Characterization of Potential Regulators of the Type Three and Type Six Secretion Systems in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is an opportunistic bacterial pathogen, capable of causing both acute and chronic infections in humans. The establishment of infection is a result of the broad pathogen-host interactions. *P. aeruginosa* possesses a variety of virulence factors to gain advantage over the host during such interactions, and among them are the complex type three (T3SS) and type six (T6SS) secretion systems, which help the pathogen to invade, colonize and survive in the host environment. Both T3SS and T6SS play important roles in cell-cell interaction and pathogenesis. Expression and function of these systems relies on various extracellular signals, such as calcium levels, phosphate concentrations and the contact with host cell. Much is still unknown about the complex regulation of the two secretion systems.

A transposon insertion library was previously constructed in our lab to identify genes involved in T3SS regulation. In this project, the gene *fimV* and a gene cluster PA3284-81 identified from the library were selected for further study. Their effect on T3SS expression was verified and the potential mechanism investigated. The results showed that *fimV* is important for *P. aeruginosa* virulence and persistence, and the knockout *fimV* mutant downregulated the expression of *exoS*, a representative of T3SS system. PA3284-81 also influenced T3SS expression, and a significant increase of ExoS protein secretion was observed when PA3284-81 was over-expressed on a plasmid. Finally, T3SS expression was found to be regulated by zinc ions; an upregulation of *exoS* was observed under conditions of zinc depletion. In addition, PA3284-81 was shown to respond to other environmental signals, such as calcium, iron and peracetic acid, possibly influencing virulence.

To investigate the regulation of the recently discovered T6SS systems, a transposon mutagenesis library was constructed. Potential regulators of the second T6SS system H2-T6SS were screened for. A Total of eight putative regulator genes were identified, in which two genes, one that downregulated (*norC*), and the other that upregulated (PA0961) H2-T6SS were investigated further.

Significant decrease in *H2*-T6SS promoter activity as well as *exoS* promoter activity was observed in *norC* mutant, suggesting *norC* is a positive regulator of both H2-T6SS and T3SS. It is possible that the *norC* identified in the H2-T6SS transposon library serves as a link between T6SS and T3SS.

In summary, this study has confirmed the involvement of *fimV* and PA3284-81 in the regulation of T3SS. The results also revealed that zinc condition affected T3SS expression, independently of PA3284-81. New factors including *norC* in T6SS regulation were also identified. These results provide a basis for further studies on the complex secretion systems in *P. aeruginosa.*

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List of Abbreviations

ABC	ATP-binding cassette
ACP	Acyl carrier protein
aGM1	Asialo-ganglioside M1 glycolipid)
AHL	Ayl homoserine lactone
AHQ	2-alkyl-4(1H)-quinolone
AI	Autoinducer
Amp	Ampicillin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
Cb	Carbenicillin
c-di-GMP	bis-(3′-5′)-cyclic dimeric guanosine monophosphate
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colonies forming unit
COPD	Chronic obstructive pulmonary disease
CPS	Counts per second
CQ	Quantification cycle
CTD	Carboxy-terminal domain
DNTPs	Deoxyribonucleotides
dsDNA	Double-strand DNA
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl) tetraacetic acid
EPS	Extracellular polymeric substances

Fe^{2+}	Iron
GAP	GTPase activating protein
GAP	GTPase activating protein
Gm	Gentamicin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HSL	Homoserine lactone
IMC	Inner membrane component
IMTEs	Miniature Inverted-repeat Transposable Elements
Kn	Kanamycin
LB	Luria-Bertani
LPS	Lipopolysaccharide
LS	Length-sensing
MCS	Multiple cloning site
MDR	Multi drug resistance
MFP	Membrane fusion protein
Ndk	Nucleoside diphosphate kinase
NETs	Neutrophil extracellular traps
NTD	Amino terminal domain
OD	Optical Density
OMF	Membrane protein or factor
PAA	Peracetic acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIA	Pseudomonas isolation agar
PQS	Pseudomonas quinolone signal

QS	Quorum sensing
RIOH	Research Institute in Oncology and Hematology
RND	Resistance– nodulation–division
SAM	S-adenosylmethionine
SBP	Solute-binding protein
SDS	Sodium dodecyl sulfate
sRNA	Small RNA
SS	Substrate-switching
T3SS	Type III Secretion System
TA	Thymine–adenine
Tat	Twin Arginine Translocation
Tc	Tetracycline
TCA	Trichloroacetic acid
TCS	Two-component system
TCS	Two-component system
TIS	Transposon insertion sequencing
Tm	Melting temperature
Tmp	Trimethoprim
Tn	Transposon
TPEN	N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine
TPP	Threonine phosphorylation pathway
Vfr	Virulence factor regulator
Zn^{2+}	Zinc

