BIOFILM AND VIRULENCE REGULATION IN THE CYSTIC FIBROSIS-ASSOCIATED PATHOGENS, *STENOTROPHOMONAS MALTOPHILIA* AND *PSEUDOMONAS AERUGINOSA*

by

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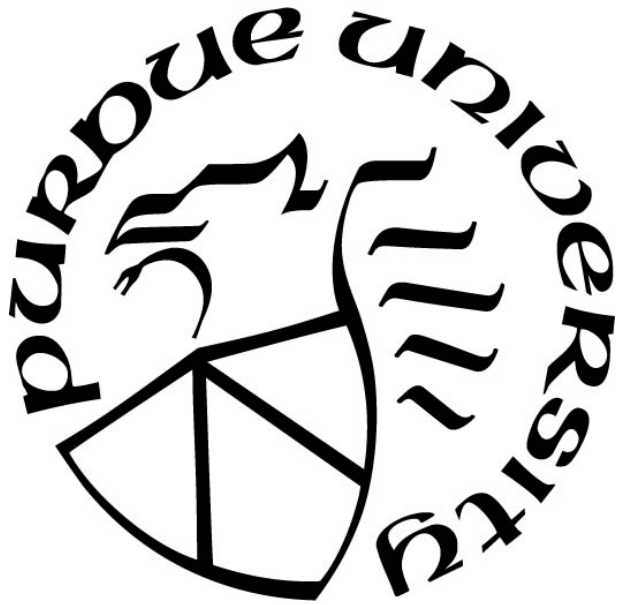

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This thesis is dedicated to my Parents and Grandparents.

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ABSTRACT

Cystic fibrosis (CF) is a fatal, incurable genetic disease that affects over 30,000 people in the United States alone (1). People with this disease have a homozygous mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) which causes defects in chloride transport and leads to build up of mucus in the lungs and disruption of function in various organs (1). CF patients often suffer from chronic bacterial infections within the lungs, wherein the bacteria persist as a biofilm, leading to poor prognosis. Two of these pathogens, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, are often found in the lungs of patients with CF and are an increasing medical concerns due to their intrinsic antimicrobial resistance (2), (3). Both species can readily form biofilms on biotic and abiotic surfaces such as intravascular devices, glass, plastic, and host tissue (4), (5). Biofilm formation starts with bacterial attachment to a surface and/or adjacent cells, initiating the acute infection stage (6). Chronic, long-term infection involves subsequent or concurrent altered genetic regulation, including a downregulation of virulence factors, resulting in the bacteria committing to a sessile lifestyle, markedly different from the planktonic one (7). Many of these genetic switches from an acute to chronic lifestyle are due to pressures from the host immune system and lead to permanently mutated strains, most likely an adaptive strategy to evade host immune responses (8). Biofilms are extremely problematic in a clinical setting because they lead to nosocomial infections and persist inside the host causing long-term chronic infections due to their heightened tolerance to almost all antibiotics (6), (9). Understanding the genetic networks governing biofilm initiation and maintenance would greatly reduce consequences for CF and other biofilm-related infections and could lead to the development of treatments and cures for affected patients. This study showed that in *S. maltophilia*, isogenic deletion of phosphoglycerate mutase (*gpmA*) and two chaperone-usher pilin subunits, *S. maltophilia* fimbrae-1 (*smf-1*) and *cblA*, lead to defects in attachment on abiotic surfaces and cystic fibrosis derived bronchial epithelial cells (CFBE). Furthermore, Δ*smf-1* and Δ*cblA* showed defects in long-term biofilm formation, mimicking that of a chronic infection lifestyle, on abiotic surfaces and CFBE as well as stimulating less of an immune response through TNF-α production. This study also showed that in *P. aeruginosa*, the Type III secretion system (T3SS), an important virulence factor activated during the acute stage of infection, is downregulated when *polB*, a stress-induced alternate DNA polymerase, is overexpressed. This downregulation is due to post-

transcriptional inhibition of the master regulatory protein, ExsA. Taken together, this project highlights important genes involved in the acute and chronic infection lifestyle and biofilm formation in *S. maltophilia* and genetic switches during the acute infection lifestyle in *P. aeruginosa*.

CHAPTER 1. CYSTIC FIBROSIS AND ASSOCIATED RESPIRATORY PATHOGENS

Cystic fibrosis (CF) is an incurable, heritable, chronic disease affecting over 100,000 people worldwide (1). CF is the most lethal and common genetic disease in the Caucasian population and half of patients with this disease do not live past 30 (10). Homozygous mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) cause defects in chloride transport and lead to build up of mucus in various organs such as the lungs and dysfunction of organs like the gastrointestinal tract and pancreatic ducts (10). In the lungs, altered mucus secretion compromises airflow, which plays a critical role in the disease pathogenesis and can ultimately lead to respiratory failure and death (10). The CF lung harbors complex and dynamic polymicrobial communities that greatly affect the condition, progression, and outcome of the patient's disease. Lung disease and inflammation caused by bacterial infections are one of the leading causes of morbidity and mortality for CF patients (11). Bacterial species that colonize CF patient lungs is very heterogeneous and becomes less diverse into adulthood compared to adolescence (12). During adolescence, *Staphylococcus aureus* are the dominant species, and going into adulthood *Achromobacter* spp., *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, and *Pseudomonas aeruginosa* are more prevalent (11). Many of these bacterial infections, including *S. maltophilia* and *P. aeruginosa*, are nearly impossible to eradicate and chronically infect CF lungs due to their intrinsic antimicrobial resistance and ability to form biofilms (11).

In the CF lung, bacteria, as well as different species of fungus, viruses, and bacteriophages coexist which has great implications on the microbial interactions within this environment. While many of the fungal species are culture-independent, DNA sequencing has identified over 30 fungal species or genera including *Candida*, *Aspergillus*, *Penicillium*, *Malassezia*, and *Kluyveromyces* (13). Coexistence of *P. aeruginosa* and *Aspergillus* has been shown to both improve and inhibit growth of both species and co-colonization by both species correlates with a worsened condition (14). Very few studies have examined the CF respiratory virome, however, infections by RNA viruses increase inflammation and trigger pulmonary exacerbations (15). Furthermore, increased inflammation facilitates and intensifies bacterial adherence, highlighting the dynamic interactions between different types of microbes (16). Bacteriophages also play an important role in CF disease progression by producing small colony variants (SCVs) that often harbor antibiotic resistance (17).

Additionally, in *P. aeruginosa* biofilms, bacteriophages play an important role in the formation of the polymer matrix (18). Taken together, microbial interactions within the CF lung are both dynamic and complex, consisting of many different microbes that can worsen the patient's prognosis.

1.1 The acute and chronic lifestyle of *Pseudomonas aeruginosa* within the CF lung

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that is commonly found in the environment. *P. aeruginosa* can cause acute and chronic infections in compromised patients and is a great concern to human health, especially in hospital settings. Infections caused by *P. aeruginosa* range from keratitis to bacteremia (19), (20). Additionally, one of the worst manifestations of *P. aeruginosa* infections is within the lungs of CF patients where the pathogen persists as a chronic infection, indicating poor prognosis and high patient morbidity and mortality (3). Inflammation and damage of the epithelial mucosa caused by chronic infections of *P. aeruginosa* can promote the growth of other bacteria, specifically *S. maltophilia* (21). Currently, it is recognized that *P. aeruginosa* has two different lifestyles that allow it to cause acute and chronic infections; one lifestyle is that of free-living cells that are predominantly cytotoxic while a second lifestyle is characterized by community behaviors, like biofilm formation, that facilitate a chronic infection (Fig. 1.1) (22). Selective pressure from the host's immune system and complex genetic networks activate the switch from an initial acute (planktonic) infection to a chronic (biofilm) infection lifestyle (22). The type III secretion system (T3SS) and expression of toxins like elastase are diminished. Likewise, virulence factors that trigger host immunity, like flagella and pili, are down regulated once the pathogen has established adherent contact with the host respiratory epithelium. On the other hand, biofilm bacteria produce vast amounts of exopolysaccharides such as alginate, leading to a highly mucoid phenotype (22). This transition in lifestyle is most likely an evolutionary adaptation that allows *P. aeruginosa* to persist and form chronic infections within the host (22). Complex genetic networks tightly regulate the planktonic-to-sessile transition and the overall process is reversible and dynamic.

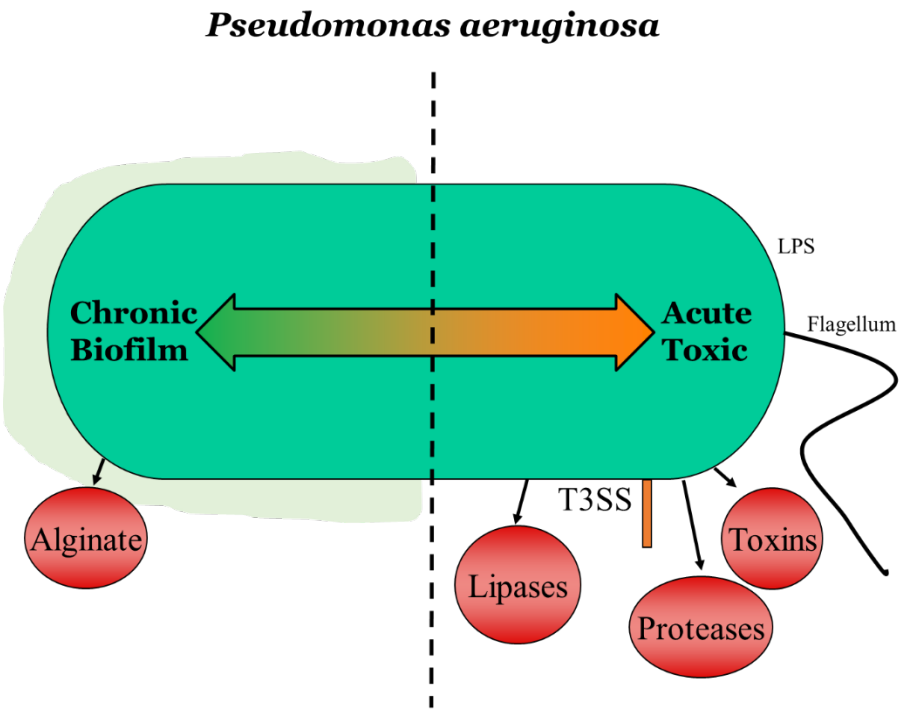

Figure 1.1 The acute and chronic lifestyle switch in *P. aeruginosa* during CF infection.

During the acute stage of infection, bacterial cell surface virulence factors such as flagella, pili, and lipopolysaccharide (LPS), aid in initial adhesion to the host epithelial layer (22). Following attachment, activation of the type III secretion system (T3SS) occurs, causing extensive damage to host cells. Effector molecules, ExoU, ExoS, ExoT, ExoY, are secreted out of the T3SS and into the host cells, causing host cell cytoskeleton rearrangement, immune evasion, and cytotoxicity, leading to tissue necrosis and allowing the pathogen to rapidly spread within the host (23). Secretion of other virulence factors including elastase, phospholipase C, pyoverdine, and pyocyanin cause additional host cell cytotoxicity and tissue destruction (23), (24). Quorum sensing (QS), two-component systems (TCs), stress response systems, and other factors tightly regulate this intricate system and production of these virulence factors (23).

Following the acute stage, *P. aeruginosa* often settles onto host tissue and infection persists in a chronic state. Growth of the bacterial cells slows and they become less cytotoxic and immunogenic by downregulating virulence factors, allowing the infection to persist in the host for decades usually as a biofilm (22). Many *P. aeruginosa* isolates from chronically infected individuals contain mutations in the major determinants of the planktonic cells, particularly T3SS function, flagellar function, and polysaccharide production (25). As a result, isolated CF strains

often exhibit a highly mucoid phenotype, producing a thick polysaccharide matrix, primarily made up of alginate, nucleic acids, and amino sugars (26), (5), (24). Altogether, these parallel lifestyles suggest a binary model of pathogenesis, in which acute infections consist of aggressively virulent cells equipped for host invasion while the biofilm state cells are adapted for chronic infections and immune evasion.

