore size). The cells were fixed in primary fixative (3% glutaraldehyde in 0.1M sodium cacodylate, 500ppm final conc. Ruthenium Red), followed by post fix (2% osmium tetroxide in 0.1M sodium cacodylate). Cells were dehydrated with sequential washes in 30, 50, 70, 90, and 100% ethanol, dried with hexamethyldisilazane:ethanol (50:50) followed by 100% HMDS. The membrane was coated with gold (15 seconds, 45milliamps) and examined by SEM. (A) PAO1, (B) PAO-B1.

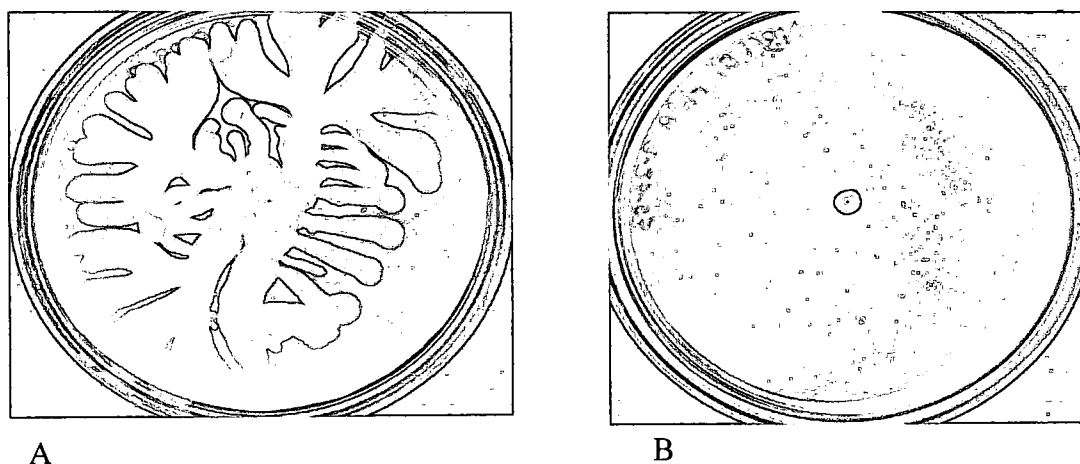


Figure 25. Macroscopic stab assay for swarming motility. Agar (0.5%) plates containing 8g/L Nutrient Broth and 5g/L glucose were stab inoculated with *P. aeruginosa* cells and incubated at 37°C for 24 hours. (A) PAO1, (B) $\Delta rhlAB$ mutant.

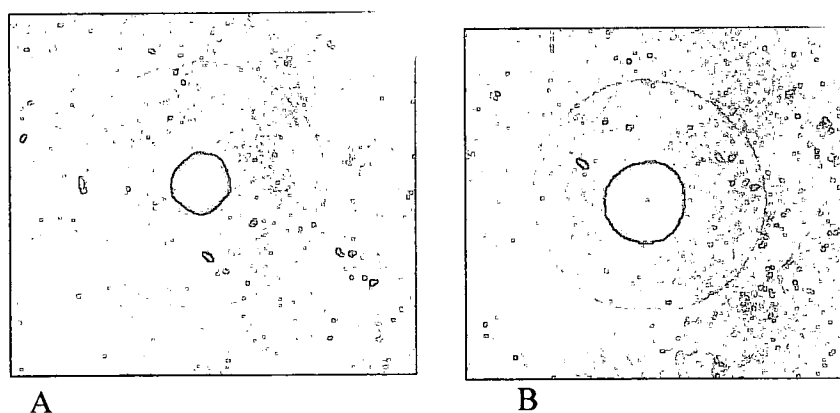


Figure 26. Macroscopic stab assay for twitching motility. LB (1% agar) plates were stab inoculated to the bottom of the plate and incubated at 37°C for 24 hours. The twitch zone (diffuse zone), at the petri dish/agar interface, is a measure of twitching motility. The center, dense zone is surface colony growth. (A) PAO1, (B) $\Delta rhlAB$ mutant.

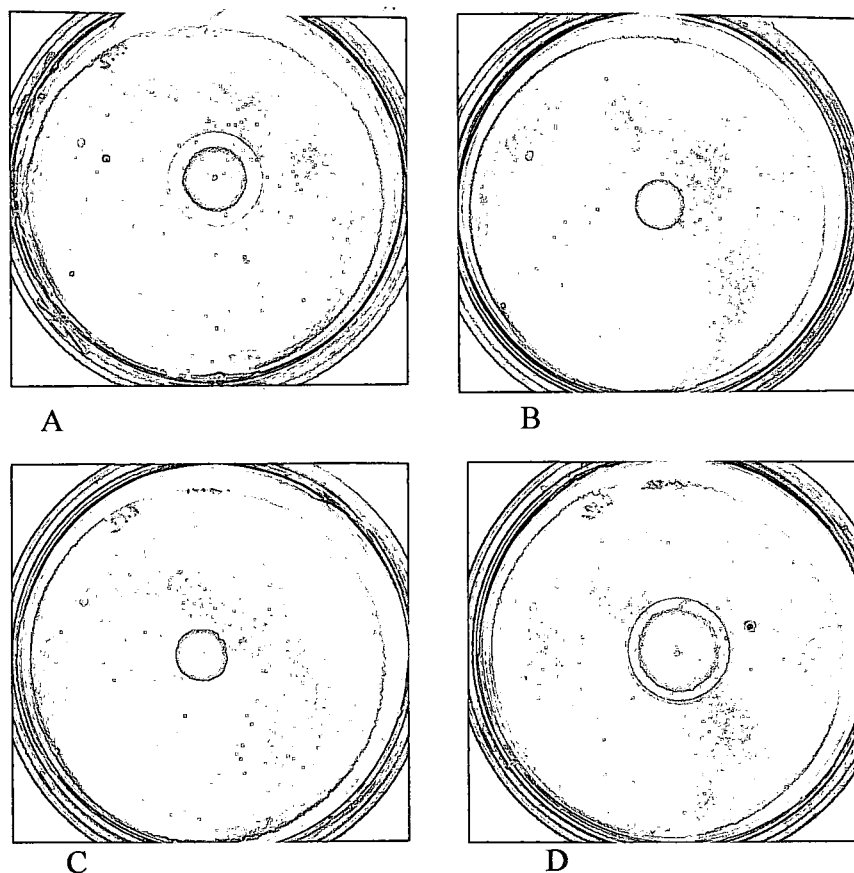


Figure 27. Rhamnolipid assay. Plates for detecting rhamnolipids were prepared as described by Kohler *et al.* (2000) with 0.0005% methylene blue. Plates were spotted with *P. aeruginosa* cells and incubated at 37°C for 24 hours, followed by incubation at room temperature for 2-5 days. The appearance of a halo surrounding the inoculation site indicates the production of rhamnolipids. (A) PAO1, (B) PAO-B1, (C) complemented PAO-B1, (D) *lasB* overexpression in PAO1.

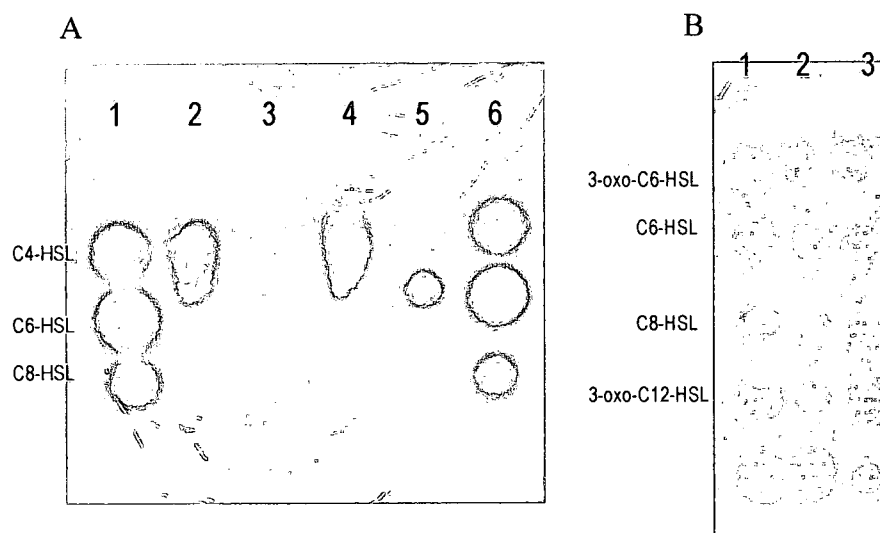


Figure 28. AHL bioassays. Late log cultures ($OD_{600}=0.9$) were extracted 2X with acidified ethyl acetate. Samples were separated by thin-layer chromatography and overlaid with an agar-bioreporter mixture. (A) *Chromobacterium violaceum* CV026 bioassay using 10 ml culture equivalents (McClellan *et al.*, 1997). Lane 1, standards; lane 2, PAO1; lane 3, PAO-B1; lane 4, *lasB* overexpression in PAO1; lane 5, complemented PAO-B1; lane 6, standards. (B) *Agrobacterium tumefaciens* NTL4 bioassay using 3 ml culture equivalents (Shaw *et al.*, 1997). Lane 1, standards; lane 2, PAO1; lane 3, PAO-B1.

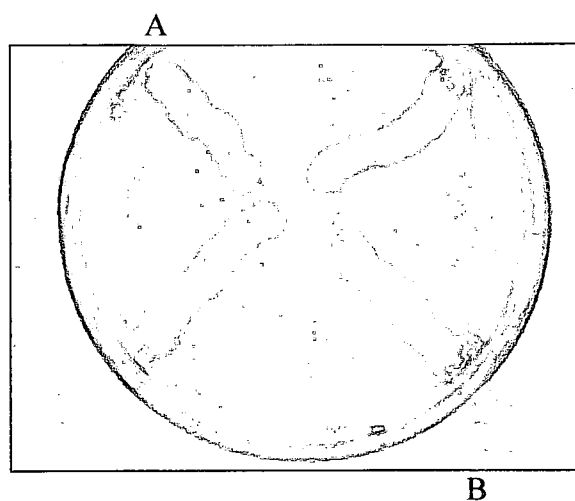


Figure 29. Elastin plate assay. LB (1.5% agar) plates were overlaid with 5ml of 0.5% elastin / 0.8% Nutrient Agar mixtures and solidified at room temperature overnight. The following day, elastin agar plates were streaked with *P. aeruginosa* wild-type and mutant cultures. Clearing zones surrounding bacterial growth indicate elastase activity. (A) MPAO1, (B) 31878.