CHAPTER I: INTRODUCTION

1.1 Introduction to *P. aeruginosa*

1.1.1 Bacteriology and Epidemiology

P. aeruginosa is a Gram-negative bacterium capable of growing in a wide range of environmental conditions, including soil, water, animals, humans, and plants. It has a genome of 6.3 Mb, which encodes a large number of genes required for regulatory systems, explaining its ability to adapt to various environments (Arai, 2011). *P. aeruginosa* can utilize numerous compounds as nutrition sources for growth and establishment of infection. It is an opportunistic pathogen that can lead to pneumonia, bloodstream, burn wound, and urinary tract infections in immunocompromised individuals. Moreover, *P. aeruginosa* infections are the leading cause of pulmonary infections in patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). *P. aeruginosa* is the predominant pathogen in CF, 80% of patients are infected by the age of 18.

P. aeruginosa forms biofilms in the host, which allows the bacterium to efficiently adhere to bio- and non-bio-surfaces, such as tissues and implanted medical-devices, leading to many complications (Lund-Palau et al., 2016). The formation of biofilms also make *P. aeruginosa* highly resistance to antibiotics, contributing to its "superbug" status (Goncalves-de-Albuquerque et al., 2016)

1.1.2 Clinical Relevance

P. aeruginosa can cause a wide range of infections in immunocompromised individuals, including respiratory tract infections such as in cystic fibrosis (CF), and hospital acquired pneumonia (HAP). CF is a life-threatening autosomal recessive genetic disease in Caucasians. A mutation in the cystic fibrosis transmembrane conductance regulator [CFTR] gene is the cause of CF disease. This dysfunction in CFTR compromises the airway lumen, encouraging the accumulation of thick mucus secretion in the respiratory tract making it difficult to clear microbial pathogens colonizing the airways. Upon infection, the host neutrophils are recruited to the lungs where neutrophil extracellular traps (NETs) are induced by pathogens such as *P. aeruginosa* to promote viscosity of airway secretions, aiding in the process of biofilm formation, escape from antimicrobials, and host defense. *P. aeruginosa* plays a significant role in the chronic infection of the upper respiratory tract in CF patients. CF patients carrying *P. aeruginosa* have a higher mortality rate, more frequent hospitalization, lower body weight, and decreased lung function compared to CF patients without *P. aeruginosa.*

Hospital acquired pneumonia is caused by the colonization of *P. aeruginosa* in the upper respiratory tract. The colonization is associated with the duration of patients stay in intensive care unit (ICU), use of antibiotics, mechanical ventilation, and/ or cross colonization from other patients (Fujitani & Sun, 2011). Ventilator associated pneumonia (VAP), is a type of hospital acquired pneumonia caused by mechanical ventilation. Patients who developed VAP due to *P. aeruginosa*, are at high risk of morbidity and mortality (Koenig & Truwit, 2006).

Moreover, urinary tract infections (UTI) caused by *P. aeruginosa* develop mostly with catheterization during the process of catheter insertion, damaging the epithelial layers and giving *P. aeruginosa* attached to the catheter, a port of entry and colonization (Mittal & Aggarwal,

2009). Burn victims are at risk of multidrug resistance (MDR) *P. aeruginosa* infection in burn wounds, due to the major breaches in the skin barrier, and tissue destruction, leading to extensive delay in recovery (Gonzalez et al., 2016). In addition, *P. aeruginosa* causes soft tissue infections such as in Follicular dermatitis, nail infection ‘’green nail syndrome’’, paronychial infection, and post-surgical infections (McNeil & Nordstrom-Lerner, 2001). Otitis externa, an infection or inflammation of the external auditory canal is associated with *P. aeruginosa* in contaminated pools (Ninkovic, Dullo, & Saunders, 2008). Furthermore, Contact-lens associated keratitis caused by contamination of contact lenses or prolonged use leads to adhesion of *P. aeruginosa* to damaged corneal epithelial cells and infection (Ramphal, McNiece, & Polack, 1981). These infections and more make *P. aeruginosa* an important nosocomial pathogen in clinical settings.