1.2 *Stenotrophomonas maltophilia* pathogenesis during CF infection

The Gram-negative opportunistic pathogen *S. maltophilia* is a ubiquitous organism that can be found in water and soil. *S. maltophilia* causes a wide spectrum of serious infections and is an important nosocomial pathogen because of its ability to grow and persist as a biofilm in irrigation solutions and invasive medical devices (27), (2). Furthermore, *S. maltophilia* is a multi-drug resistant pathogen that commonly infects the lungs of CF patients (28). Evidence suggests that *S. maltophilia* respiratory infections significantly contributes to inflammation and compromised function of the lungs (29). Within the CF lung, *S. maltophilia* biofilms correlate with increased incidence of pulmonary exacerbation and decreased lung function (30). Combined with its ability to form biofilms, *S. maltophilia* also has an array of extracellular virulence factors including proteases, lipases, fibrolysin, and DNases used to establish infections (31), (32). It also has many cell-associated virulence factors such as lipopolysaccharide, pili, and flagella that elicit an immune response and are important for mediating cell attachment and colonization of eukaryotic cells.

Some of these virulence factors are only associated with clinical isolated strains, indicating adaptive strains from selective pressures in a clinical environment. For example, the *S. maltophilia* fimbriae-1 (*smf-1*) gene is an important epithelial cell attachment mediator that is absent in environmental isolates and only present in clinical isolates (33). Considering that most environmental isolates do not form biofilms or express *smf-1*, it can be suggested that these virulence factors are present only during infection (34). Furthermore, loss of biofilm associated genes such as the quorum sensing system mediated by the diffusible signal factor (DSF), reduces levels of extracellular protease, motility, and virulence in *S. maltophilia* (35). This further emphasizes the importance of biofilm associated genes during infection and suggests that genetic networks involving biofilm and virulence associated genes may be present in *S. maltophilia*. In contrast to this idea, there are many virulence factors that are shared among all *S. maltophilia* strains, environmental and clinical (36). *S. maltophilia* encodes several serine proteases, many of

which degrade several human proteins, interleukins, and connective tissue (37), (38). Additionally, hemolysins, a class of enzymes used to lyse erythrocytes, leukocytes, mast cells, and neutrophils, are present in the genome of both environmental and clinical isolated *S. maltophilia* strains (36). This information suggests that although some virulence genes may be clinical-specific, there is a general potential for all *S. maltophilia* strains to act as opportunistic pathogens.

1.3 The need to identify the mechanisms of biofilm formation in chronic infections

Given the importance of biofilm formation and its role in pathogenesis, there is a need to further explore and identify necessary gene regulation and factors that facilitate this process. Many of these mechanisms remain unclear and the purpose of this study is to elucidate genes important for *S. maltophilia* and *P. aeruginosa* biofilm and lifestyle switches. Understanding this process will help the development of treatments and cures for patients that suffer from biofilm mediated infections.

CHAPTER 2. *STENOTROPHOMONAS MALTOPHILIA* ATTACHMENT AND BIOFILM REGULATION

2.1 Introduction

The Gram-negative bacterium *Stenotrophomonas maltophilia* causes numerous human infections that are difficult to treat due to high intrinsic antimicrobial resistance (39). *S. maltophilia* is a ubiquitous opportunistic pathogen, commonly found in water and soil and is capable of surviving in low and high nutrient environments making it optimally positioned to infect humans (31). It causes a wide range of infections including in the urinary tract, skin, and bloodstream, as well as meningitis and endocarditis (40), (41), (42). Moreover, many clinical *S. maltophilia* isolates readily form biofilms on biotic and abiotic surfaces, such as intravascular devices, glass, plastic, and host tissue (43). This is especially concerning for individuals with cystic fibrosis (CF) where *S. maltophilia* causes chronic infections in the patients' lungs often mediated by biofilm formation (11). Inhibiting biofilm formation would greatly reduce these consequences for CF and other *S. maltophilia* biofilm-related infections. However, genetic networks governing biofilm initiation and maintenance in *S. maltophilia* are poorly understood.

Many environmental factors such as pH, temperature, presence of metal ions, and antibiotics greatly effect *S. maltophilia* biofilm formation (41). Additionally, the polysaccharide synthesis genes *rmlA*, *rmlC*, and *xanB* (44) as well as diffusible signal factor (DSF)-mediated cell communication (45) affect biofilm levels. Pili, important structures that aid in bacterial attachment to adjacent cells and surfaces, also affect early and late stages of biofilm formation (46). *S. maltophilia* fimbrae-1 (Smf-1) has previously been shown to be involved with biofilm and attachment on abiotic and biotic surfaces (27). *S. maltophilia* also encodes a pilus (CBL) homologous to the Giant Cable Pilus in *Burkholderia cepacia*, another infectious CF pathogen (47). In *B. cepacia*, Giant Cable Pili facilitate the attachment to host cells and perhaps other important pathogenic activities (47). Both *smf-1* and *cblA* pilus systems are chaperone-usher pili, a family of pili named for the components involved in biogenesis of the appendage.

To identify novel *S. maltophilia* biofilm factors on abiotic and biotic surfaces, we generated and screened a transposon mutant library for mutations that lead to altered biofilm levels compared to the wild-type (WT) strain. Two mutations had strikingly reduced biofilm formation and attachment: 1) glycolytic enzyme phosphoglycerate mutase, *gpmA* (31) and 2) *smf-1.* Isogenic

deletion of *gpmA* revealed adherence defects on abiotic and biotic surfaces, suggesting a role for metabolism in biofilm attachment and/or development. Additionally, isogenic deletion of *smf-1* and the bioinformatically identified pilin gene *cblA* revealed adherence and biofilm defects on abiotic and biotic surfaces suggesting an attachment role for these two systems.

2.2 Materials and Methods

2.2.1 Bacterial strains and culture

We used *S. maltophilia* clinical isolate strain K279a (48). *Escherichia coli* S17-1 (49) was used for cloning and plasmid maintenance. Bacteria were cultured in LB medium, with gentamicin as needed for maintaining plasmids (10 µg/mL or 70 µg/mL for *E. coli* and *S. maltophilia*, respectively.

2.2.2 Transposon mutagenesis

We generated a transposon mutant library of *S. maltophilia* strain K279a by conjugation of plasmid pBT20 (containing the mariner transposon (50) from *E. coli* S17-1 into K279a. Briefly, overnight cultures of K279a and S17-1 pBT20 were mixed 1:3, 1:1, and 3:1 and spotted on LB agar plates. After 24 hr incubation at 30˚C, spots were resuspended in LB and exconjugants were selected for on LB plates with 70 µg/mL gentamicin and 5 µg/mL norfloxacin. For candidate mutants, we performed arbitrary-primed PCR using the primers listed in Table 2.1, as previously described (51), to identify the location of transposon insertion. Primers specific to the ends of the transposon and a random sequence in the chromosomal DNA, were used to amplify the location where the transposon inserted. Location of the transposon was first amplified using primer sets P238, P15 ARB1 and P237, P16 ARB6. A second round of PCR was performed using primer sets P240, P14 ARB2. Final PCR products were purified using QIAquick Spin PCR purification (Qiagen, Valencia, CA) according to manufacturer's instructions and then subsequently subjected to DNA sequence analysis.

2.2.3 Strain construction of Δ*gpmA*

The suicide vector pMQ30 (52) was used to construct an isogenic *gpmA* deletion strain by allelic replacement, as previously described (51). Briefly, ~1000bp fragments immediately

upstream and downstream of *gpmA* were generated by PCR using primers sets gpmALfor/gpmALrev, respectively (Table 2.1). Fragments were transformed into *Saccharomyces cerevisiae* INVSC1 (Invitrogen; Eugene, OR) along with BamHI-digested pMQ30, as previously described (51), (53). Recombination in *S. cerevisiae* resulted in plasmid pΔ*gpmA*. This plasmid was isolated as previously described (51), transformed into *E. coli* S17-1 by electroporation, and conjugated into K279a, as described above. Exoconjugants were selected on LB plates containing 70 μg/mL gentamicin and 5 μg/mL norfloxacin. Isolated colonies were grown overnight in LB and then plated on LB agar containing 10% sucrose to select for excision of the plasmid. Deletion mutants were confirmed using primers gpmAFor/gpmARev.

Complementation was achieved by PCR-amplifying full length *gpmA*, along with ~500bp upstream, using the primer pairs GpmACompNatForNew/GpmACompRev (Table 2.1). Fragments were transformed into *S. cerevisiae*, along with BamHI-digested pMQ132 expression vector (52), as described above, generating plasmid p*gpmA*. This plasmid was then transformed into *E. coli* S17-1 via electroporation, and successful construction was confirmed by PCR using primers p729/p730A. The Δ*gpmA* strain was then transformed with p*gpmA* by electroporation.

Table 2.1. Primers used in this study

Primer	**Sequence (5' à 3')**
Arbitrary PCR	
P14 ARB2	GGCCACGCGTCGACTAGTAC
P15 ARB1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
P16 ARB6	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
P237	GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG
P238	TATAATGTGTGGAATTGTGAGCGG
P240	ACAGGAAACAGGACTCTAGAGG
P241	CACCCAGCTTTCTTGTACAC
Deletion of Δ*gpmA*	
GpmALfor	TCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTT CCGGCGCCTGCACGATGACTTC
GpmaALrev	CACCAACGGTGGGTATCTACTGCAGGGTCTCCAACGCGAG

Table 2.1 continued

Primer	Sequence (5' → 3')
GpmaARfor	CTCGCGTTGGAGACCCTGCAGTAGATACCCACCGTTG GTG
GpmaARrev	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACA GCTTGAGCGGGTTGTAGCGCTTG
GpmArev	CCAGGTTGCGGACGTTCATG
***gpmA* Complementation**	
GpmACompfor	TACTGTTTCTCCATACCCGTTTTTTTGGGCTAGCGAATT CAGGAGGAGCGCGCTGTGACC CGTAAACTCGTACT
GpmAComprev	CTGTATCAGGCTGAAAATCTTCTCTCATCCGCCAAAAC AGTTATTTCGCCTTGCCCTGGT
GpmACompNatForNew	TTGTGTGGAATTGTGAGCGGATAACAATTTCACACAG GAATCGACTTGGCACACCGATG
P730A	TGCTTCCGGCTCGTATGTTG
P729	CAGACCGCTTCTGCGTTCTG
Deletion of Δ*smf-1*	
706LFor	TCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTT CCTCAAGATGGTCCGCTGCAAC
706LRev	CAGTGCGGGTACGGCTACGATCGCTTTTACCTAACCCT AC
706RFor	GTAGGGTTAGGTAAAAGCGATCGTAGCCGTACCCGCA CTG
706RRev	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACA GCTGGCAAAGCGCCTGATGGATC
Deletion of Δ*cblA*	
3833LFor	TCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTT CCAAGTGCGTGCCGAAGTGAAC
3833LRev	GATGCGGGCCGGCGCGGGGGAGGGTGTCTCTCTCGG ACAGG
3833RForNew	CCTGTCCGAGAGAGACACCCAAGGCGGCTACGCCGT AATC
3833RRev	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACA GCTGCCTGCAGGTAGAAGGTCTG
To test constructs	
706for	TACTTGCGTCACGGATTCAG
706rev	AGATCACTCGCGTGCCATTG
3833for	ATGCTTGAATGGCCTGCATG
3833rev	TGGCCTTCGACCAGATCTTC

2.2.4 Strain construction of Δ*smf-1* and Δ*cblA*

Isogenic deletion of Δ*smf-1* and Δ*cblA* was similar as to Δ*gpmA,* generating ~1000bp fragments immediately upstream and downstream of *smf-1* by PCR using primers sets 706LFor/Rev and 706RFor/Rev and 3833LFor/Rev and 3833RForNew/Rev respectively (Table 2.1). After electroporation into K279a and plating on 10% sucrose plates, Δ*smf-1* and Δ*cblA* deletion mutants were tested using the primers 706For/Rev 3833For/Rev respectively.

2.2.5 Biofilm and attachment assays on an abiotic surface

Attachment and biofilm formation on abiotic surfaces were assayed as previously described (51), (54). Strains were grown overnight in LB, diluted 1:100 into fresh LB, inoculated into individual wells in 96-well polystyrene microtiter plates (Greiner Bio-One; Monroe, NC), and incubated at 37°C for the indicated duration. Detection of attachment or biofilm formation was achieved by staining the plates with 0.1% crystal violet (CV) for 10 min. CV was solubilized using 30% acetic acid solution and transferred to a new 96-well polystyrene microtiter plate and quantified by measuring optical density at 550 (OD_{550}) in a SpectraMaxM2 spectrophotometer (Molecular Devices; Sunnyvale, CA).