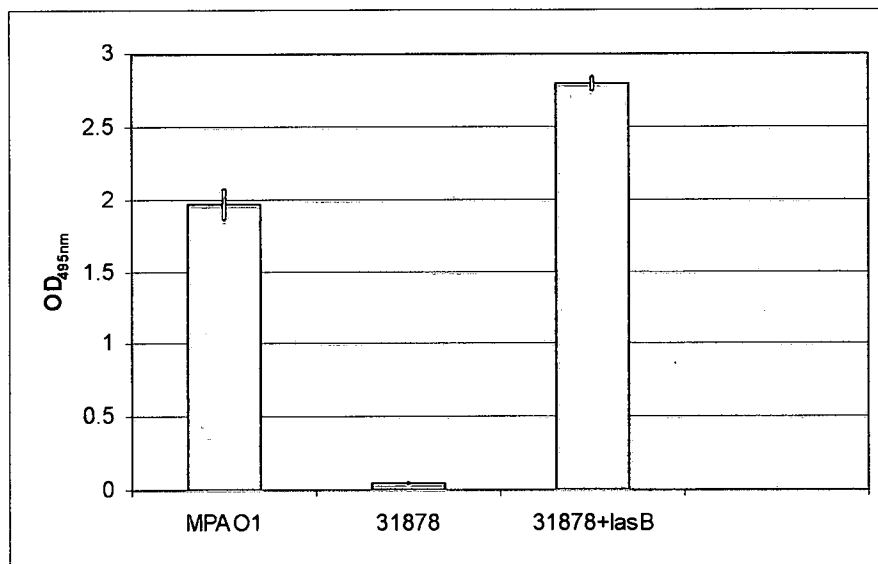


Figure 30. Elastolysis assay. Elastase production was measured using Elastin-Congo Red (ECR) substrate.

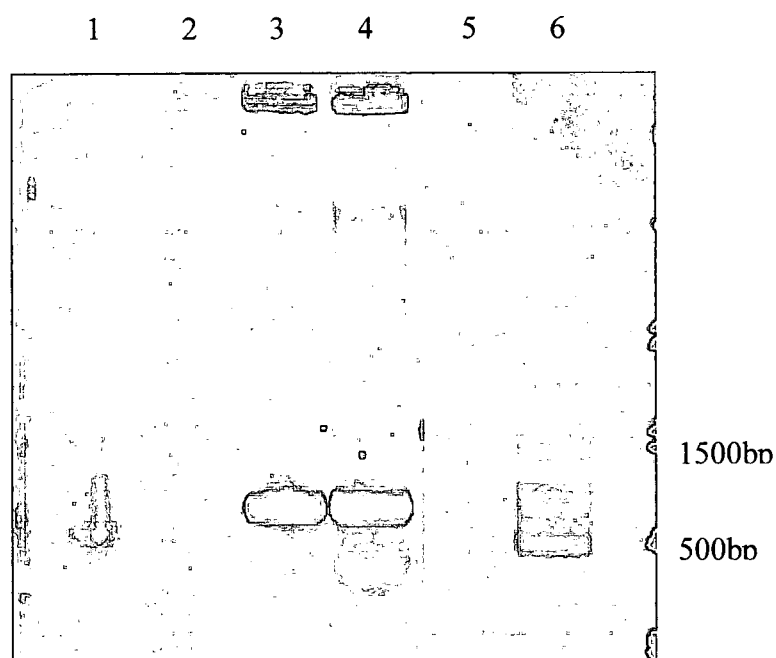


Figure 31. PCR confirmation of 31878 ($\Delta lasB$ transposon mutant). Forward and reverse primers were designed to the *lasB* gene and the *phoA* transposon insertion. PCR was performed in thermocycler using Taq polymerase. Lane 1, 100bp MW ladder; lanes 2-4, 31878 colony-PCR product; lane 5, negative control; lane 6, 100bp MW ladder.

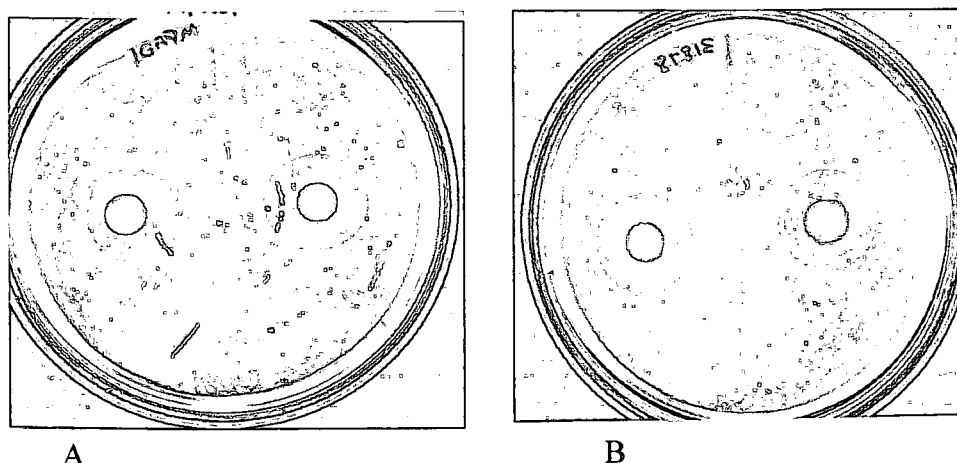


Figure 32. Macroscopic stab assay for twitching motility. LB (1% agar) plates were stab inoculated to the bottom of the plate and incubated at 37°C for 24 hours. The twitch zone (diffuse zone), at the petri dish/agar interface, is a measure of twitching motility. The center, dense zone is surface colony growth. (A) MPAO1, (B) 31878.

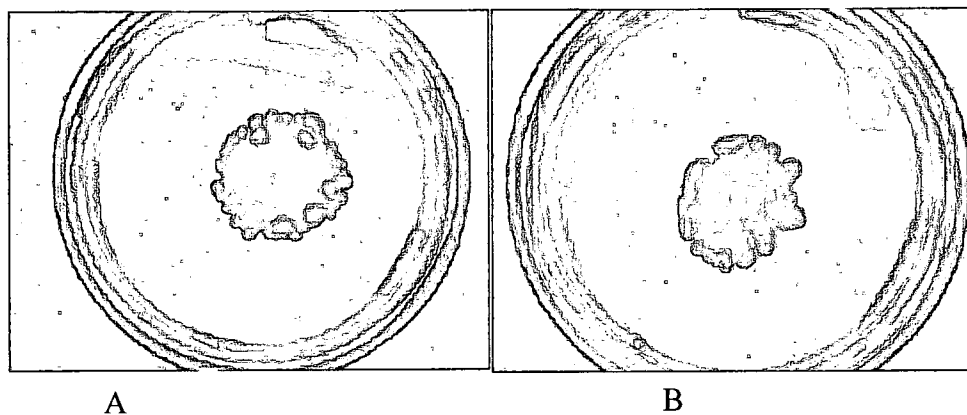


Figure 33. Macroscopic stab assay for swarming motility. Agar (0.5%) plates containing 8g/L Nutrient Broth and 5g/L glucose were stab inoculated with *P. aeruginosa* cells and incubated at 37°C. (A) MPAO1, (B) 31878.

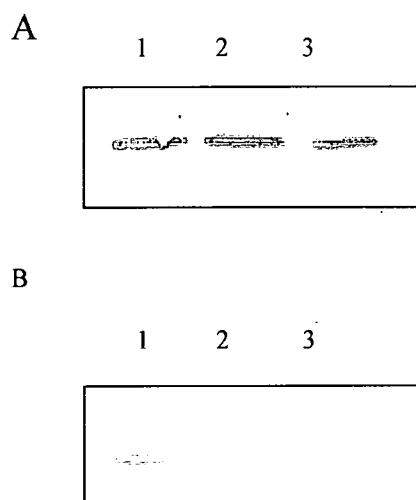


Figure 34. Western Blot analysis of surface appendages. Surface appendages were sheared off cells (grown overnight on LB 1% agar twitch plates) by passing through a 18-gauge needle of a syringe, and bacterial cells were removed by centrifugation (Durand *et al.*, 2003). Proteins were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with either A) rabbit anti-pilin primary antibody or B) rabbit anti-XcpT antibody, followed by AP-conjugated anti-rabbit secondary antibody. A) Lane 1, purified pilin; lane 2, MPAO1; lane3, 31878; B) PAO1 whole cell lysate containing XcpT; lane 2, MPAO1; lane3, 31878.

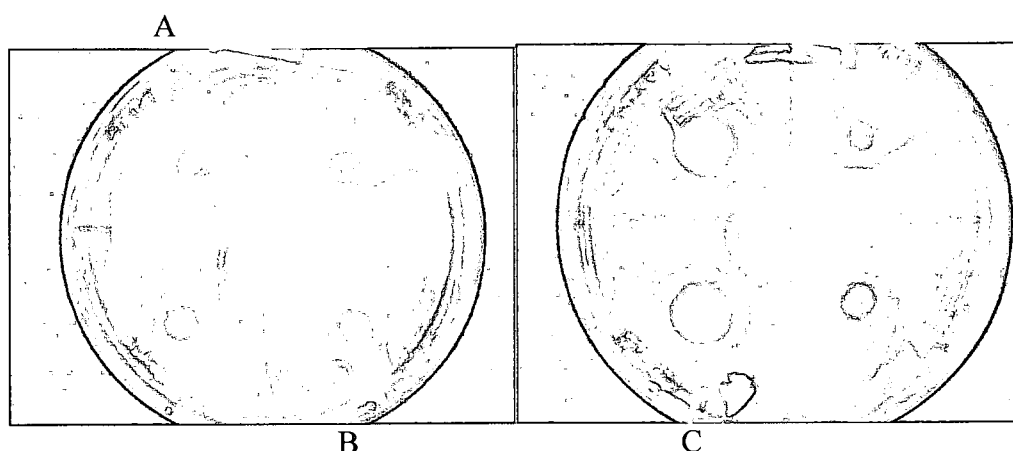


Figure 35. Rhamnolipid assay. Plates for detecting rhamnolipids were prepared as described by Kohler *et al.* (2000) with 0.0005% methylene blue. Plates were spotted with *P. aeruginosa* cells and incubated at 37°C for 24 hours, followed by incubation at room temperature for 2-5 days. The appearance of a blue halo surrounding the inoculation site indicates the production of rhamnolipids. (A) MPAO1, (B) 31878, (C) PAO1.