1.2 *P. aeruginosa* Pathogenesis

1.2.1 Motility and Attachment

P. aeruginosa has a single flagellum and pili for motility and surface interaction. Surface pili (type four pili -T4P) are responsible for adherence to host cells. Flagella are essential for lung infection as they facilitate the attachment to lung epithelial cells by binding to respiratory mucin and asialo-ganglioside M1 glycolipid (aGM1) (Adamo & Sokol, 2004). aGM1 is expressed during cell membrane repair and is thought to be the reason that *P. aeruginosa* only adheres to injured epithelial cells. This binding induces the release of interleukin (IL)-8 through a Src-Ras-ERK1/2-NF-κB pathway (Lau, Hassett, & Britigan, 2005). Upon adherence and attachment, a series of virulence factors such as T3SS, biofilm production, and secreted factors are expressed by *P. aeruginosa*.

1.2.2 Quorum Sensing

Quorum sensing (QS) is a bacterial cell–cell communication mechanism, which regulates gene expression in response to changes in population density. This communication is mediated by the signal molecule autoinducers (AI). They accumulate in the environment with the increase of bacterial cell population. By monitoring cell numbers, QS can alter gene expression and change cell functions as a group. QS controls sporulation, competence, antibiotic resistance, biofilm formation, bioluminescence, and secretion of virulence factors.

There are three basic characteristics possessed by all QS known so far (Rutherford & Bassler, 2012). First, members in a community produce AIs, which is at low level when cell density is low. AIs do not function at low concentration, but become operative at high concentrations under increased cell population. Thus, a response is generated by QS at high concentrations. Secondly, these AIs are detected through specific receptors in the cytoplasm or membrane. Lastly, this detection of AIs activates the production of AIs as well. Both Gram-negative and Gram-positive bacteria have QS, though there is a variation between the two. In Gram-positive bacteria, peptides (AIPs) are used as the signaling molecules. AIPs bind to membrane-bound two-component histidine kinase receptor when AIPs concertation is at high concentration. This binding results in the activation of the receptor's kinase activity. It undergoes auto phosphorylation, and the phosphate is passed to a cognate cytoplasmic response regulator. Afterwards, the response regulator interacts with transcription factors, modulating both transcription factors and gene expression (Lixa & Mujo, 2015). In contrast, Gram-negative bacteria utilize different AIs as signaling molecules for communication. There are two types AIs, S-adenosylmethionine (SAM), and acylhomoserinelactones (AHLs). Once AIs bind to the corresponding transcriptional regulator, they regulate gene expression of the QS regulon.

The QS system in *P. aeruginosa* is one of the most studied among all known QS systems. LuxI/LuxR Quorum Sensing is used in Gram-negative bacteria and shares homology with marine symbiotic bacterium *Vibrio fischeri.* LuxI is an AI synthase, which manufactures a diffusible acyl homoserine lactone (AHL) AI, by catalyzing a reaction between SAM and an acyl carrier protein (ACP). These AHL AIs bind to a cognate cytoplasmic LuxR-like transcriptional regulator at high concentrations. LuxR-type proteins degrade rapidly when they are not bound to AIs. Once they are bound to AIs. The LuxR protein stabilizes and changes its configuration, allowing it to bind DNA and activates gene transcription (Hodgkinson & Gross, 2016). LuxI homologs harbor a variation of the substrate binding pockets to harbor specific acyl-ACP for synthesis of specific AI. In the same way, LuxR homologs harbor binding pockets for specific AHL ligands. Several Gram-negative bacteria utilize LuxI/LuxR QS to control virulence factors. Some of these systems and pathogens include, LasI/LasR and RhlI/RhlR in *P. aeruginosa*, CviI/CviR in *Chromobacterium violaceum,* SmaI/SmaR in *Serratia marcescens, as well* as VjbR in *Brucella melitensis* (Smith, Wolfgang, & Lory, 2004).

1.2.3 Biofilm Formation

Biofilm formation results from a cluster of microcolonies in a extracellular polymeric substances (EPS) matrix attached to a surface (Donlan, 2002). Biofilms cause significant problems in health settings and they are estimated to be involved in 65–80% of microbial infections (Costerton, Stewart, & Greenberg, 1999). *P. aeruginosa* biofilms can grow in catheters, endotracheal tubes, mechanical heart valves, prosthetic joints, and voice prostheses. EPS is composed of exopolysaccharides, extracellular DNA (eDNA), and polypeptides separated by water channels (Sutherland, 2001). Biofilm formation begins with the adhesion of planktonic

bacteria to a surface, followed by forming microcolonies. These microcolonies expand and eventually form a three-dimensional microbial community.