2.2.6 Biofilm and attachment assays on a biotic surface

Attachment and biofilm formation of the strains were assayed on immortalized CF-derived bronchial epithelial (CFBE) cells as previously described (55). 24-well tissue culture plates (Falcon; Franklin, NJ) were seeded with CFBE cells at a concentration of 2 x 10^5 cells/well in minimal essential medium (MEM) (Corning Inc.; Corning, NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals; Lawrenceville, CA), 50 U/mL penicillin, and 50 U/mL streptomycin (Lonza; Walkersville, MD) (56). The plates were incubated for 7 to 10 days at 37°C and 5% CO_2. Once confluence was reached, cells were washed with 1X phosphate buffered saline (PBS) solution and inoculated with overnight-grown bacteria at a final concentration of ~1.2 x 10^7 CFU/mL in 0.5 mL MEM without phenol red, FBS, or antibiotics; this assay was then incubated for the time period specified for each experiment. At each time point, wells were washed 2-3 times with 0.5 mL PBS and treated with 1 mL of 0.1% Triton X-100 for 10 min. Lysates were harvested

into micro-centrifuge tubes, vortexed for 3 min, then serially diluted and plated on LB. Attachment/biofilm level was assessed by CFU determination from colony counts.

2.2.7 Growth kinetics

Strains were grown overnight in LB and diluted 1:100 into 100 µL fresh LB or MEM in a polystyrene microtiter plate. Plates were incubated for 12 hr or 24 hr at 37°C in a SpectraMaxM2 spectrophotometer, with 5 seconds of shaking before each OD_{600} reading every 30 min.

2.2.8 Microscopy of biotic biofilms for ΔgpmA

For microscopic examination of CFBE cells attachment, wild type and Δ*gpmA* strains were transformed with pMQ400, which constitutively expresses a TdTomato gene, conferring red fluorescence (57). Attachment of strains was assayed as described above, and after 1 hr, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at 37°C. Fixed cells were washed twice with PBS and stained with 5 µg/mL wheat germ agglutinin (WGA) conjugated with Alexa Fluor 350 (Invitrogen; Eugene, OR) and incubated for 10 min at 37°C. Labeling solution was removed and cells were washed twice with PBS. Fluorescence microscopy was performed using a Keyence BZ-X800/810 series all-in-one fluorescence microscope (Osaka; Osaka Prefecture, Japan) to obtain images at 20x magnification. 4 images were taken at different fields and stitched together using the microscope software to create a composite image.

2.2.9 Hemagglutination assay

Red blood cell (RBC) agglutination was tested for Δ*smf-1* strain. Overnight cultures of WT and Δ*smf-1*were grown on LB plates at 37 °C. Bacteria was transferred into 1mL of 1X PBS using a sterile cotton swab and matched to an OD_{600} of 1.0. Samples were spun down in microcentrifuge tubes at 15,000 rpm and pellets were resuspended in 100 µL of 1X PBS. Chicken RBC's (Lampire Biological Laboratories; Pipersville, PA) were washed to remove any cellular debris by rinsing 3-4 times in 1X PBS and resuspended to an OD_{640} of 1.3-1.5. Using a U-bottom 96-well plate (Costar; Kennebunk, ME), 25µL of 1X PBS was aliquoted into every well except the first column. 1:2 dilutions were performed by transferring 25 µL of bacteria from the first well into the subsequent wells. Following, 25 µL of RBCs were added to each well and mixed by tapping on

the side of the plate. The plate was left in the refrigerator for 1 hr and minimum dilution for agglutination was recorded.

2.2.10 Yeast agglutination assay

For the yeast agglutination, *S. cerevisiae* was grown overnight at 30 °C in YPD media. WT *S. maltophilia* and Δ*smf-1* were grown in LB at 37 °C overnight. Equal volumes of *S. cerevisiae* and the indicated bacterial strain were mixed on a microscope slide and agglutination was visualized after 15 min.

2.2.11 PCR on *S. maltophilia* CF clinical isolates

To test for *cblA* in clinical *S. maltophilia* isolates, 54 clinical CF isolates obtained from Dr. Valerie Waters at the Sick Kids Hospital in Toronto, Ontario, Canada were analyzed using PCR. Strains were grown on LB overnight at 37 °C and then chromosomal DNA was isolated by using the Puregene Yeast/Bact. Kit B (Qiagen) according to the manufacturer's instructions. DNA was stored at -20 °C until further tested. PCR was performed using the primer set 3833For/Rev (Table 2.1). Isolated K279a genome was used as positive control. Presence of a single 800 bp band on a 1% agarose gel was considered a positive result.

2.2.12 Enzyme-linked immunosorbent assay (ELISA)

Samples for detecting TNF-α secretion by CFBE cells were collected after 4 hr and 14 hr infection with either WT, Δ*smf-1*, or Δ*cblA*. Infection of cells was assayed the same as the biofilm experiments and 500 µL of supernatant was aliquoted into micro centrifuge tubes and stored at -80 °C until tested. Samples were thawed on ice and TNF-α secretion was tested with Invitrogen Human TNF-α Cytoset (Invitrogen; Frederick, MD) according to manufacturer's instructions. ELISA samples and standards were tested on a 96-well polystyrene microtiter plates (Greiner Bio-One; Monroe, NC) and TNF-α secretion was quantified by measuring optical density at 450nm (OD_{450}) in a SpectraMaxM2 spectrophotometer (Molecular Devices; Sunnyvale, CA).

2.2.13 Statistical analysis

At least three independent experiments were performed for each assay, with triplicate or quadruplicate samples each. Statistical significance was determined using Student's *t* test. A *P* value of <0.05 was considered statistically significant. Error bars represent standard deviations.

2.3 Results

2.3.1 Δ*gpmA* strain is defective for attachment

We created a transposon mutant library (~5,760 mutants) of *S. maltophilia* strain K279a and screened it for strains defective in biofilm formation. Using arbitrary-primed PCR, we mapped the transposon insertion in one of these mutants to *gpmA* (Smlt1430), which encodes the glycolytic enzyme phosphoglycerate mutase (31). In order to confirm that *gpmA* affects biofilm, we created an isogenic deletion mutant and tested it for biofilm formation. Initial attachment (2 hr time point) and early biofilm formation (4 hr time point) of Δ*gpmA* on polystyrene plates was significantly reduced compared to wild type (Fig. 2.1A and 2.1B). Complementation of Δ*gpmA* with a plasmid carrying a full-length copy of *gpmA* restored biofilm production (Fig. 2.1A and 2.1B). However, after 6 hr, there was no difference in biofilm formation suggesting that *gpmA* is required for early biofilm formation on abiotic surfaces (Fig 2.1C).

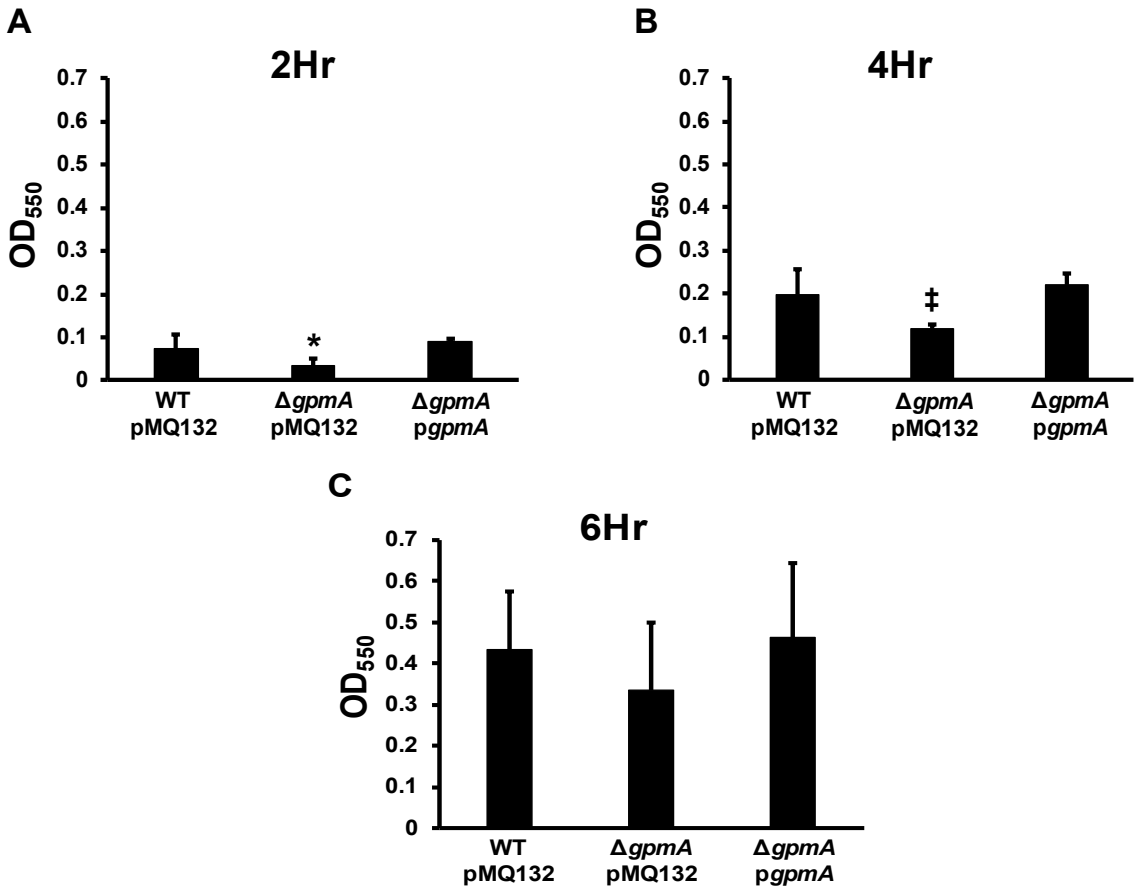

Figure 2.1. Isogenic deletion of *gpmA* leads to reduced biofilm levels at early stages on abiotic surfaces. (**A**) Attachment efficiency was quantified by measuring the intensity of CV staining of Δ*gpmA* compared to that of wild type (WT) and Δ*gpmA* complemented with p*gpmA*. pMQ132 is the empty vector, used as a control. Absorbance was measured at 550 nm (OD_{550}) as described in Materials and Methods. (**B** and **C**) Biofilm formation was measured after 4 hr and 6 hr respectively. Data are representative of three independent experiments performed in triplicate (n = 3). Error bars represent standard deviations. *$p < 0.05$ compared to WT. ‡$p < 0.001$ compared to WT Δ*gpmA*.

2.3.2 Δ*gpmA* exhibits the same growth kinetics as wild type

To rule out the possibility that attachment repression in the Δ*gpmA* strain was due to reduction in growth rate, we examined the growth kinetics of planktonic cells in LB and MEM medium. The *gpmA* mutant displayed growth equivalent with the wild type and complemented mutant strains (Fig. 2.2A and 2.2B).