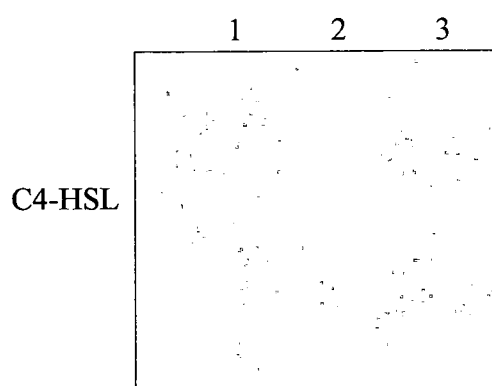


Figure 36. AHL bioassay. Late log cultures ($OD_{600}=0.9$) were extracted 2X with acidified ethyl acetate. Samples were separated by thin-layer chromatography and overlaid with an agar-*Aeromonas hydrophila* mixture. Lane 1, C4-HSL standard; lane 2, MPAO1; lane 3, 31878.

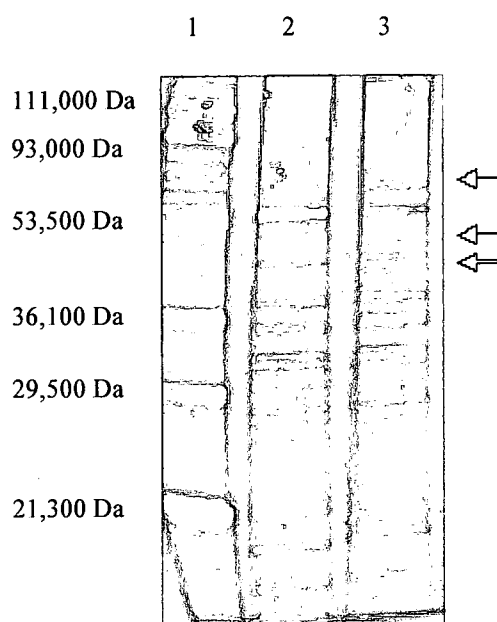


Figure 37. 12% SDS-PAGE analysis of extracellular proteins. *P. aeruginosa* cells grown overnight on LB (1.5% agar) plates were collected in CTX + 10mM MgCl₂ and passed through an 18-gauge needle of a syringe, then centrifuged to remove cells. Supernatant proteins were concentrated by 10% trichloroacetic acid (TCA) precipitation. Concentrated extracellular proteins were separated and analyzed on 12% SDS polyacrylamide gels (Biorad) and detected by silver staining. Lane 1, MW standards; lane 2, MPAO1; lane 3, 31878.

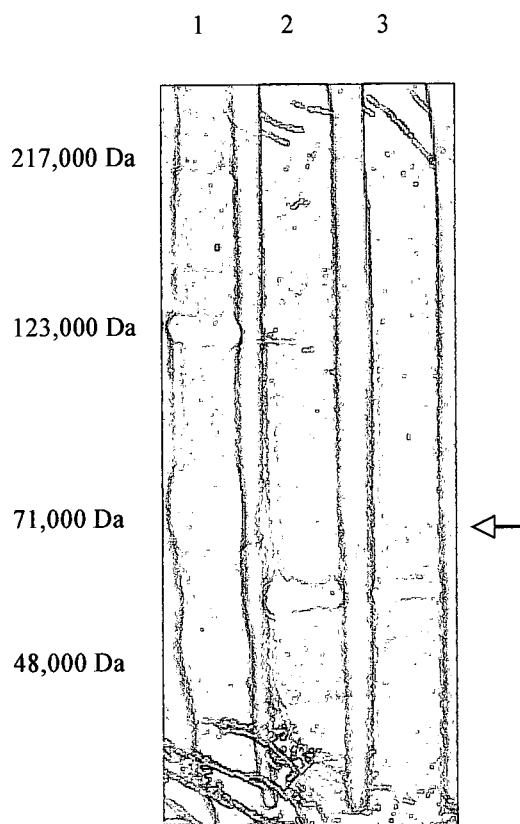


Figure 38. 7.5% SDS-PAGE analysis of extracellular proteins. *P. aeruginosa* cells grown overnight on LB (1.5% agar) plates were collected in CTX + 10mM MgCl₂ and passed through an 18-gauge needle of a syringe, then centrifuged to remove cells. Supernatant proteins were concentrated by 10% trichloroacetic acid (TCA) precipitation. Concentrated extracellular proteins were separated and analyzed on 7.5% SDS polyacrylamide gels (Biorad) and detected by silver staining (SilverSnap Stain Kit, Pierce). Lane 1, MW standards; lane 2, MPAO1; lane 3, 31878.

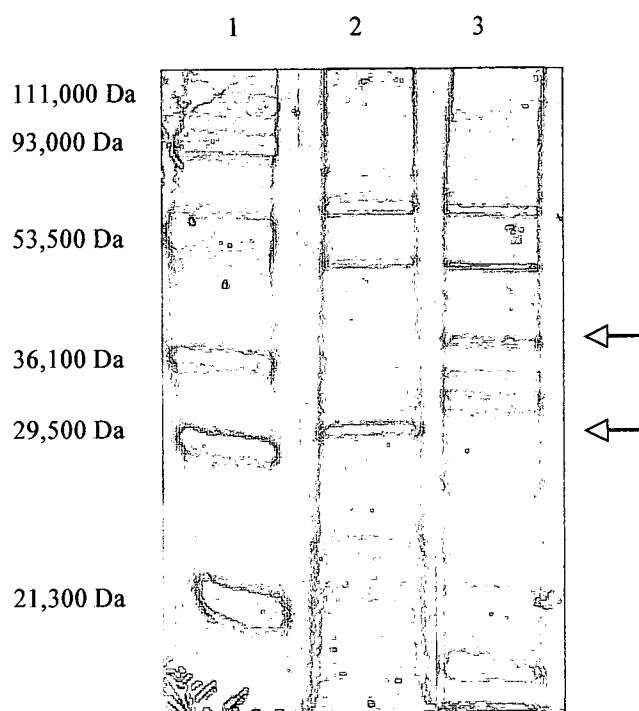


Figure 39. 12% SDS-PAGE analysis of extracellular proteins. *P. aeruginosa* cells grown overnight on LB plates or in LB broth were collected in CTX + 10mM MgCl_2 and passed through an 18-gauge needle of a syringe, then centrifuged to remove cells. Supernatant proteins were concentrated by 10% trichloroacetic acid (TCA) precipitation. Concentrated extracellular proteins were separated and analyzed on 12% SDS polyacrylamide gels (Biorad) and detected by silver staining (SilverSnap Stain Kit, Pierce). Lane 1, MW standards; lane 2, broth-grown MPAO1; lanes 3, plate-grown MPAO1.

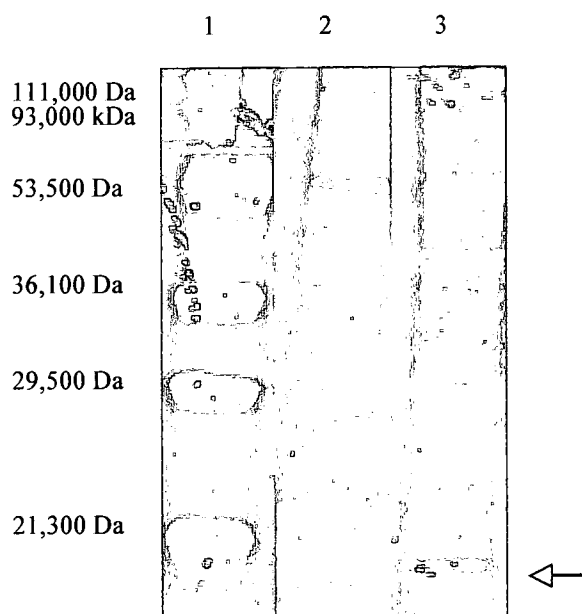


Figure 40. 15% SDS-PAGE analysis of extracellular proteins. *P. aeruginosa* cells grown overnight on LB plates or in LB broth were collected in CTX + 10mM MgCl₂ and passed through an 18-gauge needle of a syringe, then centrifuged to remove cells. Supernatant proteins were concentrated by 10% trichloroacetic acid (TCA) precipitation. Concentrated extracellular proteins were separated and analyzed on 15% SDS polyacrylamide gels (Biorad) and detected by silver staining (SilverSnap Stain Kit, Pierce). Lane 1, MW standards, lane 2, broth-grown MPAO1; lane 3, plate-grown MPAO1.

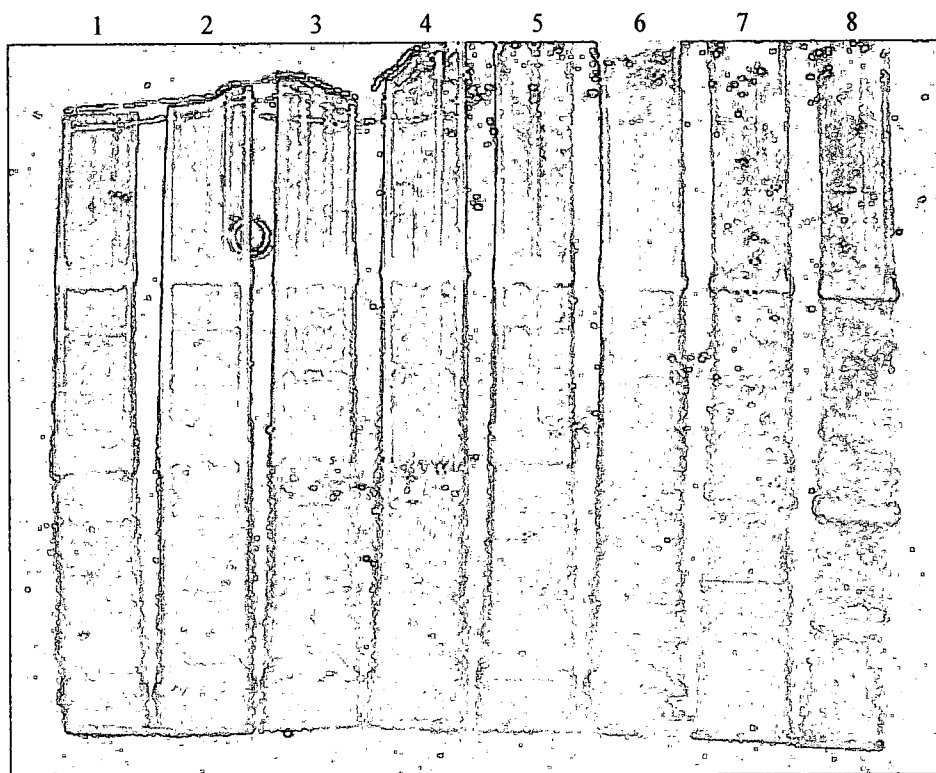


Figure 41. 12% SDS-PAGE analysis of extracellular proteins. *P. aeruginosa* cells grown overnight on LB plates or in LB broth were collected in CTX + 10mM MgCl₂ and passed through an 18-gauge needle of a syringe, then centrifuged to remove cells. Supernatant proteins were concentrated by 10% trichloroacetic acid (TCA) precipitation. Concentrated extracellular proteins were separated and analyzed on 12% SDS polyacrylamide gels (Biorad) and detected by Sypro Ruby staining (Invitrogen). Lanes 1 and 3, plate-grown MPAO1, lanes 2 and 4, plate-grown 31878; lanes 5 and 6, plate-grown MPAO1, lanes 7 and 8, broth-grown MPAO1.