In *P. aeruginosa*, alginate, Pel, and Psl polysaccharides define the structure and stability of the biofilm structure (Ryder, Byrd, & Wozniak, 2007). Alginate is a type of polymer composed of D-mannuronic acid and L-guluronic acid contributing to water and nutrition conservation in the matrix. Pel polysaccharide is a glucose-rich matrix, and Psl is composed of a repeated pentasaccharide consisting of D-mannose, L-rhamnose, and D-glucose. Both Psl and Pel are involved in the early stages of biofilm formation (Friedman & Kolter, 2004). Global regulatory systems are involved in regulating *P. aeruginosa* biofilm formation, include QS systems and two-component regulatory systems, such as GacS/GacA, RetS/LadS, and bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) (de Kievit, 2009).

The GacS/GacA, RetS/LadS systems are involved in regulation of virulence and biofilm formation. Upon autophosphorylation of the transmembrane sensor kinase GacS, a phosphate group is transferred to the response regulator GacA which, in return, upregulates the small regulatory RNAs, RsmZ and RsmY. These two small RNAs sequester RNA-binding regulatory protein RsmA limiting its availability. RsmA is a global regulator of T3SS and a repressor of the *psl* locus (Brencic et al., 2009). Moreover, RsmA negatively impact the synthesis of C4-HSL and 3-oxo-C12-HSL signal molecules for *las* and *rhl* systems (Kay et al., 2006). The hybrid sensor histidine kinase RetS represses biofilm formation through activating RsmA, whereas LadS antagonizes RetS effect (Ventre et al., 2006). RetS/LadS interaction with the GacS/GacA occurs through the modulation of GacS and its action of GacA phosphorylation. Therefore, two component systems GacS/GacA and RetS/LadS control the transition of the *P. aeruginosa* from acute to chronic infection (Goodman et al., 2004). Furthermore, a hybrid sensor kinase PA1611

modulates the transition of acute and chronic phase in *P. aeruginosa* through RsmAYZ. PA1611 interacts with RetS limiting its activity on acute factors under chronic conditions, controlling virulence via RetS-PA1611 and RetS-GacS (Weina Kong et al., 2013).

bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), an intracellular second messenger, controls the production of polysaccharides in *P. aeruginosa*. High levels of c-di-GMP promote alginate and Pel biosynthesis through binding to PelD and Alg44 (Lee et al., 2007). On the other hand, low c-di-GMP levels promote enhanced motility and biofilm dissemination (Hickman, Tifrea, & Harwood, 2005). Thus, biofilm formation is controlled by a complex regulatory network contributing to *P. aeruginosa* pathogenicity, and leading to many complications in immunocompromised individuals.

1.2.4 Secreted Virulence Factors

P. aeruginosa secretes a wide range of virulence factors upon its interaction with host targets. Phenazines, proteases, phospholipases, exotoxin-A, rhamnolipid, and nitrite reductase are all secreted by *Pseudomonas* (Kanthakumar et al., 1996). Phenazines are secondary metabolites, consisting of 1-hydroxyphenazine and phenazine-1-carboxylic acid, as well as 2 hydroxyphenazine and phenazine-1-carboxylic acid. Bioactive phenazine, pyocyanin influences bacterial pathogenicity in CF by 1) the generation of reactive oxygen species and cell damage. 2) modulating epithelial cytokine production and signaling pathways (Rada et al., 2013). Elastases (LasA and LasB), alkaline protease and protease IV exoproteases cause degradation of host defense and immunoregulatory proteins (Mariencheck & Alcorn, 2003). Exotoxin A is an inhibitor of protein synthesis. It is controlled by QS and is influenced by iron levels in the environment (Beare & For, 2003). It causes mitochondrial dysfunction, DNA degradation and

cell apoptosis. Rhamnolipids are rhamnose-containing glycolipid biosurfactants secreted in the stationary phase, which induces leukocytes and macrophages killing, intracellular junction disruption, and inhibition of phagocytosis (Read et al., 1992). These acute and chronic secreted virulence factors contribute to the bacterium establishment of infection and pathogenesis.