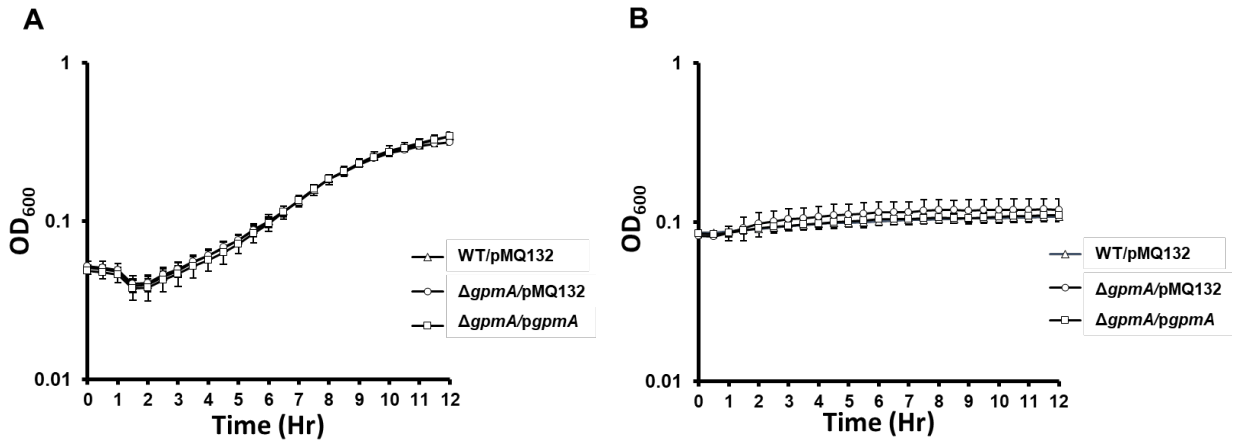

Figure 2.2. Mutant strains grow equally as well as the wild type in LB and MEM. No differences were observed in the growth kinetics of wild type (WT) pMQ132, Δ*gpmA* pMQ132, and Δ*gpmA* p*gpmA* in both LB (**A**) and MEM (**B**). Absorbance was measured at 600 nm (OD_{600}) over the course of 12 hr. The results are representative of three independent experiments carried out in triplicate (n=3) and error bars represent standard deviations. No statistically significant difference was found between strains in either medium.

2.3.3 Δ*gpmA* is defective for attachment to airway epithelial cells

S. maltophilia forms biofilms in the airways of cystic fibrosis patients (58), therefore, the effect *gpmA* has on attachment and biofilm on airway epithelial cells was investigated. Thus, bacteria attached to cystic fibrosis bronchial epithelial (CFBE) (59) cells after incubation with wild type, Δ*gpmA*, and complemented mutant strains was quantified. After 1 hr of incubation, Δ*gpmA* exhibited an approximately 100-fold reduction in binding compared to wild type (Fig. 2.3A). Complementation of the mutant restored the wild type phenotype (Fig. 3A). However, there was no significant differences between wild type and Δ*gpmA* at 4 hr and 6 hr time points (Fig. 2.3B and 2.3C). Importantly, there was a significant difference between Δ*gpmA* and the complemented strain at 4 hr and 6 hr (Fig. 2.3B and 2.3C). By fluorescence microscopy, numerous wild type biofilm microcolonies were evident on CFBE cells after 1 hr incubation (Fig. 3D), but fewer Δ*gpmA* clusters were seen at the same time point.

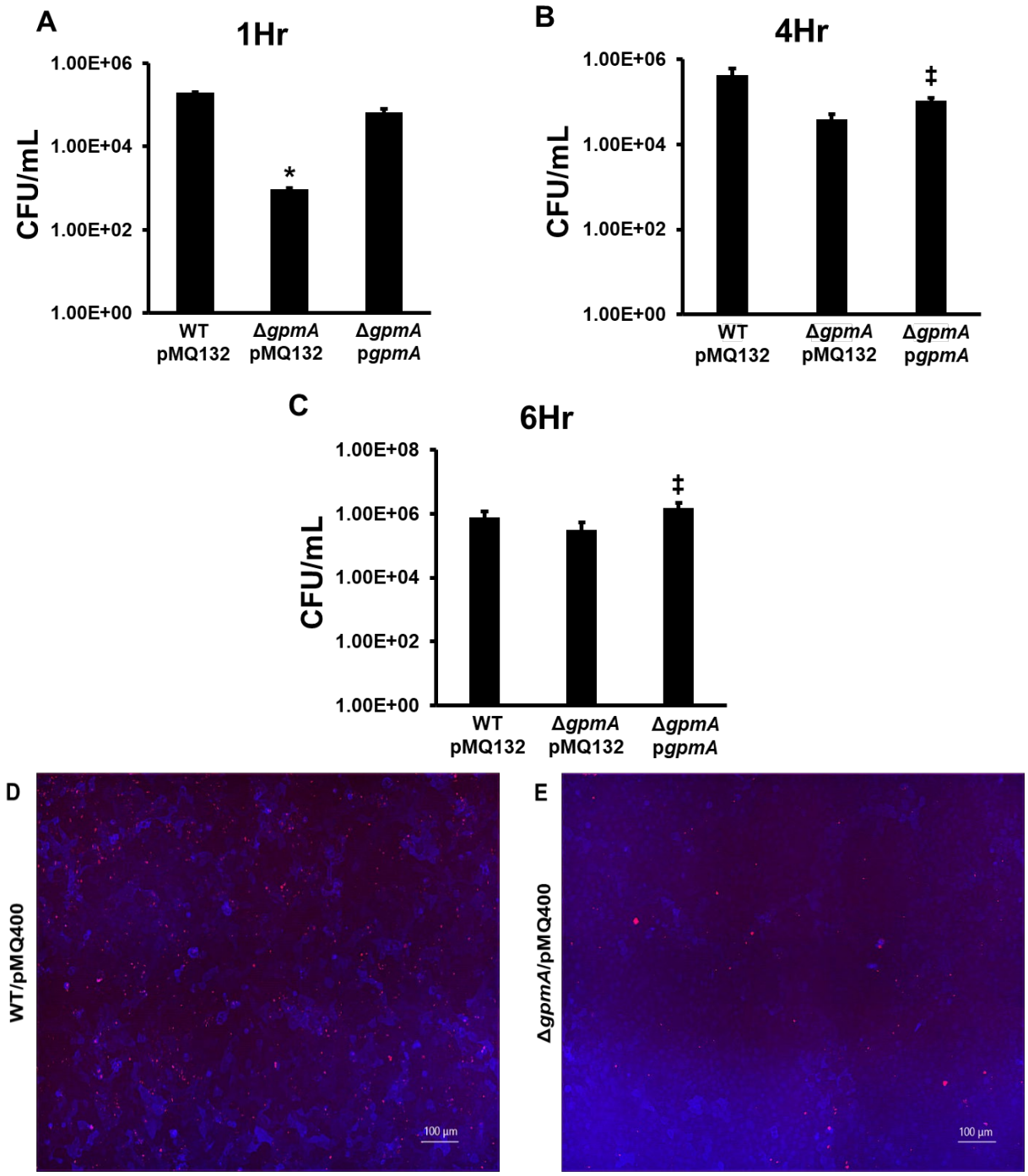

Figure 2.3. Δ*gpmA* is defective for attachment on CFBE airway cells. (**A**) Attachment efficiency was quantified by measuring CFU/mL after 1 hr incubation at 37 °C. (**B** and **C**) Biofilm levels were measured at 4 hr and 6 hr respectively. The results are representative of three independent experiments carried out in triplicate (n = 3) and error bars represent standard deviation. *$p < 0.05$ compared to WT. ‡$p < 0.05$ compared to Δ*gpmA*. (**D** and **E**) Composite images from fluorescence microscopy of CFBE cells incubated with WT (**D**) or Δ*gpmA* (**E**) for 1 hr. pMQ400 contains a constitutive TdTomato gene, conferring red fluorescence to the bacteria. CFBE cell membranes were stained with WGA-Alexa Fluor 350 (blue).

2.3.4 Δ*smf-1* and Δ*cblA* have defects in abiotic attachment and biofilm formation

In a biofilm screen of the *S. maltophilia* transposon mutant library, we identified additional mutants with defects in biofilm formation were identified. In one of these clones, the mutation mapped (through arbitrary-primed PCR) to *smf-1*, which encodes for the pilin subunit of Smf-1 pili (27). To confirm defects in attachment and biofilm formation, we isogenically deleted the *smf-1* gene. An isogenic deletion of *cblA* was generated, which encodes the pilin subunit of CBL pili; this pilus gene cluster was identified bioinformatically. Both Δ*smf-1* and Δ*cblA* strains exhibited defects in initial attachment (2 hr time point) and long-term biofilm formation (24 hr time point) (Fig. 2.4A and 2.4B).

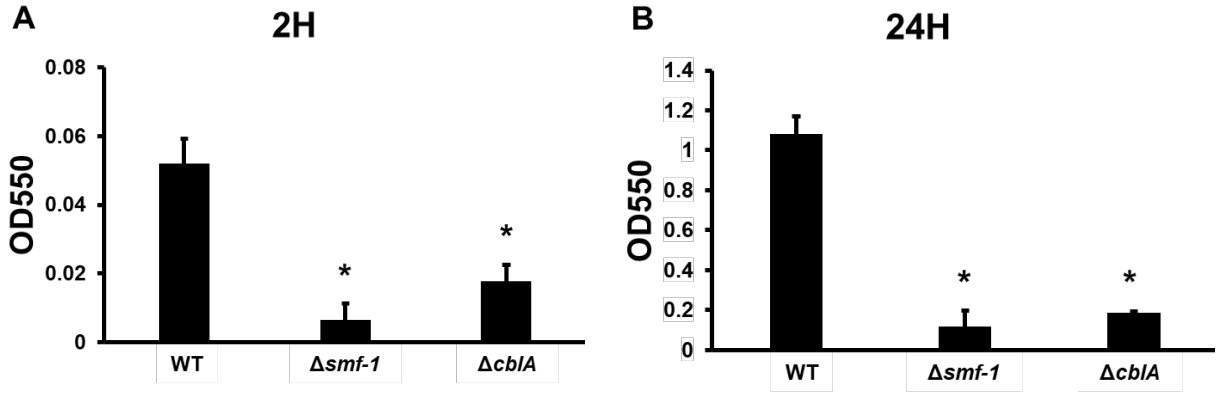

Figure 2.4. Isogenic deletion of *smf-1* and *cblA* leads to reduced attachment abiotic surfaces. (**A**) Attachment efficiency was quantified by measuring the intensity of CV staining of Δ*smf*-1 and Δ*cblA* compared to that of wild type (WT). Absorbance was measured at 550 nm (OD_{550}) as described in Materials and Methods. (**B**) Biofilm formation was measured after 24 hr. Data are representative of three independent experiments performed in triplicate (n = 3). Error bars represent standard deviations. *$p < 0.05$ compared to WT.

2.3.5 Δ*smf-1* and Δ*cblA* show fewer pili when visualized on TEM

Negatively stained TEM images of wild-type *Stenotrophomonas maltophilia* K279a revealed many hair-like appendages ubiquitously covering the cell. However, images of Δ*smf-1* and Δ*cblA* show many fewer appendages with Δ*smf-1* completely lacking pili and Δ*cblA* having a few appendage**s,** though many fewer than WT (Fig. 2.5).