DISCUSSION

PAO-B1 ($\Delta lasB$ mutant) cells obtained from the lab of Barbara Iglewski were elastase-deficient and were found to have several variant phenotypes from wild-type PAO1 cells. Macroscopic motility assays demonstrated that PAO-B1 mutant cells were reduced in twitching motility and swarming motility, but not swimming motility (Figures 8, 9, 10). Therefore, we concluded that *lasB* was essential for normal twitching and swarming motility, but did not play a role in swimming motility. A β -galactosidase assay showed that *lasB* expression did not vary according to these different motility conditions (Figure 12). Therefore, the involvement of *lasB* in twitching and swarming motility, but not swimming motility, could not be attributed to variation in the expression of *lasB*.

Western Blot analysis was performed on isolated surface pilin, and it was discovered that PAO-B1 mutant cells possessed a higher level of surface pilin than wild-type cells under twitch conditions (Figure 13B). By using transmission electron microscopy we discovered that PAO-B1 mutant cells were hyperpiliated in a peritrichous fashion (Figure 14). These results suggested the importance of LasB elastase in regulating the number and arrangement of TFP on the surface of *P. aeruginosa* cells. Other examples of hyperpiliated *P. aeruginosa* mutants that are also twitch-defective have been discovered (Deziel *et al.*, 2001). Pili-specific phage were used to test the functionality of the TFP. Based on these studies, it was evident that pili on PAO-B1

mutant cells were defective in retraction (Figure 16). Besides being somehow involved in regulating the number and location of pili assembled on the surface of the cell, LasB elastase appeared to be involved in the functioning of TFP.

The hyperpiliation observed on PAO-B1 cells by electron microscopy was interesting in light of recent findings that *P. aeruginosa* cells assembled surface structures called pseudopili when *xcpT* was artificially overexpressed (Durand *et al.*, 2003). The pseudopili were composed of XcpT subunits, homologous to TFP composed of PilA subunits. Some of the hyperpiliation seen on PAO-B1 cells may be attributed to not just pili but also pseudopili. Elastase is secreted through the Xcp secretion pathway (thus interacting with the Xcp secretion machinery), and perhaps the absence of elastase somehow led to *xcpT* overexpression. Or perhaps twitch conditions lead to *xcpT* overexpression. Besides appendages composed of entirely XcpT (pseudopili) or PilA (pili), appendages may also be composed of XcpT and PilA complexes. It has been shown that XcpT and PilA can form heterodimers (Hong-Mei *et al.*, 1997).

To investigate the possibility for XcpT-containing surface-appendages, Western Blot analysis using anti-XcpT antibody was performed on isolated surface appendages from twitch plate-grown cells. This showed the presence of XcpT in isolated surface appendages from PAO1 and PAO-B1 mutant cells (Figure 19). Our results demonstrated that wild-type cells could produce surface appendages containing XcpT even when XcpT was not being artificially overexpressed. Growth at an interface, such as we have done here by growing them on twitch plates, may trigger the natural overexpression of XcpT and result in appendages containing XcpT in addition to PilA.

Due to the numerous similarities between *Pseudomonas aeruginosa* and *Myxococcus xanthus*, we speculated that LasB elastase may be involved in the composition or function of the extracellular matrix (ECM). Congo Red and Alcian Blue dyes were used to assay for polysaccharides in the extracellular matrix. We found that compared to wild-type PAO1, PAO-B1 mutant cells did not possess the specific polysaccharides that bind Congo Red, but actually possessed more of the acidic polysaccharides that bind Alcian Blue (Figures 22, 23). Interestingly, elastase was involved in the correct composition of the matrix. Without elastase the matrix lacked some polysaccharides while possessing a larger amount of other polysaccharides. This could possibly be due to the secretion of elastase coordinating with the secretion of some polysaccharides, or to the action of elastase as a protease in the matrix.

If elastase plays a role in the composition of the ECM, then it is likely that elastase also plays a role in ECM structure. Through the use of scanning electron microscopy we directly observed the structure of the extracellular matrix of wild-type and mutant cells and observed the presence of long, thin fibrils extending between wild-type cells (Figure 24). In contrast, mutant cells displayed an ECM that appeared clumpy and did not extend from cell to cell. Besides an altered matrix composition, PAO-B1 cells exhibited an altered arrangement of the matrix. Elastase was found to be important in the composition and structure of the matrix. Elastase is known to degrade at least one ECM protein, CbpD, resulting in two degradation products (Foldes *et al.*, 2000). Elastase has long been known to act as a virulence factor that degrades human tissues. If elastase has substrates in the matrix and not just in human hosts, it would be expected that the composition and structure of the ECM would be affected by elastase.

Using transmission electron microscopy, we concluded that elastase plays a role in the production of flagella used in swarming motility based on our finding that unlike PAO1 cells, PAO-B1 mutant swarm cells possessed just a single polar flagellum instead of multiple polar flagella (Figure 21). Besides flagella, other factors have been determined to be necessary for swarming, such as the biosurfactant known as rhamnolipids (Kohler *et al.*, 2000). We assayed for rhamnolipids in PAO1 and PAO-B1 cells and discovered that PAO-B1 cells were greatly reduced in levels of rhamnolipids (Figure 27). However, the same was true in complemented PAO-B1 cells containing the *lasB* complementing plasmid. Based on the deficiency in rhamnolipids (the synthesis of which is dependent on quorum-sensing), we investigated the levels of the quorum-sensing system autoinducers in wild-type and mutant cells. We discovered a lack of C4-HSL in PAO-B1 cells (Figure 28). However, again we did not observe restoration in complemented PAO-B1 cells. Complemented mutant cells did not display restoration of rhamnolipids or C4-HSLs from greatly reduced mutant levels to full wild-type levels. The rhamnolipid and C4-HSL assays were the first instances in which a mutant phenotype could not be even partially complemented.

In summary, complemented PAO-B1 cells were fully restored in elastase activity, partially restored in twitching and swarming motility, but not restored in rhamnolipid and AHL production. The lack of rhamnolipids and C4-HSL in complemented PAO-B1 cells led us to believe that PAO-B1 mutant cells had a second-site mutation that was responsible for the phenotypes seen in PAO-B1 cells that could not be restored by complementation with the *lasB* containing plasmid, pRB1804SF. We were informed that the PAO-B1 mutant cells were constructed in a wild-type background that was only much

later determined to have an overexpression of the MexEF efflux pump. An overexpression of *mexEF* has been found to affect the production of virulence factors (including LasB elastase), levels of rhamnolipids, and levels of C4-HSL (Kohler *et al.*, 2001). In fact, *mexEF* overexpression led to a decrease in transcription of *rhlAB* and *rhlI*, the genes responsible for rhamnolipid and C4-HSL synthesis. This would explain the decreased levels of both of these molecules in PAO-B1 cells that overexpress *MexEF*. However, if our experiments had been comparing PAO-B1 cells to their parent cells, like we thought, we would not have seen any differences in the levels of rhamnolipids and C4-HSL.

We were also informed that “wild-type” strains of PAO1 differed widely. Some had the *MexEF* overexpression and some did not. The *mexEF* overexpression leads to a large decrease in rhamnolipid production. If the wild-type we had been working with was the parent of PAO-B1 and contained the *mexEF* overexpression, we would have found low levels of rhamnolipid production no different from PAO-B1. However, the wild-type PAO1 we were working with produced high levels of rhamnolipids (Figure 27). The results of the rhamnolipid and AHL assays, together with the information provided to us about the PAO-B1 and wild-type strains, led us to believe that we were not working with the parent of PAO-B1. Instead, we had a wild-type PAO1 that differed in respect to not possessing the *mexEF* overexpression. Therefore, wild-type cells (no *mexEF* overexpression) would show high levels of rhamnolipids and C4-HSL, but PAO-B1 mutant cells (*mexEF* overexpression) would show low levels of rhamnolipids and C4-HSL. This is what our results have shown (Figures 27, 28).

To determine if any of the phenotypic differences we had observed in PAO-B1 mutant cells were due to the *lasB* mutation and not overexpression of *mexEF*, we tested different isogenic Δ *lasB* mutants provided to us by the University of Washington transposon mutant library. The mutants were constructed in the wild-type MPAO1 strain which is known to overexpress *mexEF*. The first step was to determine which of the several Δ *lasB* mutants provided to us were truly deficient in elastase activity. We assayed the mutants for elastase activity on elastin plates and on the Elastin-Congo Red (ECR) conjugate used previously in the elastase assay performed with the PAO-B1 mutant. We discovered that Δ *lasB* mutant 31878 produced a reduced clearing zone on elastin plates, indicating less elastase activity than MPAO1 (Figure 29). The presence of a remaining slight clearing of elastin in the absence of LasB elastase can be attributed to the protease activity of LasA (Toder *et al.*, 1991). The elastolysis assay testing for the activity of LasB elastase on ECR showed the complete absence of elastase activity in 31878 mutant cells (Figure 30). Complemented 31878 mutant cells, constructed by transformation of 31878 mutant cells with the *lasB* complementing plasmid, exhibited a full restoration of elastase activity. This indicated that the *lasB* gene in 31878 cells was disrupted.

Final verification of the *phoA* transposon insertion in the *lasB* gene was accomplished by PCR confirmation using primers designed to the *lasB* gene and the *phoA* transposon sequences. The genomic primer was designed to begin at base pair 11 in the *lasB* gene, while the transposon was inserted at base pair 520 in the *lasB* gene. The PCR product should have been 647 base pairs due to 509 base pairs of *lasB* gene sequence plus 138 base pairs of transposon sequence in between the two primers. The

PCR product from 31878 mutant cells corresponded to the expected size product for an insertion of the *phoA* transposon in the *lasB* gene (Figure 31).