1.2.5 Secretion Systems

Secretion systems are cross-membrane exporting systems that are important for survival, colonization, and infection. They deliver virulence factors (exoproteins- effector proteins) into the host cytoplasm or into the environment. There are seven bacterial secretion systems known so far from T1SS to T7SS. T1SS through T6SS are present in Gram-negative bacteria, and T7SS have been discovered in mycobacteria. These systems differ from each other in the context of structure and functionality. For example, some systems secret toxins into the environment, while other systems secrete their toxins directly into the host cells to alter host physiology (Green & Mecsas, 2016). In addition, secreted proteins can be secreted through the inner, and outer membrane in one step as done by T3SS, T1SS, T4SS and T6SS. Whereas, other proteins are secreted into the periplasmic space first through general secretion pathway (Sec) or the twin arginine translocation (Tat) pathway (two step secretion pathways), then excreted to outer membrane as in T2SS, T5SS. In the two-step secretion mechanism, the Sec pathway is the most important export pathway. It is composed of Sec proteins, SecA (ATPase) and SecYEG. The other Tat (Twin Arginine Translocation) pathway is made of TatABC proteins and it is not considered essential as Sec (Tseng, Tyler, & Setubal, 2009).

Type one secretion system

The system is composed of three essential membrane proteins, ATP-binding cassette transporters (ABC transporter), a membrane fusion protein (MFP) and an outer membrane protein or factor (OMF). They all harbor an independent sec secretion system at either the N-terminus or the C-terminus. The system is similar to the resistance– nodulation–division (RND) in terms of structure. RND is a family of multidrug efflux pumps, and participates in antibiotic resistance. These two systems together form tripartite double-membrane-spanning channel with an inner membrane component (IMC), a periplasmic adaptor protein (membrane fusion protein (MFP)) and TolC (OM protein channel). It is proposed that substrates bind to the IMC and are translocated to periplasmic cavity of the MFP through the IM by using ATP hydrolysis for energy. Then, the binding of substrates allows the IMC–MFP complex to associate with TolC in the outer membrane (Delepelaire, 2004). This association triggers the opening of TolC and release of substrate into the extracellular area. T1SS exports a variety of proteins from the cytoplasm into the extracellular environment, such as haemolysins and leukotoxins. These proteins play a role in pathogenesis (haemolysin A) as well as antibacterial activity. Other secreted proteins are important for the acquisition of nutrients, for example as extracellular proteases and lipases or HasA (Saier, 2006).

Type two secretion system

Type two secretion systems include the export of hydrolyzing enzymes such as pullulanase and pseudolysin. T2SS harbors a pseudopilus that is limited within the periplasm, and is composed of five pseudopilin subunits. This pseudopilus is also similar to type four pili (T4P) in regard of structure and function. The difference between them is the fact that T4P

extends to the extracellular space through OM to ensure adhesion, motility, and DNA transport (Korotkov, Sandkvist, & Hol, 2012). SecYEG translocon (proteins responsible for the translocation of polypeptides through membranes) inserts the pseudopilin into the IM, which are cleaved by GspO23 peptidase. GspG, the major complex in pseudopilus stays anchored to IM until it is removed from the IM and polymerized into a pseudopilus. Lastly, an assembly of minor pseudopilins, GspH, GspI, GspJ and GspK at the tip of GspG filament completes the formation of pseudopilus structure. Afterwards, the substrates are translocated by SecYEG translocon to the periplasm in the form of unfolded proteins, or they could be translocated as folded proteins by the Tat transporter (Costa et al., 2015). Similar to the T1SS secretion system, it is suggested that T2SS use ATP hydrolysis for energy to assemble the pseudopilus as well as to export substrates via the OM. The interaction between pseudopilus with GspC and GspD supports the insertion of substrates into the secretion channel. Next, the pseudopilus extends in a piston manner and exports the substrates through the channel without extending to the extracellular environment (Sandkvist, 2001).

Type Four Secretion System

Type four secreting system or Type IV Conjugation and Virulence-Related Secretory Pathway (IVSP) is a unique system between all the other secretion systems. It can mediate DNA, and effector protein (toxins) translocation in bacterial and eukaryotic target cells. It is found in gram negative and gram positive bacteria. T4SS participates in plasmid borne antibiotic resistance genes through mediating conjugation of DNA. The System is comprised by 12 proteins, VirB1–11 and VirD4, forming a macromolecular complex. VirB1, a periplasmic lytic transglycosylase that degrades the peptidoglycan layer, which is needed for pilus formation. Three ATPases provide energy source for this system, VirB4, VirB11 and VirD4. EM structure

of T4SS helped in understanding the structure of the system. It showed a core OM complex connected to IM complex through a central stalk. The OM complex is composed of 14 copies of VirB7, VirB9 and VirB10. (Costa et al., 2015). VirB7 and the CTDs of VirB9 and VirB10 form the outer layer. Whereas, NTDs of VirB9 and VirB10 form the inner layer. VirB10 extends in the inner part of OM complex leading to the formation of OM pores. The IM cytoplasm is compromised of two barrels, carrying six VirB4 subunits. It is suggested that T4SS switches between pilus formation mode and substrate translocation mode. VirB11 associates with VirB4 in the pilus formation mode, while VirB11 associates with VirD4 in substrate translocation mode. Activation of this switch is proposed to occur during the binding of a receptor on recipient cells. Though is a lack of knowledge about the reliability of this proposal (Fronzes et al., 2009).