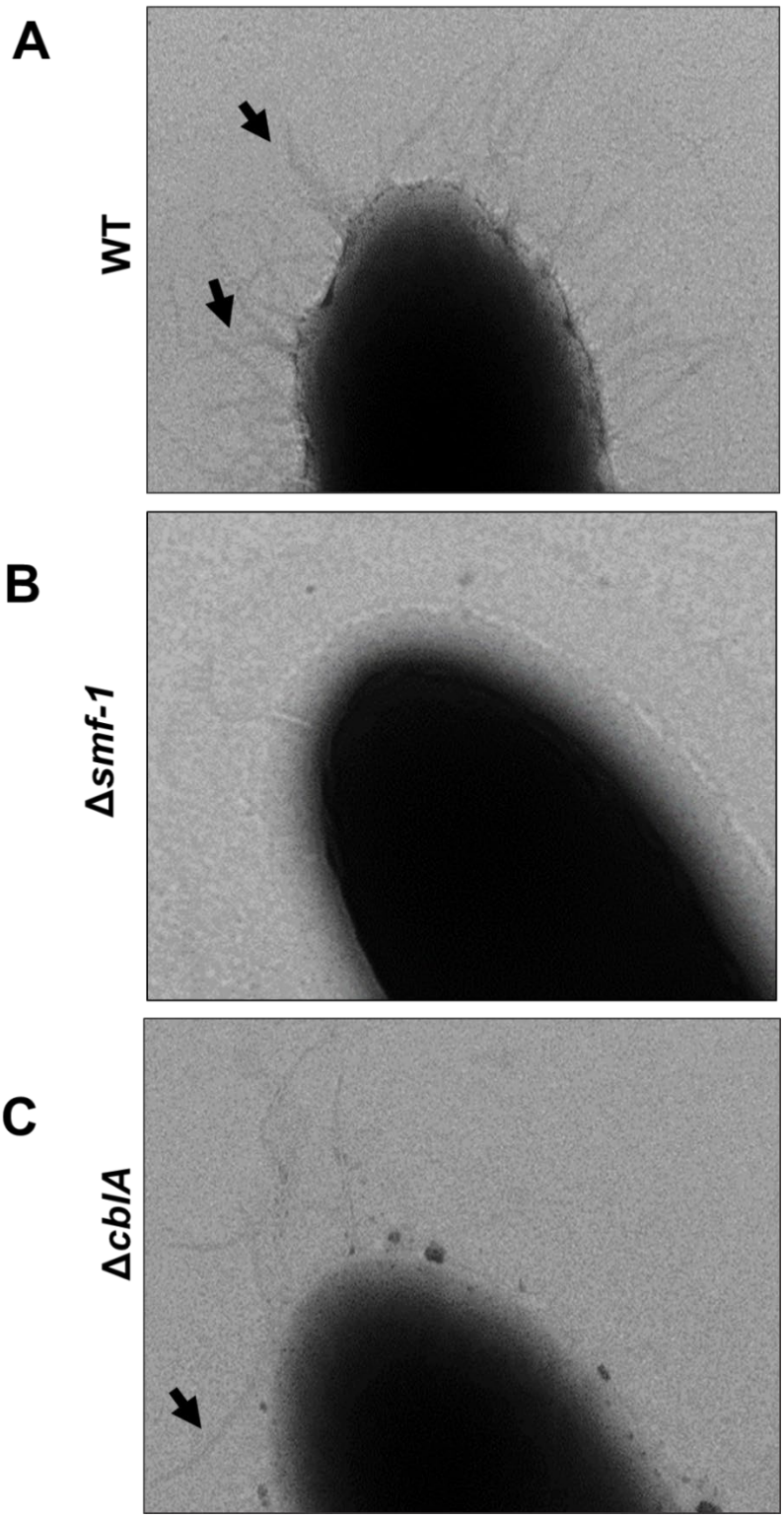

Figure 2.5. TEM images of (A) WT, (B) Δ*smf-1*, and (C) Δ*cblA*. Negatively stained TEM images show lack of pili in Δ*smf-1* and Δ*cblA* compared to WT. Arrows indicate pili appendages.

2.3.6 Δ*smf-1* showed defects in yeast and red blood cell agglutination

It has been previously reported that, *smf-1* has been associated with agglutination of chicken red blood cells (RBC) (27). To further confirm that the Δ*smf-1* strain lacked functional activity, yeast and blood cell agglutination assays were performed. Yeast agglutination has not been shown previously to be associated with this pilus system. These data suggests that the Δ*smf-1* shows much less agglutination of both yeast cells and RBC compared to WT (Fig. 2.6A and 2.6B). In the RBC assay, WT agglutination was observed until the 1:16 dilution whereas Δ*smf-1* failed to agglutinate even in the most concentrated sample.

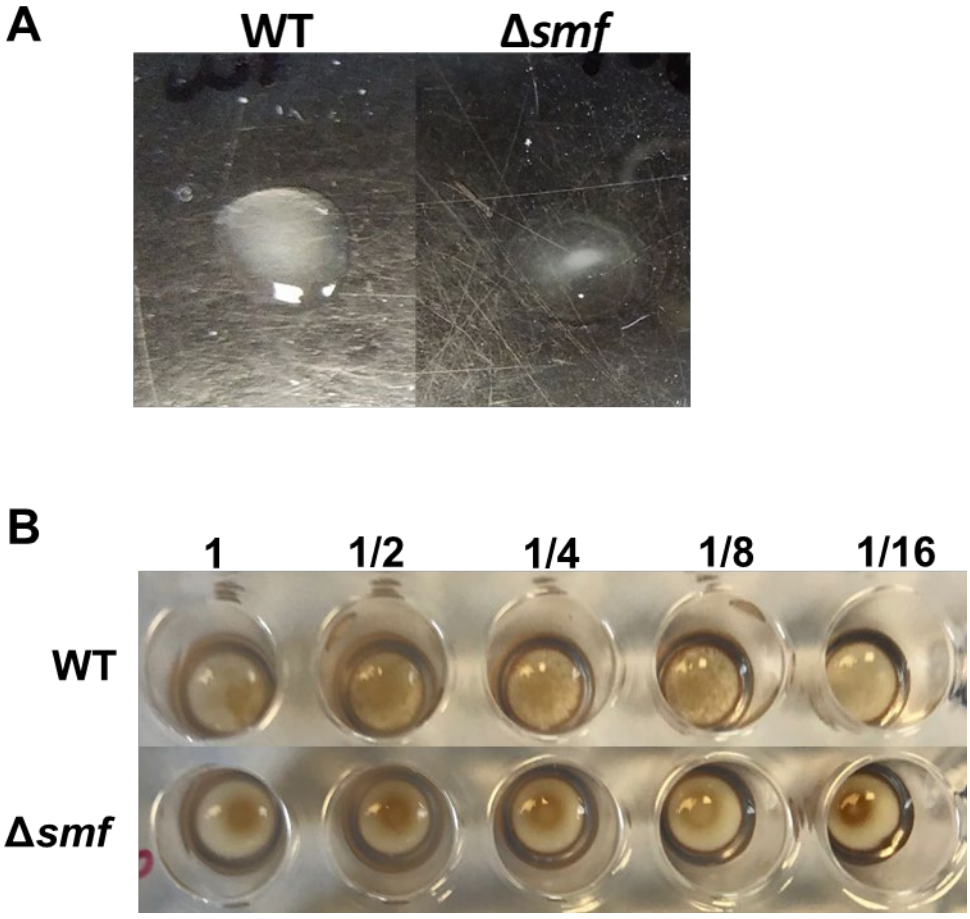

Figure 2.6. Agglutination of yeast and red blood cells is defected in Δ*smf-1* strain. (**A**) Yeast agglutination of WT and Δ*smf-1* (**B**) Hemagglutination of WT and Δ*smf-1* using chicken red blood cells; top numbers show dilution factors.

2.3.7 *cblA* is present in many of the CF clinical isolates

A collection of 52 CF *S. maltophilia* clinical isolates were screened for the *cblA* gene. If *cblA* affects clinical phenotypes, then it should be present in most clinical isolates. Using PCR to screen for the c*blA* gene, we found that 63% (33/52) of clinical isolates had the *cblA* gene encoded in their genome suggesting that *cblA* is a clinically relevant gene (Fig. 2.7).

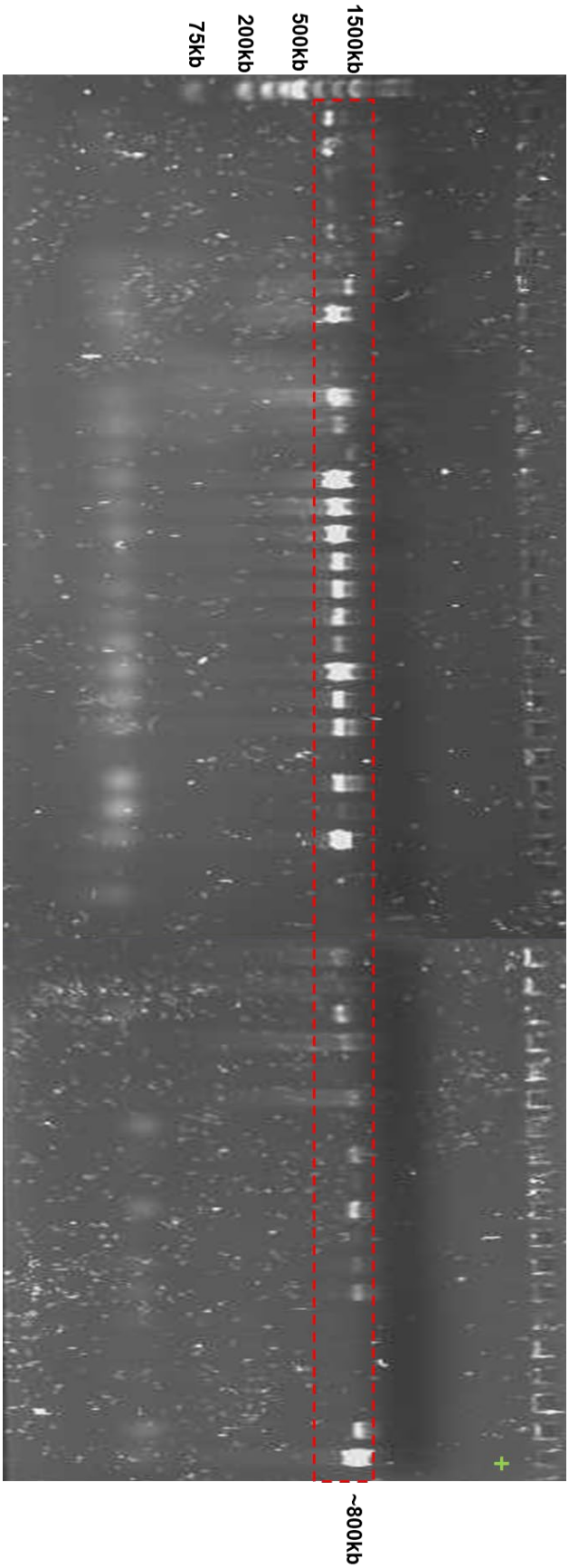

Figure 2.7. CF clinical isolates indicate presence of *cblA* gene. The *cblA* gene is around 800bp and highlighted in the red dotted line box, amplified using 3833F/3833R primers. The last well shows positive control (K279a genome DNA: "+") and left side is the ladder with the indicated fragment size. This image is a composite of two separate gels.

2.3.8 Growth kinetics of Δ*smf-1* and Δ*cblA* were the same compared to wild-type

To rule out the possibility that attachment repression in the Δ*smf-1* and Δ*cblA* strains was due to reduction in growth rate, we examined the growth kinetics of planktonic cells in LB. Both mutants displayed growth equivalent with the wild type and complemented mutant strains (Fig. 2.8).

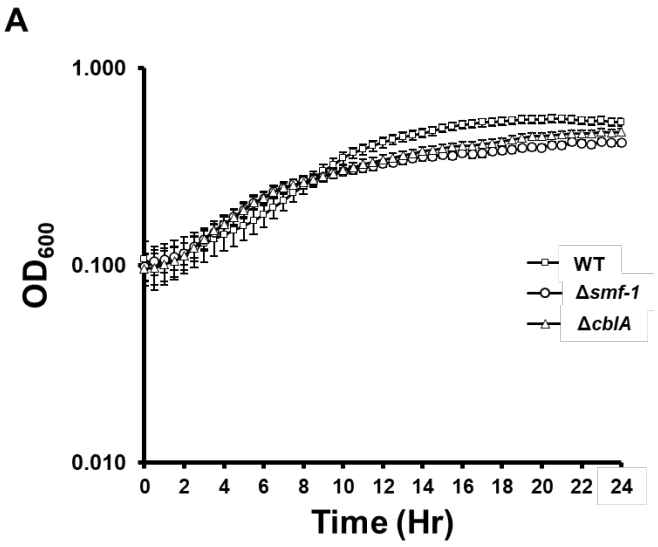

Figure 2.8. Mutant strains grow equally as well as the wild type in LB. No statistical differences were observed between the growth kinetics of wild type (WT), Δ*smf-1*, and Δ*cblA* in LB.

2.3.9 Δ*smf-1* and Δ*cblA* show defects in attachment and biofilm formation on airway cells.

Bacteria attached to CFBE cells after initial attachment (1 hr time point) and long-term biofilm (14 hr time point) were quantified. Later time points could not be quantified due to increased cell death. Both Δ*smf-1* and Δ*cblA* strains showed about a an approximate 100- fold decrease in attachment after 1 hr incubation and 14 hr incubations. Δ*smf-1* displayed a was slightly more defected in attachment to CFBE cells compared to Δ*cblA* (Fig. 2.9A and 2.9B).