Following verification of elastase-deficient 31878 *lasB* mutant cells, we began the process of determining if any of the phenotypes we had attributed to LasB elastase from assays using PAO-B1 mutant cells could still be attributed to *lasB*. Twitching and swarming motility assays were performed comparing MPAO1 wild-type cells and 31878 mutant cells. We found that neither twitching motility nor swarming motility were reduced in 31878 mutant cells compared to MPAO1 cells (Figures 32, 33). This indicated that *lasB* was not essential for motility. The previous differences seen in motility in PAO-B1 cells could be attributed to overexpression of the MexEF efflux pump, or any other difference possibly existing between PAO-B1 mutant cells and PAO1 wild-type cells that were not the background parent strain of PAO-B1. However, MPAO1, 31878, and PAO-B1 cells (which all overexpress *mexEF*) twitch equivalently to PAO1 cells (which do not overexpress *mexEF*). Therefore, it can be concluded that *mexEF* overexpression does not affect twitching motility. The aberrant twitching motility seen in PAO-B1 mutant cells must have been due to another mutation.

Since twitching motility was not affected by a *lasB* mutation, as shown by motility assays comparing MPAO1 and 31878 cells, we believed that type IV pili production essential for twitching motility would not be affected either. We confirmed this when we performed a Western Blot analysis of surface pilin and XcpT. We did not discover any differences between MPAO1 and 31878 cells (Figures 34, 35). Therefore, *lasB* did not affect the assembly of TFP. LasB elastase also did not affect the function of TFP since 31878 cells twitch equivalently to MPAO1 cells.

The possibility of reduced swarming motility in PAO-B1 cells being caused by *mexEF* overexpression is likely and consistent with the findings of Kohler *et al.* (2001). They found that MexEF overexpression results in reduced transcription of *rhlAB*, which would lead to reduced rhamnolipids, which Kohler *et al.* (2000) found to be essential for swarming. MPAO1, 31878, and PAO-B1 cells (which all overexpress *mexEF*) were reduced in swarming motility compared to PAO1 cells (which do not overexpress *mexEF*). Therefore, it appears that the decrease in swarming motility in PAO-B1 cells was due to the overexpression of *mexEF*.

Since swarming motility were not affected by a *lasB* mutation, as shown by motility assays comparing MPAO1 and 31878 cells, we believed that rhamnolipid production essential for swarming would not be affected either. We confirmed this when we performed a rhamnolipid plate assay that showed equivalent rhamnolipid production between MPAO1 and 31878 cells (Figure 35). We also discovered that MPAO1 and 31878 cells produced severely reduced levels of rhamnolipids compared to PAO1 cells used previously. This is consistent with the fact that the MPAO1 wild-type strain used by the University of Washington for the transposon mutant library was obtained from B. Iglewski's lab and contained the *mexEF* overexpression, leading to reduced levels of the biosurfactant. It also shows that the PAO1 wild-type strain that was originally sent to us by Dr. Iglewski's lab and used previously in our lab to compare to PAO-B1 cells did not possess the *mexEF* overexpression because it produces high levels of rhamnolipids compared to strains with the overexpression. This finding also confirms that PAO1 wild-type used in previous assays with PAO-B1 was indeed not the wild-type strain that contained the *mexEF* overexpression in which PAO-B1 was created. AHL levels

between MPAO1 and 31878 were also found to be equivalent, indicating no role of *lasB* in AHL synthesis (Figure 36). Again, this finding is consistent with the discovery that *mexEF* overexpression leads to reduced C4-HSL levels (Kohler *et al.*, 2001). In conclusion, the reduced levels of rhamnolipids and C4-HSL in PAO-B1 cells could be attributed to the overexpression in *mexEF*.

It was still reasonable to hypothesize that LasB elastase plays a role in the composition and structure of the extracellular matrix because of its function as a protease. It has been shown to act on at least one other ECM protein, CbpD (Folders *et al.*, 2000). The absence of LasB elastase in the ECM of 31878 would likely affect the composition and structure of the ECM if elastase has substrates in the ECM. Therefore, we sought to elucidate differences in the matrix proteins of wild-type and $\Delta lasB$ mutant cells. Isolated extracellular protein mixtures were separated by 1D-gel electrophoresis (SDS-PAGE) and visualized by staining proteins in the gel. We verified that the extracellular protein profile of 31878 cells differed from that of MPAO1 cells. Several differences were found between 36 and 53kDa, just above 53,500Da, and just under 71kDa (Figures 37, 38). It is possible that the protein components of the ECM differ from MPAO1 to 31878 cells because of elastase acting on substrates and forming degradation products, or because of the interaction of elastase with other proteins during secretion. Many extracellular proteins are secreted via the Type II secretion system, and elastase may interact with these proteins during secretion.

Interestingly, we also discovered differences in extracellular proteins between wild-type plate-grown and broth-grown cells at 36,100Da, 29,500Da, and just below 21,300Da (Figures 39, 40). In some of these cases there is a band present in one sample

that appears to be absent in the other sample. However, in the sample where the band appears to be absent, there is another band present that is a slightly different molecular weight. The differences in banding patterns may be due to the presence or absence of proteins, or due to modifications in proteins that caused a molecular weight change and therefore shifted the bands slightly higher or lower. It will be interesting to determine the exact extracellular protein differences between plate-grown and broth-grown cells because it may lead to the discovery of proteins involved in adhering to or moving across surfaces.

The next step towards identification of the differentially expressed proteins includes extraction of proteins from the gel, trypsin digestion, and analysis by mass spectrometry (MS). Silver staining is incompatible with MS analysis, and so we attempted to use SyproRuby staining to detect the proteins. SyproRuby stain does not interfere with mass spectrometry, and we could therefore subsequently extract, digest, and identify the protein differences by MS analysis. However, we could not get enough resolution with SyproRuby staining to discern individual bands that consist of just one individual protein (Figure 41). The protein mixtures must be separated completely so that individual proteins can be extracted from the gel and identified.

Determining the identity of differentially expressed extracellular proteins in *ΔlasB* mutant cells is important for elucidating previously undiscovered functions of LasB elastase. It could lead to the identification of novel substrates of LasB and novel cellular roles for LasB in the functioning of *P. aeruginosa* cells. Future work stemming from this project will include analysis and identification of differentially expressed extracellular proteins by mass spectrometry (MS). Isolated extracellular proteins must be separated by

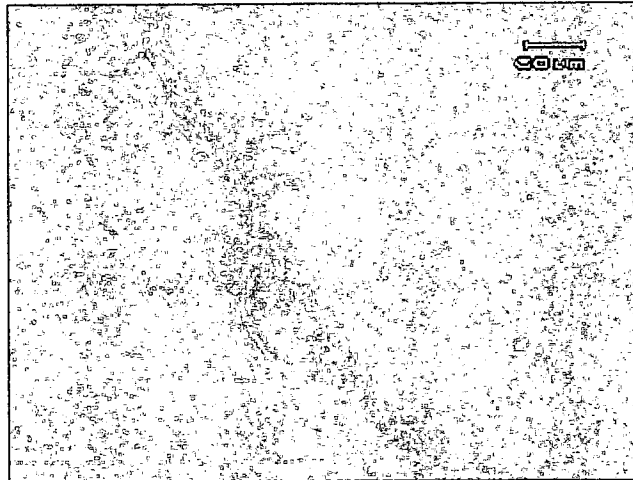
2D-gel electrophoresis to obtain optimal separation and resolution of proteins for mass spectrometry identification. Another aspect of the matrix to investigate is its structure. Since differences exist between MPA1 and 31878 matrix proteins, the structure of the matrix would likely vary also. MPAO1 and 31878 cells' matrix structure should be compared by scanning electron microscopy. The differences viewed by SEM in matrix structure between PAO1 and PAO-B1 cells may be due to LasB elastase. It seems likely that these differences may still be seen between MPAO1 and 31878 cells. Characterizing the matrix of *P. aeruginosa* cells is an interesting research area because of the importance of the ECM in biofilm formation, and thus host infection.

Work completed outside of this thesis has shown that plate-grown *P. aeruginosa* MPAO1 and 31878 cells also differed in their ability to colonize *Candida albicans*, another human pathogen. *P. aeruginosa* and *C. albicans* coexist within the human host (Hermann *et al.*, 1999), and *P. aeruginosa* has been shown to limit growth of *C. albicans* (Kerr, 1994; Burns *et al.*, 1999). We found that while large groups of wild-type MPAO1 cells colonized *C. albicans*, 31878 mutant cells were deficient in colonization (Appendix 1). This may be due to differences in the ECM proteins. Identification of matrix protein differences between wild-type and mutant may lead to the discovery of a protein essential for colonization of *C. albicans* by *P. aeruginosa*.

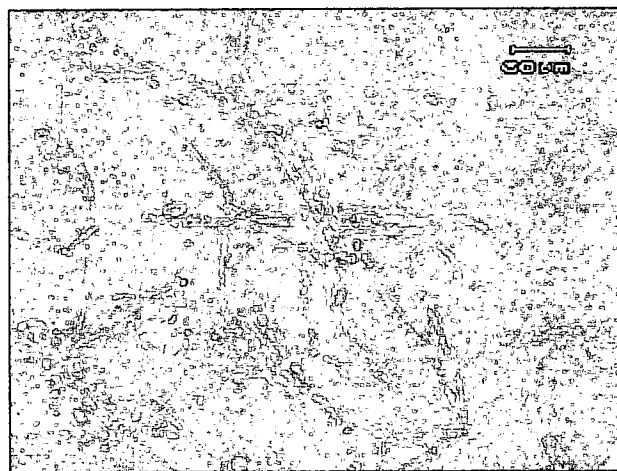
Our results have generated another interesting area of research to investigate as well. We discovered the presence of XcpT (pseudopili subunits) on the surface of *P. aeruginosa* PAO1 wild-type cells grown at a solid-solid interface. To our knowledge, this is the first report of this occurrence in a situation other than under artificial *xcpT* overexpression. Perhaps XcpT-containing pseudopili or complexed XcpT/PilA-

containing pili are assembled and used during twitching motility across solid surfaces. It could be determined whether twitch conditions lead to an overexpression of XcpT. It would be interesting to investigate further the purpose of XcpT-containing surface appendages and when they are produced.

APPENDIX 1



A



B

Figure 42. Colonization of *Candida albicans* by *Pseudomonas aeruginosa*. *C. albicans* cells grown overnight were washed and resuspended in YNB media before the addition of plate-grown *P. aeruginosa* cells. The co-culture was incubated statically overnight at 37°C and viewed using light microscopy. (A) MPAO1 + *C. albicans*, (B) 31878 + *C. albicans*.