Type five secretion system

This system, also known as the autotransporter system, is considered the simplest of all. This single polypeptide is composed by the fusion of the substrate and secretion pore into a single polypeptide(van Ulsen & Rahman, 2014). Thus, this single polypeptide can initiate and drive its secretion through the OM. T5SS also functions as virulence factor secretion system. It is important for cell-cell adhesion, and plays a role in biofilm formation. Moreover, it needs the SecYEG translocon to transfer unfolded autotransporter polypeptide through the IM to the periplasm. The T5SS is composed of a passenger domain (secreted domain) folded or semi folded, and a translocator' or 'β–domain', transmembrane domain (Houben, Korotkov, & Bitter, 2014).

1.3 Type Three Secretion System (T3SS)

T3SS is a needle like structure. It injects effector proteins directly into the cytoplasm of target host cells to evade the host immune system and facilitate bacterial survival or to inhibit host cellular functions. It is present in animal and plant pathogenic or non-pathogenic symbiotic bacteria.

1.3.1 T3SS Characteristics

The structure of T3SS consists of a basal body, the needle and translocon. There are several proteins which form or assist in forming the structure of the complex, including PscC, PscN, PscF, PscP, PcrV, PopD, PopB (Lara-Tejero & Kato, 2011). The basal body is a ring-shaped body that crosses inner membrane (PscJ- lipoprotein), and outer membrane (PscC compounds). PscN is controlled by PscL, and is thought to be an ATPase that fuels the system (Yip et al., 2005). The needle is 60–100 nm in length, assembled by polymerization of PscF protein. Additionally, PscP protein determines and controls the needle length (Marlovits et al., 2006) **(Figure 1.1)**. It is believed that the needle plays a role in sensing host cell to activate T3SS (Marlovits et al., 2004). A translocon/injectisome in T3SS is responsible for transporting effector proteins (ExoS, ExoY, ExoT, ExoU) from the needle to the host cytoplasm (Galan & Wolf-Watz, 2006). Hydrophilic protein PcrV is required for translocator assembly (Costa et al., 2015). Permeabilization pore forming proteins PopD and PopB are hydrophobic translocator proteins, which function in host cells under acidic conditions (PH). Yet the exact mechanism controlling needle length and substrate switching is still a subject of debate. Bergeron et al suggested a mechanism for sensing of needle length in T3SS through the ruler protein PscP (Bergeron et al., 2016). Ruler proteins are associated with the secretion of effector proteins in T3SS at early stages. Deletion of this protein results in aberrant needle length, and the formation of multiple

hooks in flagellum. Studies on YscP ruler protein of *Shigella* T3SS revealed a conserved two domain structure. A length-sensing (LS) domain at the N terminal in charge of needle length, and a substrate-switching (SS) domain at the C terminal responsible for substrate switching through assembling T3SS structure and toxin secretion. Bergeron et al reported that PscP ruler protein of *P. aeruginosa* consists of a ball-and-chain structure, with autoprotease PscU associated with LS domain and SS domain anchored to the tip of the needle. They hypothesized that the growth strength of the needle exerted on the SS domain leads to the detachment of the N-terminal anchor form the autoprotease. This mechanism allows for sensing of needle length by the ruler protein.