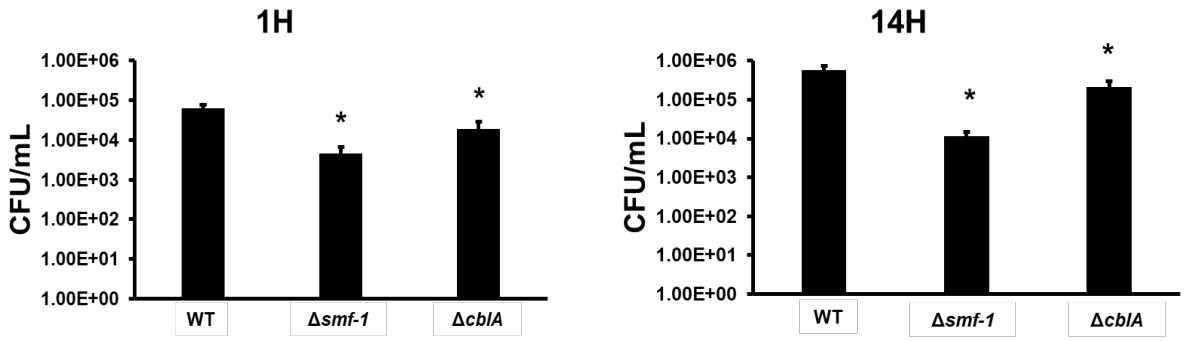

Figure 2.9. Δ*smf-1* and Δ*cblA* is defective for attachment and biofilm on CFBE airway cells. (**A**) Attachment efficiency was quantified by measuring CFU/mL after 1 hr incubation at 37 °C. (**B**) Biofilm levels were measured at 14 hr. The results are representative of three independent experiments carried out in triplicate (n = 3) and error bars represent standard deviation. *$p < 0.05$ compared to WT.

2.3.10 Δ*smf-1* and Δ*cblA* elicit less of an immune response compared to WT after 14hr infection.

After infecting the CFBE cells with WT and the mutant strains, ELISAs were performed on the supernatant at either 4 hr or 14 hr infection time points. After 4 hr, TNF-α secretion was not significantly different between WT and mutant strain (Fig. 2.10A). However, there was significantly less TNF-α secretion at 14 hr time point when infected with either Δ*smf-1* or Δ*cblA* strains compared to WT (Fig. 2.10B).

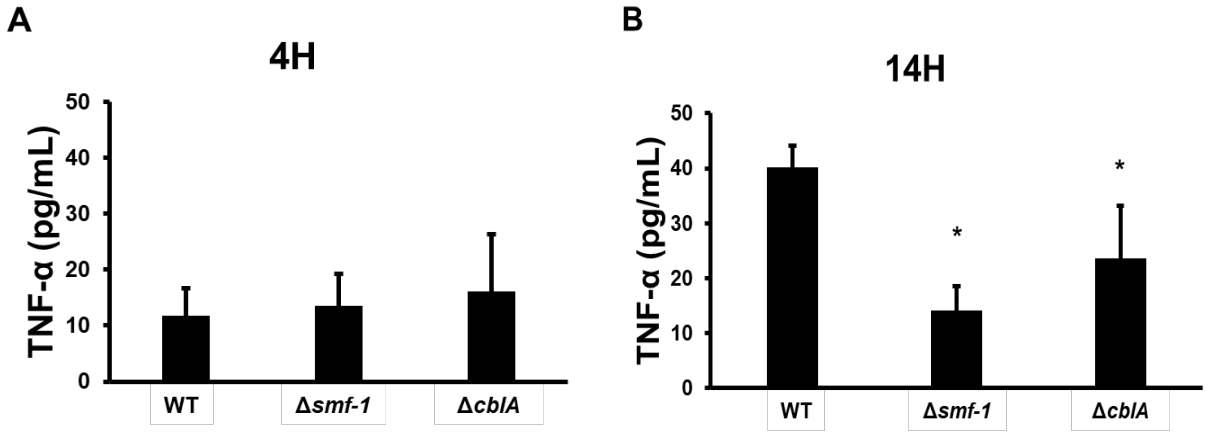

Figure 2.10. Δ*smf-1* and Δ*cblA* induce less TNF-α expression when infected on CFBE cells after 14 hr. (**A**) TNF-α expression after 4 hr infection showed no significant difference when infected with Δ*smf-1* and Δ*cblA* compared to WT. (**B**) After 14 hr infection, TNF-α secretion was significantly reduced when CFBE cells were infected with Δ*smf-1* and Δ*cblA* compared to WT. The results are representative of three independent experiments carried out in triplicate (n = 3) and error bars represent standard deviation. *$p < 0.05$ compared to WT.

2.3 Discussion

2.3.1 Role of *gpmA* in attachment on abiotic and biotic surfaces

The molecular mechanisms by which *S. maltophilia* initiates and maintains biofilm are poorly understood. This study, found that *gpmA* mediates *S. maltophilia* adherence to an abiotic surface and to human airway cells (Fig. 2.1 and Fig. 2.3). The *gpmA* gene encodes the glycolysis enzyme phosphoglycerate mutase, catalyzing the interconversion of 3-phosphoglycerate and 2-phosphoglycerate (60). Importantly, *S. maltophilia* contains genes for a complete Entner-Doudoroff alternative glycolysis pathway, and so it is likely to survive disruption of genes in the later stage of "standard" glycolysis. Indeed, the observed adherence phenotype is not due to a growth deficiency (Fig. 2.2) thus indicating a specific defect in either detecting the surface or synthesis of an adhesion molecule.

Previous studies have shown the importance of GpmA involvement in adherence of other organisms. Interestingly, phosphoglycerate mutase is upregulated in *Staphylococcus aureus* biofilm formation versus planktonic growth, highlighting its importance across different pathogenic species (61). In *Streptococcus suis,* phosphoglycerate mutase binds to host fibronectin and collagen type I (62). Similarly, in the yeast *Candida albicans*, phosphoglycerate mutase (Gpm1p) can localize to the cell wall, where it can facilitate binding to host proteins, possibly as a mechanism of immune evasion during infection (63). It is unknown whether *S. maltophilia* GpmA is similarly localized to the cell surface, though this action could directly explain the attachment defect in the Δ*gpmA* strain. Furthermore, a separate *S. maltophilia* transposon mutagenesis screen identified additional glycolysis/gluconeogenesis enzymes (glyceraldehyde 3-phosphate dehydrogenase and pyruvate dehydrogenase) as involved in biofilm formation, suggesting a unique role for carbohydrate metabolism in *S. maltophilia* biofilm formation, possibly through effects on extracellular matrix production (64). Alternatively, *gpmA* might contribute to production of a metabolite important for synthesis of an adhesive molecule. As in other biofilm forming bacteria, pili and flagella have been implicated in *S. maltophilia* adherence (39). Thus, it is possible that *gpmA* affects fimbrial or flagellar synthesis.

2.3.2 Pili mediated attachment and biofilm

The factors that mediate *S. maltophilia* attachment and biofilm formation on abiotic and biotic surfaces are poorly understood. In many bacteria, initial adherence to host epithelial cells is of key significance for initial colonization and infection, often mediated by pilus systems (32). Pili are also highly immunogenic, playing a significant role in the virulence and pathogenesis during infections. This study highlights two important pilus mediators, *smf-1* and *cblA*, that are required for *S. maltophilia* biofilm initiation and long-term formation on abiotic surfaces and CFBE cells (Fig. 2.4 and Fig. 2.9). Deletion of *cblA* and *smf-1* lead to almost complete absence of pili appendages visualized on negatively stained TEM images compared to WT, indicating that these two pilus systems are the primary appendages on *S. maltophilia* (Fig. 2.5). Hemagglutination of chicken red blood cells (RBC) has previously been described (27) to be mediated by Smf-1 and, indeed, the Δ*smf-1* strain had defects in RBC agglutination confirming the phenotype of the strain (Fig. 2.6B). Furthermore, yeast agglutination has been shown to be mediated by pili appendages in bacteria (65), this study showed that deletion of *smf-1* resulted in defects in yeast agglutination, suggesting *smf-1* has a role in yeast agglutination (Fig. 2.6A).

Biofilm formation involves many factors including attachment to host cells and surfaces, attachment to adjacent bacterial cells, and the formation of the polysaccharide matrix. This study demonstrates that *cblA* is a clinically relevant gene and is found in 33/52 (63%) of our CF *S. maltophilia* clinical isolates (Fig. 8). Considering *cblA* deletion of *cblA* leads to defects in attachment and biofilm formation, it can be suggested that this pilus system is important during CF infection and mediates initial and long-term colonization on host cells. Previous studies have also shown this to be true for *smf-1* because it is only present in *S. maltophilia* clinical isolates (33). Taken together, these data suggest that *smf-1* and *cblA* genes are likely genes involved in *S. maltophilia* CF pathogenesis and play a major role in cell attachment and biofilm formation during infection. In the attachment and biofilm assays, Δ*smf-1* and Δ*cblA* effect attachment and biofilm, though to different extents. The Δ*smf-1* strain displayed a greater attachment and biofilm formation defect on abiotic surfaces and CFBE cells than Δ*cblA*. While both showed a significant difference compared to WT, there was a clear difference in the extent of the defect, suggesting that these pilus systems are involved in different steps of the biofilm process. A previous study suggested that *smf-1* was involved with attachment to adjacent cells and host cells (27). In *Burkholderia cepacia*, CblA has been shown to promote attachment and transmigration on squamous epithelial cells, and

given their homology, this may suggest that CblA plays similar roles in *S. maltophilia* biofilm formation (66). It is possible that both genes are working in parallel to mediate either adjacent bacterial cell attachment, host cell attachment, or both.

Pili appendages are very immunogenic to the host and can initiate secretion of a plethora of interleukins by host cells. Studies have shown that Smf-1 can stimulate the innate immune response by secretion of IL-1β, TNF-α, and IL-8 in mice bladders (67). IL-1β data was inconclusive in this study and no difference was seen during infection by mutant versus WT (data not shown). This study also did not evaluate IL-8 secretion considering *S. maltophilia* encodes proteases that can degrade IL-8 (37). TNF-α secretion was measured using ELISAs after 4 hr and 14 hr infections on CFBE cells. Compared to WT, Δ*cblA* and Δ*smf-1* strains were significantly less immunogenic and elicited less secretion of TNF-α by CFBE cells after 14 hr infections (Fig. 2.10B). This difference could account for decreased CFBE cell attachment, or it could be because of lack of pilus appendages.

2.3.3 Conclusion: *Stenotrophomonas maltophilia* attachment and biofilm factors

Overall, this study highlights a glycolysis enzyme, *gpmA*, and two important pilus systems that mediate critical steps during the biofilm process on abiotic surfaces and host epithelial cells. Moreover, these data link biofilm formation with carbohydrate metabolism in *S. maltophilia*, suggesting a unique avenue for attacking this deadly pathogen. It can also be concluded that both *smf-1* and *cblA* are mediating different stages of the biofilm process since deletion of each one resulted in different attachment or biofilm capacity. Understanding the roles of these pilus systems can result in future treatments for patients suffering from *S. maltophilia* infections. Attachment and biofilm formation to both such surfaces have important implications in nosocomial settings involving patient tissues, surgical equipment, and prosthetic devices. Thus, these findings have significant clinical importance, given the frequency and seriousness of *S. maltophilia* biofilm infections.

CHAPTER 3. POLB REGULATION OF THE TYPE III SECRETION SYSTEM IN *PSEUDOMONAS AERUGINOSA*

3.1 Introduction

Pseudomonas aeruginosa is a critical pathogen that can be acquired from the environment or hospitals and can cause a vast array of infections including keratitis, acute pneumonia, bacteremia, and burn infections (20). One of the worst manifestations of *P. aeruginosa* infections is in CF lungs where it causes acute and chronic infections and is nearly impossible to eradicate due to its intrinsic antimicrobial resistance and ability to form biofilms (3), (5). The versatility and severity of *P. aeruginosa* infections is attributed to the many virulence factors that are used to destroy host cells, including pyoverdine, pyocyanin, and the type III secretion system (T3SS) (24). During the acute phase of infection, *P. aeruginosa* uses T3SS, a macromolecular syringe-like structure, to inject toxins that ultimately destroy the host cell. ExoU, ExoS, ExoT, and ExoY, the effector toxins released by T3SS, induce cell death by disrupting the cell host cytoskeleton, cell membrane, and cAMP levels thereby enhancing disease severity (20).