REFERENCES

- Adler, J. 1966. Chemotaxis in bacteria. *Science* 153:708-16.
- Albus, A. M., E. C. Pesci, L. J. Runyen-Janecky, S. E. H. West, and B. H. Iglewski. 1997. Vfr controls quorum sensing in *Pseudomonas aeruginosa*. *J. Bacteriol.* 179:3928-35.
- Alm, R. A., J. P. Hallinan, A. A. Watson, and J. S. Mattick, 1996. Fimbrial biogenesis genes of *Pseudomonas aeruginosa*: *pilW* and *pilX* increase the similarity of type 4 fimbriae to the GSP protein-secretion systems and *pilYI* encodes a gonococcal PilC homologue. *Mol. Microbiol.* 22:161-73.
- Alm, R. A., and J. S. Mattick. 1995. Identification of a gene, *pilV*, required for type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*, whose product possesses a prepilin-like leader sequence. *Mol. Microbiol.* 16:485-96.
- Alm, R. A., and J. S. Mattick. 1996. Identification of two genes with prepilin-like leader sequences involved in type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 178:3809-17.
- Alm, R. A., and J. S. Mattick. 1997. Genes involved in the biogenesis and function of type-4 fimbriae in *Pseudomonas aeruginosa*. *Gene* 192:89-98.
- Arnold, J. W., and L. J. Shimkets. 1988. Cell surface properties correlated with cohesion in *Myxococcus xanthus*. *J. Bacteriol.* 170:5771-5777.
- Arnold, J. W., and L. J. Shimkets. 1988. Inhibition of cell-cell interactions in *Myxococcus xanthus* by congo red. *J. Bacteriol.* 170:5765-5770.
- Ball, G., E. Durand, A. Lazdunski, and A. Filloux. 2002. A novel type II secretion system in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 43:475-485.

- Bally, M., A. Filloux, M. Akrim, G. Ball, A. Lazdunski, and J. Tommassen. 1992. Protein secretion in *Pseudomonas aeruginosa*: characterization of seven *xcp* genes and processing of secretory apparatus components by prepilin peptidase. *Mol. Microbiol.* 6:1121-1131.
- Behmlander, R., and M. Dworkin. 1994. Biochemical and structural analyses of the extracellular matrix fibrils of *Myxococcus xanthus*. *J. Bacteriol.* 176: 6295-6303.
- Bever, R. A., and B. H. Iglewski. 1988. Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. *J. Bacteriol.* 170:4309-14.
- Bitter, W., and J. Tommassen. 1999. Ushers and other doorkeepers. *Trends Microbiol.* 7:4-6.
- Bjorn, M. J., P. A. Sokol, and B. H. Iglewski. 1979. Influence of iron on yields of extracellular products in *Pseudomonas aeruginosa* cultures. *J. Bacteriol.* 138:193-200.
- Bleves, S., R. Voulhoux, G. Michel, A. Lazdunski, J. Tommassen, and A. Filloux. 1998. The secretion apparatus of *Pseudomonas aeruginosa*: identification of a fifth pseudopilin, XcpX (GspK family). *Mol. Microbiol.* 27:31-40.
- Bodey, G. P., R. Bolivar, V. Fainstein, and L. Jadeja. 1983. Infections caused by *Pseudomonas aeruginosa*. *Rev. Infect. Dis.* 5:279-313.
- Boyd, J. M. and S. Lory. 1996. Dual function of PilS during transcriptional activation of the *Pseudomonas aeruginosa* pilin subunit gene. *J. Bacteriol.* 178:831-39.
- Boyd, J. M. 2000. Localization of the histidine kinase PilS to the poles of *Pseudomonas aeruginosa* and identification of a localization domain. *Mol. Microbiol.* 36:153-62.
- Bradley, D. E. 1980. A function of *Pseudomonas aeruginosa* PAO pili: twitching motility. *Can. J. Microbiol.* 26:146-54.
- Braun, P., A. de Groot, W. Bitter, and J. Tommassen. 1998. Secretion of elastinolytic enzymes and their propeptides by *Pseudomonas aeruginosa*. *J. Bacteriol.* 180:3467-3469.
- Braun, P., J. Tommassen, and A. Filloux. 1996. Role of the propeptide in folding and secretion of elastase of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 19:297-306.

- Braun, P., A. de Groot, W. Bitter, and J. Tommassen. 1998. Secretion of elastinolytic enzymes and their propeptides by *Pseudomonas aeruginosa*. J. Bacteriol. 180:3467-69.
- Burns, J., J. Van Dalfsen, R. Shawar, K. Otto, R. Garber, and J. Quan. 1999. Effect of intermittent administration of inhaled tobramycin on respiratory microbial flora in patients with cystic fibrosis. J. Infect. Dis. 179:1190-96.
- Calfee, M. W., J. P. Coleman, and E. C. Pesci. 2001. Interference with *Pseudomonas* quinolone signal synthesis inhibits virulence factor expression by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 98:11633-11637.
- Carpenter, B., and O. Cerf. 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. J. Appl. Bacteriol. 75:499-511.
- Castric, P., F. J. Cassels, and R. W. Carlson. 2001. Structural characterization of the *Pseudomonas aeruginosa* 1244 pilin glycan. J. Biol. Chem. 276:26479-85.
- Chiang, P. and L.L. Burrows. 2003. Biofilm formation by hyperpilated mutants of *Pseudomonas aeruginosa*. J. Bacteriol. 185: 374-2378.
- Chugani, S. A., M. Whiteley, K. M. Lee, D. D'Argenio, C. Manoil, and E. P. Greenberg. 2001. QscR, a modulator of quorum sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 98:2752-57.
- Comolli, J. C., A. R. Hauser, L. Waite, C. B. Whitchurch, J. S. Mattick, and J. N. Engel. 1999. *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity in vitro and virulence in a mouse model of acute pneumonia. Infect. Immun. 67:3625-30.
- Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284:1318-22.
- Cowell, B. A., S. S. Twining, J. A. Hobden, M. S. Kwong, and S. M. Fleiszig. 2003. Mutation of *lasA* and *lasB* reduces *Pseudomonas aeruginosa* invasion of epithelial cells. Microbiol. 149:2291-2299.
- Croft, L., S. A. Beatson, C. B. Whitchurch, B. Huang, R. L. Blakeley, and J. S. Mattick. 2000. An interactive web-based *Pseudomonas aeruginosa* genome database: discovery of new genes, pathways, and structures. Microbiology 146:2351-64.
- Darzens, A. 1993. The *pilG* gene product, required for *Pseudomonas aeruginosa* pilus production and twitching motility, is homologous to the enteric, single domain response regulator CheY. J. Bacteriol. 175:5934-44.

- Darzins, A. 1994. Characterization of a *Pseudomonas aeruginosa* gene cluster involved in pilus biosynthesis and twitching motility: sequence similarity to the chemotaxis proteins of enterics and the gliding bacterium *Myxococcus xanthus*. *Mol. Microbiol.* 11:137-53.
- Darzins, A., and M. A. Russell. 1997. Molecular genetic analysis of type-4 pilus biogenesis and twitching motility using *Pseudomonas aeruginosa* as a model system – a review. *Gene* 192:109-15.
- Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280:295-98.
- DeKievit, T. R., p. c. Seed, L. Passador, J. Nezezon, and B. H. Iglewski. 1999. RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*. *J. Bacteriol.* 181:2175-84.
- Deziel, E., Y. Comeau, and R. Villemur. 2001. Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpilated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *J. Bacteriol.* 183: 1195-1204.
- Diggel, S. P., K. Winzer, A. Lazdunski, P. Williams, and M. Camara. 2002. Advancing the quorum in *Pseudomonas aeruginosa*: MvaT and the regulation of *N*-acyl homoserine lactone production and virulence gene expression. *J. Bacteriol.* 184:2576-86.
- Doring, G., H.-J. Obernesser, and K. Botzenhart. 1981. Extracellular toxins of *Pseudomonas aeruginosa*. II. Effect of two proteases on human immunoglobulins IgG, IgA and secretory IgA. *Zentralbl. Bakteriell. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A* 249:89-98.
- Dunne, W. M. 2002. Bacterial adhesion: Seen any good biofilms lately? *Clin. Microbiol.* 15:155-166.
- Durand, E., A. Bernadac, G. Ball, A. Lazdunski, J. N. Sturgis, and A. Filloux. 2003. Type II protein secretion in *Pseudomonas aeruginosa*: the pseudopilus is a multifibrillar and adhesive structure. *J. Bacteriol.* 185:2749-58.
- Eberl, L., M. K. Winston, C. Sternberg, G. S. Stewart, G. Christiansen, S. R. Chhabra, B. Bycroft, P. Williams, S. Molin, and M. Givskov. 1996. Involvement of *N*-Acyl-L-homoserine lactone autoinducers in controlling the multicellular behavior of *Serratia liquefaciens*. *Mol. Microbiol.* 20:127-36.

- Feldman, M. R., R. Bryan, S. Rajan, L. Scheffler, S. Brunnert, H. Tang, and A. Prince. 1998. Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect. Immun.* 66:43-51.
- Ferrandez, A., A. C. Hawkins, D. T. Summerfield, and C. S. Harwood. 2002. Cluster II che genes from *Pseudomonas aeruginosa* are required for an optimal chemotactic response. *J. Bacteriol.* 184:4374-83.
- Filloux, A., G. Michel, and M. Bally. 1998. GSP-dependent protein secretion in gram-negative bacteria: the Xcp system of *Pseudomonas aeruginosa*. *FEMS Microbiol. Rev.* 22:177-198.
- Filloux, A., M. Bally, G. Ball, M. Akrim, J. Tommassen, and A. Lazdunski. 1990. Protein secretion in Gram-negative bacteria: transport across the outer membrane involves common mechanisms in different bacteria. *EMBO J.* 9:4323-29.
- Folders, J., J. Tommassen, L. C. van Loon, and W. Bitter. 2000. Identification of a chitin-binding protein secreted by *Pseudomonas aeruginosa*. *J. Bacteriol.* 182:1257-1263.
- Fuqua, C., M. R. Parsek, and E. P. Greenberg. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* 35:439-68.
- Gallagher, L. A., S. L. McKnight, M. S. Kuznetsova, E. C. Pesci, and C. Manoil. 2002. Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J. Bacteriol.* 184:6472-6480.
- Gambello, M. J., and B. H. Iglewski. 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *J. Bacteriol.* 173:3000-09.
- Glessner, A., R. S. Smith, B. H. Iglewski, and J. B. Robinson. 1999. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of twitching motility. *J. Bacteriol.* 181:1623-29.
- Gustin, J. K., E. Kessler, and D. E. Ohman. 1996. A substitution of His-120 in the LasA protease of *Pseudomonas aeruginosa* blocks enzymatic activity without affecting propeptide processing or extracellular secretion. *J. Bacteriol.* 178:6608-17.
- Gygi, D., M. M. Rahman, H. C. Lai, R. Carlson, J. Guard-Petter, and C. Hughes. 1995. A cell-surface polysaccharide that facilitates rapid population migration by differentiated swarm cells of *Proteus mirabilis*. *Mol. Microbiol.* 17:1167-75.