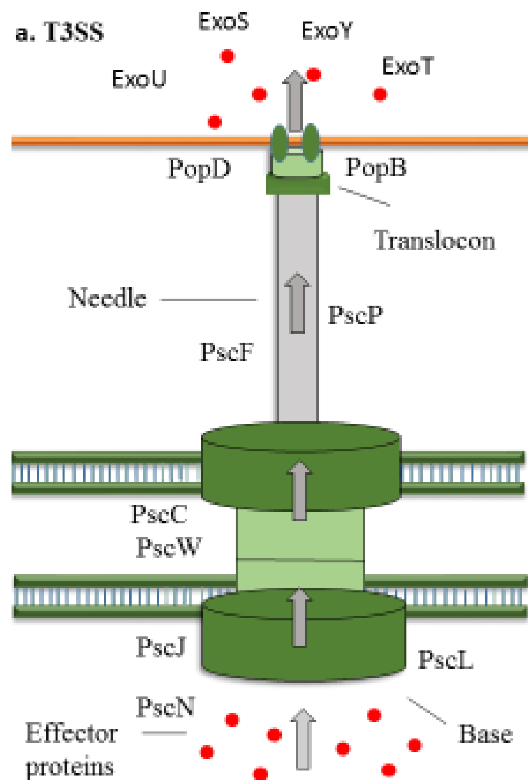


Figure 1.1 Structural organization of T3SS in Gram-negative bacteria

1.3.2 T3SS Regulation

1.3.2.1 ExsADCE Regulatory Cascade

Regulation of T3SS can be either intrinsic or extrinsic. It happens at different levels: transcriptional, post-transcriptional, and post-translational. The expression of T3SS effector proteins is regulated by the major transcriptional activator ExsA in response to environmental signals (Zheng et al., 2007). Expression of T3SS is regulated in response to environmental signals. Such signals include contact with host cell, and low extracellular Ca^{2+}. ExsA belongs to the AraC-family. It has an amino terminal domain (NTD) mediates ligand binding and oligomerization, as well as a carboxyl-terminal domain (CTD) of DNA-binding proteins. It activates the transcription of T3SS through direct binding to its promoters. ExsA triggers the activation of *exsCEBA* operon and binds to the promoters of T3SS genes by recognizing the consensus sequence (TNAAAANA)(Hovey & Frank, 1995). Upon binding to the promoter, ExsA can facilitate transcription by enhancing RNA polymerase recruitment. A protein-protein interaction network ExsCED, regulates ExsA at the post-translational level. The activation and regulation of ExsA activity is controlled by three proteins ExsD, ExsC and ExsE. In non-inducing condition, ExsC binds to ExsE in a 2:1 ratio and ExsD binds to ExsA (Diaz & King, 2011). Under inducing conditions, ExsE is secreted from T3SS, and ExsC binds with ExsD in a 2:2 ratio. Thus, ExsD binding with ExsC releases ExsA and allows the freed ExsA to activate T3SS gene transcription **(Figure 1.2)**. Extrinsic regulators are global regulators which include cAMP/Vfr, RsmAFYZ system, MucA/AlgU/AlgZR, and GacSA/RetS/LadS signaling cascade. T3SS gene expression has also been likened to quorum sensing.