Regulation of T3SS activity consists of complicated genetic networks that tightly regulate this critical virulence factor. The T3SS regulon in *P. aeruginosa* contains ~40 genes that are all controlled by a master regulatory protein, ExsA (68). During non-inducing conditions, ExsA is bound and inhibited by ExsD, the anti-activator while ExsC is inhibited by ExsE (68). Low calcium concentration, the presence of serum, and host cell contact are factors known to activate ExsA and initiate T3SS expression (68). Activation of this system leads to a partner-switching mechanism where ExsD binds to the anti-anti-activator, ExsC, therefore liberating ExsA to bind to the T3SS promoter sites and initiate transcription (Fig. 3.1) (68). It is important to note that ExsA also regulates its own transcription, via the $P_{exsCEBA}$ promoter site resulting in a positive feedback loop (68). Regulation of *exsA* transcriptional activation is also stimulated by Vfr, a global regulator of virulence factor expression, that is controlled by cAMP concentrations (69). Additionally, EexsA transcript is stabilized by the protein RsmA which leads to more ExsA protein, an example of a positive feedback loop (70). ExsA inhibition is has also been shown to be influenced by environmental factors. For example, the magnesium transporter *mgtE*, can inhibit *exsA* post-transcriptional activity via inhibition of RsmA through the small RNAs, *rsmYZ* (71). Additionally, stress response genes such as the alternative sigma factor AlgU (AlgtT) has been shown to

downregulate T3SS expression (72). Further understanding the genetic networks regulating T3SS activity can help develop therapeutics or cures to *P. aeruginosa* infections.

It was previously determined, that overexpression of the stress-induced polymerase, PolB, downregulates cytotoxicity via reducing pyocyanin levels and increases biofilm formation (51). Little is known about *polB*, however, one study showed that it is homologous to the DNA repair system, Pol II, in *Escherichia coli* and is activated during DNA damage (73). Considering T3SS is an important virulence system in *Pseudomonas aeruginosa*, it can be hypothesized that *polB* could also inhibit T3SS activity. Overexpression of *polB* lead to a decrease in T3SS promoter (P_{T3SS}) activity in PA14, a traditional lab strain, and in PA103, a strain with hyperactive T3SS activity. It was found that overexpression of *polB* inhibits T3SS transcription via post-transcriptional inhibition of the T3SS activator, *exsA*. However, this inhibition is *exsD* and *rsmYZ* independent suggesting an alternate post-transcriptional ExsA inhibitory pathway.

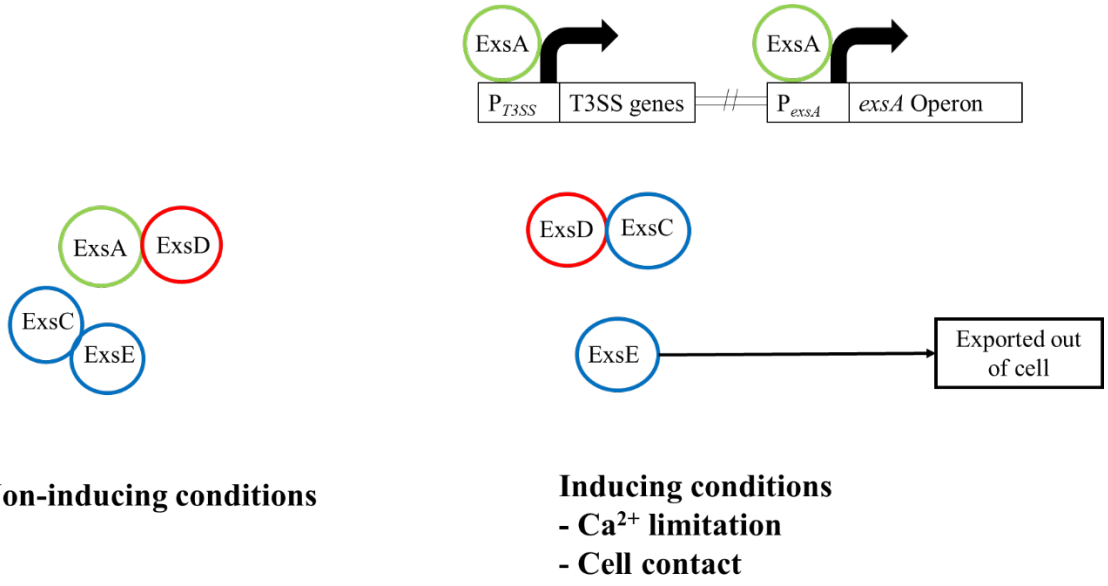

Figure 3.1. Schematic of ExsA activation and inhibition of T3SS.

3.2 Materials and Methods

3.2.1 Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 3.1 (71). Expression of *polB* was performed by transforming the strains with plasmid p*polB*, containing the *polB* gene downstream

from the P_{araBAD} promoter on vector pMQ72 (51). Plasmids were maintained in *Escherichia coli* S17-1 (74) cultured on LB agar plates or LB containing 10 µg/mL gentamicin. Isolation of the plasmid from *E. coli* was accomplished by using the QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions and transformed into the *P. aeruginosa* strains using electroporation. Transformed *P. aeruginosa* strains were cultured in Vogel Bonner minimal (VBM) medium (75) plates with 60 µg/mL gentamicin. Plasmids were confirmed by PCR primers p729 and p730 (53).

Table 3.1. Strains used in this study.

Strain	Description	References
PA14 P_{T3SS} -*lacZ*	WT strain with ExsA-dependent P_{T3SS}-*lacZ* reporter chromosomally integrated at CTX site. Shows transcriptional T3SS activity.	(71)
PA103 P_{T3SS} -*lacZ*	WT strain with hyperactive T3SS activity with ExsA-dependent P_{T3SS}-*lacZ* reporter chromosomally integrated at CTX site. Shows transcriptional T3SS activity.	(75)
PA103 *exsA-lacZ*	WT strain with P_{lacUV5}-driven *exsA* translational reporter integrated in the CTX site.	(75)

3.2.2 β-Galactosidase assays

Strains were grown to an OD_{600} of 1.0 and β-galactosidase activity was measured as previously described (75). The substrate, ortho-nitrophenyl-β-D-galactopyranoside (ONPG), was used in all β-galactosidase assays involving transcriptional reporters. For translational reporters, chlorophenol red β-D-galactopyranoside (CPRG) was used as the substrate.

3.2.3 RNA isolation and qRT-PCR

Strains were cultured as described above and 1mL of culture was collected at an OD_{600} of 1. Cells were pelleted and washed with 1X PBS and RNA was isolated using the RNeasy Plus Kit (Qiagen) according to the manufacturer's instructions. Modifications were made to the protocol as described earlier (53). cDNA was synthesized using the Superscript III first-strand synthesis for RT-PCR (Invitrogen; Eugene, OR), according to the manufacturer's instructions. Controls for

DNA contamination was tested by performing cDNA synthesis in the absence of reverse transcriptase. Quantitative Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed as previously reported (53) using the LightCycler 480 Instrument (Roche). Primers exsARTfor and exsARTrev were used which amplify *exsA* from nucleotides 436 to 676. Samples were normalized to the *fbp* transcript using primers PA5110for and PA5110rev.

3.2.4 Strain construction of PA103 Δ*exsD*

The plasmid for isogenic deletion of *exsD*, pSMC296, was used as previously described (74). This plasmid was maintained in *E. coli* S17-1 and conjugated into PA103 PA103 P_{T3SS}-*lacZ*. Exoconjugants were selected on LB plates containing 80 µg/mL gentamicin and 20 µg/mL nalidixic acid. Isolated colonies were grown overnight in LB and then plated on LB agar containing 10% sucrose to select for excision of the plasmid. Deletion mutants were confirmed using primers ExsDfor (GCGACATGAGCATCGTCGAC)/ExsDrev (CAGCAACAGGACGCTCTGTC).

3.3 Results

3.3.1 *polB* inhibits T3SS activity in PA14 and PA103

β-galactosidase assays were used to measure transcriptional activity of T3SS in PA14 and PA103 using the transcriptional reporter P_{T3SS}-*lacZ*. In PA14, overexpression of *polB* resulted in a 20% reduction in β-galactosidase activity at the T3SS promoter site compared to WT (Figure 3.2A). Similarly, in PA103, β-galactosidase activity at the T3SS promoter site resulted in 45% less activity than WT when *polB* was overexpressed (Figure 3.2B).

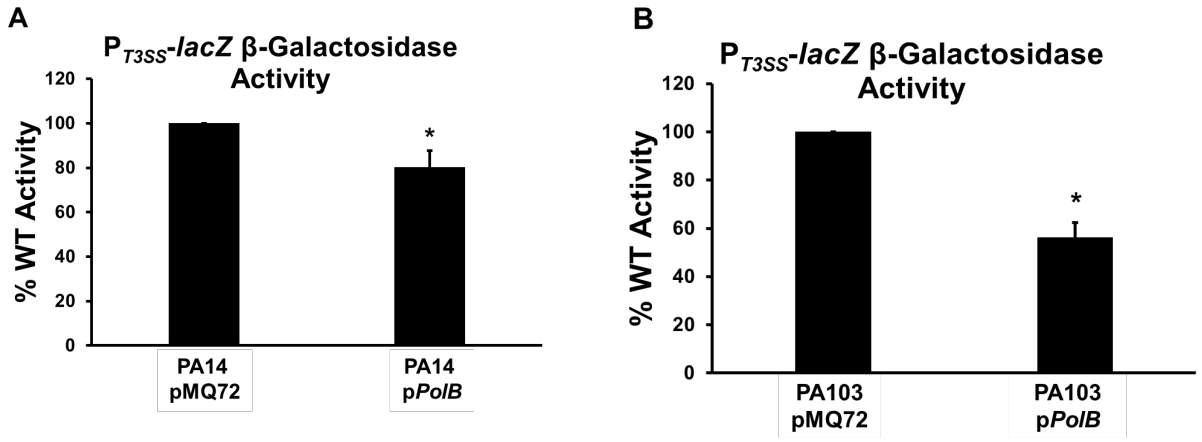

Figure 3.2. Overexpression of *polB* inhibits T3SS gene expression. (**A**) PA14 under T3SS inducing conditions and in (**B**) PA103 compared to empty vector (pMQ72). The results are representative of three independent experiments carried out in triplicate (n = 3) and error bars represent standard deviation. *$p < 0.05$ compared to PA103 pMQ72.

3.3.2 *polB* inhibits T3SS activity via post-transcriptional regulation of *exsA*

To determine the mechanism in which *polB* inhibits *exsA* activity, qRT-PCR was used to measure relative *exsA* transcript. Compared to PA103 with the empty vector, overexpression of *polB* did not repress *exsA* transcript abundance (Fig. 3.3A). Using an *exsA* translational reporter (75), β-galactosidase assays were used to measure *exsA* translation. ExsA translational activity was significantly reduced when *polB* was overexpressed in PA103 (Fig. 3.3B).

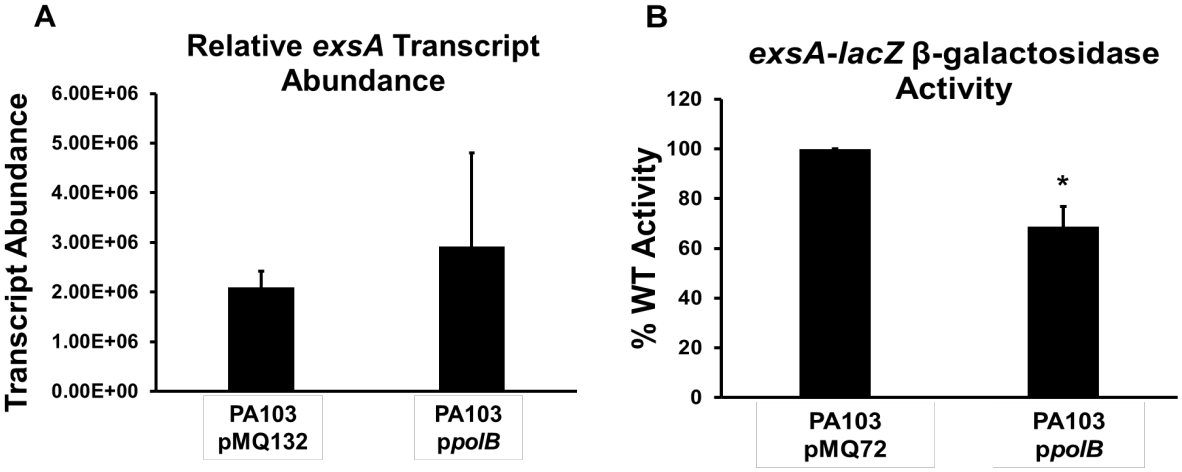

Figure 3.3. *polB* overexpression decreases *exsA* translation, but not transcript abundance. Overexpression of *polB* (**A**) does not affect *exsA* transcript abundance in PA103 but (**B**) inhibits *exsA* translational activity compared to empty vector (pMQ72). The results are representative of three independent experiments carried out in triplicate (n = 3) and error bars represent standard deviation. *$p < 0.05$ compared to PA103 pMQ72.