- Hazes, B., P. A. Sastry, K. Hayakawa, R. J. Read, and R. T. Irvin. 2000. Crystal structure of *Pseudomonas aeruginosa* PAK pilin suggests a main-chain-dominated mode of receptor binding. *J. Mol. Biol.* 299:1005-17.
- Hazlett, L. D., M. M. Moon, A. Singh, R. S. Berk, and X. L. Rudner. 1991. Analysis of adhesion, piliation, protease production and ocular infectivity of several *P. aeruginosa* strains. *Curr. Eye Res.* 10:351-62.
- Heck, L. W., P. G. Alarcon, R. M. Kulhavy, K. Morihara, M. W. Russell, and J. F. Mestecky. 1990. Degradation of IgA proteins by *Pseudomonas aeruginosa* elastase. *J. Immunol.* 144:2253-57.
- Heck, L. W., K. Morihara, W. B. McRae, and E. J. Miller. 1986. Specific cleavage of human type III and IV collagens by *Pseudomonas aeruginosa* elastase. *Infect. Immunol.* 51:115-18.
- Hentzer, M., H. Wu, J.B. Anderson, K. Riedel, T.B. Rasmussen, N. Bagge, N. Kumar, M.A. Schembri, Z. Song, P. Kristoffersen, M. Manefield, J.W. Costerton, S. Molin, L. Eberl, P. Steinberg, S. Kjelleberg, N. Hoiby, and M. Givskov. 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J.* 22:3803-15.
- Hermann, C., J. Hermann, U. Munzel, and R. Ruchel. 1999. Bacterial flora accompanying *Candida* yeasts in clinical specimens. *Myoceses.* 42:619-27.
- Heurlier K., V. Denervaud, G. Pessi, C. Reimmann, and D. Haas. 2003. Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 185:2227-35.
- Hobbs, M., and J. S. Mattick. 1993. Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage, and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. *Mol. Microbiol.* 10:233-43.
- Hong, Y., and B. Ghebrehiwet. 1992. Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components Clq and C3. *Clin. Immunol. Immunopathol.* 62:133-38.
- Hong-Mei, L., S.T. Motley, and S. Lory. 1997. Interactions of the components of the general secretion pathway: role of *Pseudomonas aeruginosa* type IV pilin subunits in complex formation and extracellular protein secretion. *Mol. Microbiol.* 25:247-59.

- Jacobs, M. A., A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. Raymond, R. Levy, L. Chun-Rong, D. Guenther, D. Bovee, M. V. Olson, and C. Manoil. 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. PNAS. 100:14339-44.
- Joklik, W. K., H. P. Willett, D. B. Amos, and C. M. Wilfert. 1992. *Pseudomonas*, In W. K. Joklik (ed.), Zinsser Microbiology, 20th ed. Appleton and Lange, Norwalk, Conn. pp. 576-583.
- Jones, C. H., P. N. Danese, J. S. Pinkner, T. J. Silhavy, and S. J. Hultgren. 1997. The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. EMBO J. 16:6394-6406.
- Kaiser, D. 1979. Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA 76:5952-56.
- Kaiser, D., and C. Crosby. 1983. Cell movement and its coordination in swarms of *Myxococcus xanthus*. Cell Motil. 3:227-245.
- Kato, J., T. Nakamura, A. Kuroda, and H. Ohtake. 1999. Cloning and characterization of Chemotaxis genes in *Pseudomonas aeruginosa*. Biosci. Biotechnol. Biochem. 63:155-61.
- Kearns, D. B., P.J. Bonner, D. R. Smith, and L. J. Shimkets. 2002. An extracellular matrix-associated zinc metalloprotease is required for dilauroyl phosphatidylethanolamine chemotactic excitation in *Myxococcus xanthus*. J. Bacteriol. 184:1678-1684.
- Kearns, D. B., J. B. Robinson, and L. J. Shimkets. 2001. *Pseudomonas aeruginosa* exhibits directed twitching motility up phosphatidylethanolamine gradients. J. Bacteriol. 183:763-7.
- Keizer, D.W., C. M. Slusky, M. Kalisiak, A. P. Campbell, M. P. Crump, P. A. Sastry, B. Hazes, R. T. Irvin, and B. D. Sykes. 2001. Structure of a pilin monomer from *Pseudomonas aeruginosa*: implications for the assembly of pili. J. Biol. Chem. 276:24186-93.
- Kerr, J. R. 1994. Suppression of fungal growth exhibited by *Pseudomonas aeruginosa*. J. Clin. Microbiol. 32:525-7.
- Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. Pechere. 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. 182:5990-96.

- Latifi, A., M. K. Winson, M. Foglino, B. W. Bycroft, G. S. A. B. Stewart, A. Lazdunski, and P. Williams. 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Mol. Microbiol.* 17:333-43.
- Latifi, A., M. Foglino, K. Tanaka, P. Williams, and A. Lazdunski. 1996. A hierarchical quorum sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR to expression of stationary phase sigma factor RpoS. *Mol. Microbiol.* 21:1137-46.
- Lauer, P., N. H. Albertson, and M. Koomey. 1993. Conservation of genes encoding components of a type IV pilus assembly/two-step protein export pathway in *Neisseria gonorrhoeae*. *Mol. Microbiol.* 8:357-68.
- Lautrop, H. 1961. *Bacterium anitratum* transferred to the genus *Cytophaga*. *Int. Bull. Bacteriol. Nomencl.* 11:107-8.
- Lawrence, J. R., P. J. Delaquis, D. R. Korber, and D. E. Caldwell. 1987. Behavior of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. *Microb. Ecol.* 14:1-14.
- Ledgham, F., C. Soscia, A. Chakrabarty, A. Lazdunski, and M. Foglino. 2003. Global regulation in *Pseudomonas aeruginosa*: the regulatory protein AlgR2 (AlgQ) acts as a modulator of quorum sensing. *Res Microbiol.* 154:207-13.
- Ledgham F., I. Ventre, C. Soscia, M. Foglino, J. N. Sturgis, and A. Lazdunski. 2003. Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhIR. *Mol Microbiol.* 48:199-210.
- Li, Y., H. Sun, X. Ma, A. Lu, R. Lux, D. Zusman, and W. Shi. 2003. Extracellular polysaccharides mediate pilus retraction during social motility of *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. USA* 100:5443-5448.
- Lindum, P. W., U. Anthoni, C. Christophersen, L. Eberl, S. Molin, and M. Givskov. 1998. *N*-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of *Serratia liquefaciens* MG1. *J. Bacteriol.* 180:6384-88.
- Lory, S., and M. S. Strom. 1997. Structure-function relationship of type-IV prepilin peptidase of *Pseudomonas aeruginosa* - a review. *Gene* 192:117-21.
- Mahajan-Miklos, S., L.G. Rahme, and F.M. Ausubel. 2000. Elucidating the molecular mechanisms of bacterial virulence using non-mammalian hosts. *Mol. Microbiol.* 37:981-8.

- Maier, B., L. Potter, M. So, H.S. Seifert, and M.P. Sheetz. 2002. Single pilus motor forces exceed 100 pN. *Proc. Natl. Acad. Sci. USA* 99:16012-16017.
- Martin, P. R., M. Hobbs, P. D. Free, Y. Jeske, and J.S. Mattick. 1993. Characterization of *pilQ*, a new gene required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 9:857-68.
- Masduki, A., J. Nakamura, T. Ohga, R. Umezaki, J. Kato, and H. Ohtake. 1995. Isolation and characterization of chemotaxis mutants and genes of *Pseudomonas aeruginosa*. *J. Bacteriol.* 177:948-52.
- Mattick, J. S. 2002. Type IV pili and twitching motility. *Annu. Rev. Microbiol.* 56:289-314.
- McIver, K., E. Kessler, and D. E. Ohman. 1991. Substitution of active-site His-223 in *Pseudomonas aeruginosa* elastase and expression of mutated *lasB* alleles in *Escherichia coli* show evidence for autoproteolytic processing of proelastase. *J. Bacteriol.* 173:7781-89.
- McIver, K. S., E. Kessler, J. C. Olson, and D. E. Ohman. 1995. The elastase propeptide functions as an intramolecular chaperone required for elastase activity and secretion in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 18:877-89.
- McKenney, D., K. E. Brown, and D. G. Allison. 1995. Influence of *Pseudomonas aeruginosa* exoproducts on virulence factor production in *Burkholderia cepacia*: evidence of interspecies communication. *J. Bacteriol.* 177:6989-92.
- McKnight, S. L., B. H. Iglewski, and E. C. Pesci. 2000. The of *Pseudomonas* quinolone signal regulates *rhl* quorum-sensing in *Pseudomonas aeruginosa*. *J. Bacteriol.* 182:2702-08.
- Merz, A.J., M. So, and M.P. Sheetz. 2000. Pilus retraction powers bacterial twitching motility. *Nature* 407:98-102.
- Miller, M. B., and B. L. Bassler. 2001. Quorum sensing in bacteria. *Ann. Rev. Microbiol.* 55:165-199.
- Moriyama, K. 1964. Production of elastase and proteinase by *Pseudomonas aeruginosa*. *J. Bacteriol.* 88:745-57.
- Moriyama, K., and J. Y. Homma. 1985. In *Bacterial Enzymes and Virulence*. I. A. Holders (ed.), CBC Press, Boca Raton, FL, pp. 41-79.
- Nealson, K. H., T. Platt, and J. W. Hastings. 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol.* 104:313-22.