3.3.3 *polB* bypasses the RsmYZ mediated inhibition of ExsA

Another post-transcriptional inhibition of ExsA is mediated through the *rsmYZ* and RsmA pathway (Fig. 3.4). RsmA can be sequestered by the small RNAs, *rsmYZ,* which leads to decreased ExsA translation. Overexpression of *polB* in PA103 Δ*rsmYZ* with the T3SS P_{T3SS}-*lacZ* transcriptional reporter still leads to inhibition of T3SS activity (Fig. 3.5).

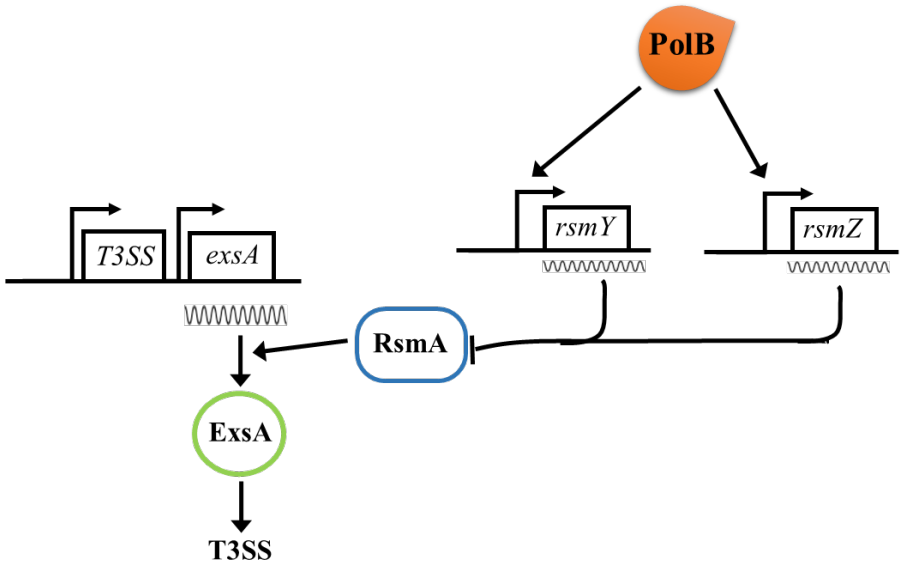

Figure 3.4. Possible pathway *polB* activates to inhibit ExsA transcript stability. Modified from Chakravarty, et al., 2017.

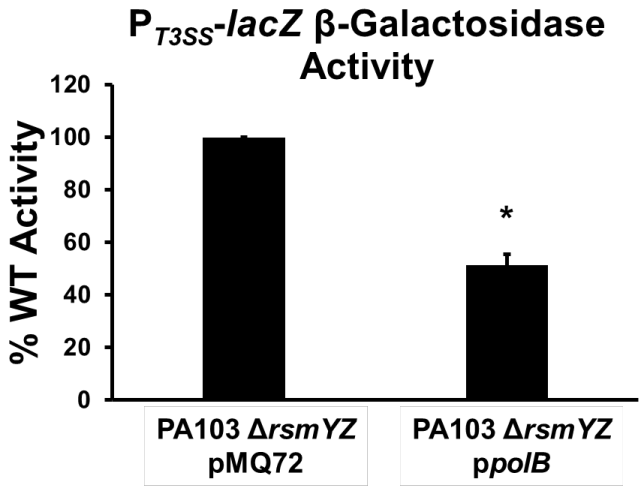

Figure 3.5. *polB* bypasses the *rsmYZ* mediated inhibition of T3SS. Inhibition of T3SS activity is present in PA103 Δ*rsmYZ* when *polB* is overexpressed compared to empty vector (pMQ72). The results are representative of three independent experiments carried out in triplicate (n = 3) and error bars represent standard deviation. *p < 0.05 compared to WT.

3.3.4 *polB* bypasses the *exsD* mediated inhibition of ExsA

To rule out mechanisms in which *polB* could inhibit ExsA post-transcriptional activity, *exsD* was isogenically deleted in PA103 containing the T3SS P_{T3SS}-*lacZ* transcriptional reporter. When *polB* was overexpressed in PA103 Δ*exsD* strain, T3SS transcriptional activity was still inhibited (Fig. 3.6).

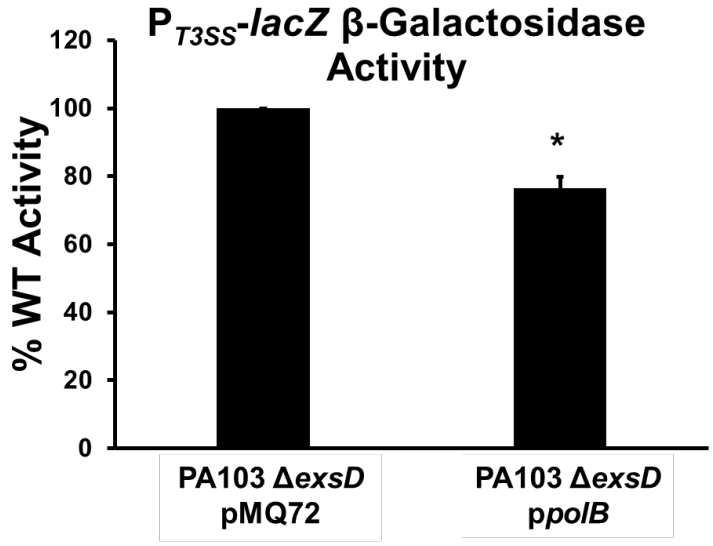

Figure 3.6. *polB* bypasses the *exsD* mediated inhibition of *exsA*. In PA103 Δ*exsD* with the transcriptional T3SS reporter P_{exsD}-*lacZ,* overexpression of polB still inhibits T3SS transcriptional activity compared to empty vector (pMQ72). The results are representative of three independent experiments carried out in triplicate (n = 3) and error bars represent standard deviation. *p < 0.05 compared to PA103 Δ*exsD* pMQ72.

3.4 Discussion

The type III secretion system (T3SS) in *Pseudomonas aeruginosa* is a tightly regulated and important virulence factor that can cause extensive damage to host cells. It is not surprising that 8% of the *P. aeruginosa* genome encodes multiple regulatory genes and pathways, including those that respond to various environmental signals (76). Some of these regulatory mechanisms include downregulation of virulence factors in response to stress, most likely as a survival mechanism for this microorganism. For example, the alternative sigma factor AlgU (AlgT) is expressed during chronic infection state and oxidative stress, downregulates T3SS expression (72), (77). Furthermore, metabolic dysregulation and nutritional stress also inhibits T3SS (78). This current study exhibits a different stress-related gene, *polB*, and its novel role in downregulating T3SS expression. The gene, *polB*, encodes for an alternate DNA polymerase that is activated during

DNA damage. Prior research showed that *polB* decreased cytotoxicity through inhibition of pyocyanin levels and increased biofilm formation (51). Overexpression of *polB* in *trans* showed a downregulation of T3SS activity in PA14 and PA103, a strain with hyper-active T3SS activity (Fig. 3.2) (75). This study also showed that downregulation of T3SS activity is due to inhibition of post-transcriptional regulation of the master activator protein, *exsA* (Fig. 3.3). qRT-qPCR analysis showed no difference in *exsA* transcript abundance when *polB* was overexpressed (Fig. 3.3A). However, the translational reporter shows downregulation of ExsA translation (Fig. 3.3B). Inhibition of *exsA* by *polB* is, however, independent of *rsmYZ* and *exsD* activation, two post-transcriptional translational inhibitors of ExsA (Fig. 3.5 and Fig. 3.6).

This study highlights an important inhibitory pathway for *exsA* activity. From this, it is known that *polB*, a stress-induced polymerase, downregulates *exsA* in some sort of post-transcriptional mechanism, probably in response to DNA damage. *rsmYZ* was explored due to its activation from biofilm production and downstream effect of T3SS repression, considering *polB* increased biofilm activity (51), (75). There is another T3SS inhibitory pathway, through *ptrB*, that is activated by DNA damage (79). It would be interesting to test if *polB* inhibits *exsA* post-transcriptional activity through this pathway. Alternatively, *polB* may be affecting expression of some upstream regulators in the *exsA* activation pathway. Many CF *P. aeruginosa* isolates exhibit non-functioning DNA repair mechanisms thereby enhancing the genetic mutations in a stressful environment (80), (81). Possibly, *polB* could mutate an upstream regulatory pathway that impairs T3SS activity via ExsA post-transcription.

From this study, it is now known that overexpression of *polB*, a stress-induced alternate DNA polymerase, inhibits T3SS by downregulating *exsA* post-transcriptional activity. Transcript abundance of *exsA* was not affected; however, the translational reporter shows repression of *exsA*. This study showed that *polB* bypasses the *exsD* and *rsmYZ* mediated pathways of *exsA* translation, so there is likely another, unknown mechanism in which *polB* is affecting post-transcriptional activity.

CHAPTER 4. STUDY CONCLUSION AND FUTURE DIRECTIONS

Cystic fibrosis (CF) is an incurable genetic disease and is caused by dysfunction of chloride transport which leads to mucus build-up within the lungs (1). This mucus is a favorable environment for bacteria to grow and colonize and CF patients suffer from chronic bacterial infections, primarily mediated by biofilms. These bacterial infections cause long-term inflammation within the lungs, and ultimately, death of the patient (11). Many of these bacterial species that colonize the lungs are acquired from the environment and once they colonize the CF lungs, they go through genetic changes mediating a switch from a planktonic to a sessile lifestyle (22), (82). Understanding these genetic networks and factors that mediate biofilm formation will help develop treatments and cures for patients suffering from these infections. The research presented focused upon two species of bacteria, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*. Both of these opportunistic Gram-negative pathogens cause severe chronic infections and they are associated with morbidity and mortality in CF patients (4), (3).

Early biofilm formation starts with attachment to a surface or host. Three attachment mediators this study focused on regarding *S. maltophilia* attachment were the glycolysis enzyme phosphoglycerate mutase (*gpmA*), and two chaperone usher pilus systems, *smf-1* and *cblA*. This study showed a role for all three of these genes in attachment to abiotic surfaces and cystic fibrosis derived bronchial epithelial cells (CFBE). Additionally, *smf-1* and *cblA* played a role in mediating long-term biofilm formation on abiotic surfaces and CFBE cells and were less immunogenic, eliciting less TNF-α secretion, compared to WT after 14 hr infection. However, both pilus systems affected attachment and biofilm formation at different extents, suggesting that they have different roles in the biofilm process. Whether that be attachment to bacterial cells, host cells, or a role in transmigration, further studies can investigate the role of each pilus system and elucidate the specific role of *smf-1* and *cblA*.

During chronic infections, *P. aeruginosa* goes through a lifestyle switch from one that is predominantly cytotoxic and a second lifestyle where biofilm forming communities facilitate a persistent infection (22). In the cytotoxic lifestyle, or acute lifestyle, *P. aeruginosa* is hypervirulent and expresses many virulence factors such as the Type III secretion system (T3SS), which is downregulated during the chronic phase (22). This lifestyle switch is thought to be triggered by the immune system and stress-induced environments (22). This study focused on the stress-

induced alternate DNA polymerase, *polB*, and its role in inhibiting T3SS activity by inhibiting post-transcriptional activity of the master activator protein, ExsA (73). Further studies can elucidate how exactly this post-transcriptional regulation works and what genetic networks *polB* is acting through to downregulate T3SS activity.

Both studies highlight the dynamic networks of *S. maltophilia* and *P. aeruginosa* within the host and important factors that mediate biofilm formation and lifestyle switches. Understanding genetic networks regulating biofilm formation and virulence factors can lead to treatments and cures for patients that suffer from these types of infections.

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