- Nicas, T. I., and B. H. Iglewski. 1985. The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. *Can. J. Microbiol.* 31:387-392.
- Nouwens, A. S., M. D. Willcox, B. J. Walsh, and S. J. Cordwell. 2002. Proteomic comparison of membrane and extracellular proteins from invasive (PAO1) and cytotoxic (6206) strains of *Pseudomonas aeruginosa*. *Proteomics* 2:1325-1346.
- Nouwens, A. S., S. A. Beatson, C. B. Whitchurch, B. J. Walsh, H. P. Schweizer, J. S. Mattick, and S. J. Cordwell. 2003. Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. *Microbiology* 149:1311-1322.
- Nunn, D., S. Bergman, and S. Lory. 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. *J. Bacteriol.* 172:2911-19.
- Nunn, D. N., and S. Lory. 1991. Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. *Proc. Natl. Acad. Sci. USA* 88:3281-3285.
- Nunn, D. N., and S. Lory. 1993. Cleavage, methylation, and localization of the *Pseudomonas aeruginosa* export proteins XcpT, -U, -V, and -W. *J. Bacteriol.* 175:4375-4382.
- Ochsner, U. A., and J. Reiser. 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 92:6424-28.
- Okamoto, S. and M. Ohmori. 2002. The cyanobacterial PilT protein responsible for cell motility and transformation hydrolyzes ATP. *Plant Cell Physiol.* 43:1127-1136.
- O'Toole, G. A., and R. Kolter. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* 30:295-304.
- Parmely, M., A. Gale, M. Clabaugh, R. Horvat, and W. W. Zhou. 1990. Proteolytic inactivation of cytokines by *Pseudomonas aeruginosa*. *Infect. Immun.* 58:3009-14.
- Parsek, M. R., and E. P. Greenberg. 2000. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. *PNAS.* 97: 8789-93.
- Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski. 1993. Expression of the *Pseudomonas aeruginosa* virulence genes requires cell-cell communication. *Science* 260:1127-30.

- Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg. 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc. Natl. Acad. Sci. USA* 91:197-201.
- Pearson, J. P., L. Passador, B. H. Iglewski, and E. P. Greenberg. 1995. A second *N*-acyl homoserine lactone signal produced by *P. aeruginosa*. *Proc. Natl. Acad. Sci. USA* 92:1490-94.
- Pearson, J. P., E. C. Pesci, and B. H. Iglewski. 1997. Poles of *Pseudomonas aeruginosa* *las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J. Bacteriol.* 179:5756-5767.
- Pesci, E. C., J. P. Pearson, P. C. Seed, and B. H. Iglewski. 1997. Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J. Bacteriol.* 179:3127-32.
- Pesci, E. C., J. B. J. Milbank, J. P. Pearson, S. McKnight, A. S. Kende, E. P. Greenberg, and B. H. Iglewski. 1999. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 96:11229-11234.
- Pesci, G., F. Williams, Z. Hindle, K. Heurlier, M. T. G. Holden, M. Camara, D. Haas, and P. Williams. 2001. The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J. Bacteriol.* 183:6676-83.
- Planet, P. J., S. C. Kachlany, R. DeSalle, and D. H. Figurski. 2001. Phylogeny of genes for secretion NTPases: identification of the widespread *tadA* subfamily and development of a diagnostic key for gene classification. *Proc. Natl. Acad. Sci. USA* 98:2503-2508.
- Pollack, M. 1990. *Pseudomonas aeruginosa*, In G. L. Mandell, R. G. Douglas, and J. E. Bennett (ed.), *Principles and practices of infectious diseases*. Churchill Livingstone, Edinburgh, Scotland, pp. 1673-1691.
- Potera, C. 1999. Forging a link between biofilms and disease. *Science* 283:1837, 1839.
- Potvin, E., D. Lehoux, I. Kukavica-Ibrulj, K. Richard, F. Sanschagrin, G.W. Lau, and R.C. Levesque. 2003. *in vivo* functional genomics of *Pseudomonas aeruginosa* for high throughput screening of new virulence factors and antibacterial targets. Abstract #88 *Pseudomonas* 2003 International Meeting, Quebec, CA September 6-10.
- Rashid, M. H., and A. Kornberg. 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 97:4885-90.

- Rahme, L.G., F.M. Ausubel, H. Cao, E. Drenkard, B.C. Gourmneroy, G.W. Lau, S. Mahajan-Miklos, J. Plotnikova, M.W. Tan, J. Tsongalis, C.L. Walendziewica, and R.B. Tompkins. 2000. Plants and animals share functionally common bacterial virulence factors. *Proc. Natl. Acad. Sci. USA* 97:8815-21.
- Reimmann, C., M. Beyeler, A. Latifi, H. Winteler, M. Foglino, A. Lazdunski, and D. Haas. 1997. The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol. Microbiol.* 24:309-13.
- Reimmann, C., N. Ginet, L. Michel, C. Keel, P. Michaux, V. Krishnapillai, M. Zala, K. Heurlier, K. Triandafillu, H. Harms, G. Defago and D. Haas. 2002. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology* 148:923-932.
- Rosenbluh, A., and M. Eisenbach. 1992. Effect of mechanical removal of pili on gliding motility of *Myxococcus xanthus*. *J. Bacteriol.* 174:5406-5413.
- Russel, M. 1998. Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems. *J. Mol. Biol.* 279:485-99.
- Russell, M. A., and A. Darzins. 1994. The *pilE* gene product of *Pseudomonas aeruginosa*, required for pilus biogenesis, shares amino acid sequence identity with the N-termini of type 4 prepilin proteins. *Mol. Microbiol.* 13:973-85.
- Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W., and D. G. Davies. 2001. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J. Bacteriol.* 184:1140-5
- Schultz, D. R., and K. D. Miller. 1974. Elastase of *Pseudomonas aeruginosa* inactivation of complement components and complement-derived chemotactic and phagocytic factors. *Infect. Immun.* 10:128-35.
- Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg. 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J. Bacteriol.* 185:2066-2079.
- Semmler, A., C. Whitchurch, and J. Mattick. 1999. A re-examination of twitching motility in *Pseudomonas aeruginosa*. *Microbiology* 145:2863-2873.
- Shi, W., F. K. Ngok, and D. R. Zusman. 1996. Cell density regulates cellular reversal frequency in *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. USA* 93:4142-46.

- Skerker, J. M., and H. C. Berg. 2001. Direct observation of extension and retraction of type IV pili. *Proc. Natl. Acad. Sci. USA* 98:6901-4.
- Smith, J. J., S. Travis, E. P. Greenburg, and M. J. Welsh. 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* 85:229-236.
- Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrenner, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., Olson, M. V. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406:959-64.
- Strom, M. S., D. N. Nunn, and S. Lory. 1993. A single bifunctional enzyme, PilD, catalyzes cleavage and *N*-methylation of proteins belonging to the type IV pilin family. *Proc. Natl. Acad. Sci. USA* 90: 2404-2408.
- Sutherland, I.W. 2001. The biofilm matrix – an immobilized but dynamic microbial environment. *Trends Microbiol.* 9:222-7.
- Sun, H., Yang, Z. & Shi, W. 1999. Effect of cellular filamentation on adventurous and social gliding motility of *Myxococcus xanthus*. *Microbiology* 96:15178-15183.
- Tang, H. B., E. DiMango, R. Bryan, M. Gambello, B. H. Iglewski, J. B. Goldberg, and A. Prince. 1996. Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. *Infect. Immunol.* 64:37-43.
- Thompson L. S., J. S. Webb, S. A. Rice, and S. Kjelleberg. 2003. The alternative sigma factor RpoN regulates the quorum sensing gene *rhII* in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett.* 220:187-95.
- Toder, D.S., M.J. Gambello, and B.I. Iglewski. 1991. *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of *lasR*. *Mol Microbiol.* 5:2003-10.
- Tommassen, J., A. Filloux, M. Bally, M. Murgier, and A. Lazdunski. 1992. Protein secretion in *Pseudomonas aeruginosa*. *FEMS Microbiol. Rev.* 9:73-90.
- Wagner, V.E., D. Bushnell, L. Passador, A.I. Brooks, and B.H. Iglewski. 2003. Microarray analysis of the *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J. Bacteriol.* 185:2080-2095.

- Ward, M. J., and D. R. Zusman. 1997. Regulation of directed motility in *Myxococcus xanthus*. *Mol. Microbiol.* 24:885-93.
- Watson, A. A., R. A. Alm, and J. S. Mattick. 1996. Identification of a gene, *pilF*, required for type 4 fimbrial biogenesis and twitching motility in *Pseudomonas aeruginosa*. *Gene* 180:49-56.
- Whitchurch, C. B., M. Hobbs, S. P. Livingston, V. Krishnapillai, and J. S. Mattick. 1991. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialized protein export system in eubacteria. *Gene* 101:33-44.
- Whitchurch, C. B., R. A. Alm, and J. S. Mattick. 1996. The alginate regulator AlgR and an associated sensor FimS are required for twitching motility in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 93:9839-43.
- Whitehead, N. A., A. M. Barnard, H. Slater, N.J. Simpson, and G. P. Salmond. 2001. Quorum-sensing in gram-negative bacteria. *FEMS Microbiol. Rev.* 25:365-404.
- Whiteley, M., M. R. Parsek, and E. P. Greenberg. 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 96:13904-09.
- Whiteley, M., K. M. Lee, and E. P. Greenberg. 2000. Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*. *J. Bacteriol.* 182:4356-60.
- Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, V. Chapon, G. P. C. Salmond, B. W. Bycroft, A. Lazdunski, G. S. A. B. Stewart, and P. Williams. 1995. Multiple *N*-acyl homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *P. aeruginosa*. *Proc. Natl. Acad. Sci. USA* 92:9427-31.
- Wolfgang, M., J. P. M. van Putten, S. F. Hayes, D. Dorward, and M. Kooney. 2000. Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. *EMBO J.* 19:6408-18.
- Wozniak, D.J., T.J.O. Wyckoff, M. Starkey, R. Keyser, P. Azadi, G.A. O'Toole, and M.R. Parsek. 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc. Natl. Acad. Sci. USA* 100:7907-12.
- Wreitland, B., and T. Wadstrom. 1977. Purification and properties of a protease with elastase activity from *Pseudomonas aeruginosa*. *J. Gen. Microbiol.* 103:319-27.
- Wu, S. S., and D. Kaiser. 1995. Genetic and functional evidence that type IV pili are required for social gliding motility in *Myxococcus xanthus*. *Mol. Microbiol.* 18:547-558.

Yang, Z., X. Ma, L. Tong, H. B. Kaplan, L. J. Shimkets, and W. Shi. 2000. *Myxococcus xanthus dif* genes are required for biogenesis of cell surface fibrils essential for social gliding motility. J. of Bacteriol. 182: 5793-5798.

ZoBell, C. E. 1943. The effect of solid surfaces on bacterial activity. J. Bacteriol. 46:39-56.

R002592087