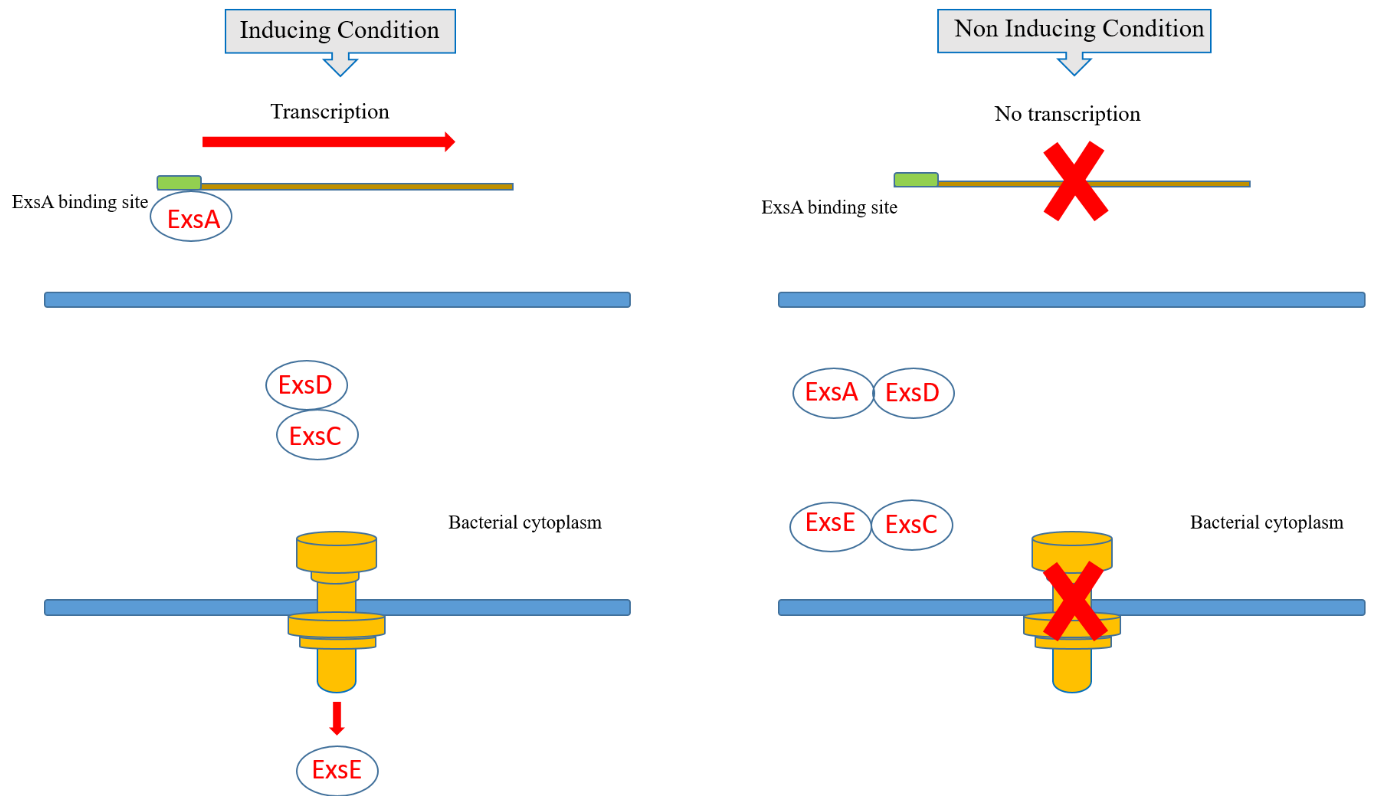


Figure 1.2 Transcription and activation of T3SS

T3SS activation through the interactions between the four proteins: ExsA, ExsC, ExsD and ExsE. When, ExsE accumulates and binds ExsC, allowing ExsD to bind ExsA blocking the transcription of the T3SS genes. When ExsE is secreted outside the bacterial cell, it allows ExsC to strongly bind to ExsD, which frees ExsA, and results in the transcription of T3SS genes by the transcriptional regulator ExsA allowing the secretion of effector proteins ExoS, ExoT, ExoU, ExoY.

1.3.2.2 GacS/A-RsmA Pathway

It has been established that high levels of RsmA (a posttranscriptional regulatory protein) lead to the expression of T3SS. Whereas, Low levels of RsmA result in the expression of PsI, thus inhibition of T3SS expression. GacA/GacS/RsmYZ pathway regulates the T3SS. RetS induces gene expression in T3SS through inhibiting the expression of RsmYZ, thus initiating T3SS regulation. In contrast, LadS/ GacS downregulate T3SS through RsmA. It was also suggested through several studies that the T3SS expression regulation is carried out by RetS/GacSA/LadS through cAMP/Vfr pathway (Diaz, King, & Yahr, 2011). This pathway is considered a major regulatory pathway controlling the expression of the T3SS and virulence factors related to acute and chronic infections.

1.3.2.3 cAMP-Dependent Pathway

CyaB and CyaA both participate in increasing 3′,5′-cyclic AMP (cAMP) cellular levels in T3SS inducing condition (limited calcium condition) to control T3SS genes as well as other genes regulated by calcium in the host environment through Vfr (Marsden & Intile, 2016). CyaB a class III membrane-associated adenylate cyclase, and CyaA a member of the cytoplasmic class I adenylate cyclase family, modulate cAMP production in *P. aeruginosa*. cAMP functions as a cofactor for Vfr, virulence regulator factor and transcriptional regulator. Vfr is a member of cAMP receptor family controlling the regulation of many genes associated with T3SS, T4P, flagellar biogenesis, pyocyanin production, exotoxin A, and protease through the LasR/LasI quorum-sensing system. Mutants defective in cAMP signalling pathway in acute pneumonia mouse model exhibited attenuated colonization and pathogenesis (Smith et al., 2004).

1.3.2.4 Two Component System (TCS)

Two-component systems (TCS) are a large family of signal-transduction systems in bacteria, archaea and eukaryote (Wolanin, Thomason, & Stock, 2002). The TCS is composed of a histidine kinase sensor that senses environmental signals and auto-phosphorylates a histidine residue and a response regulator. The system modulates gene expression in response to these external signals. Environmental signals could be related to nutrition deficiency, osmolality, oxygen limitation, phosphate deficiency, and antimicrobial peptides. *P. aeruginosa* is a versatile pathogen inhabiting in a wide range of habitats and hosts. It has over 130 TCS encoding genes , but most of them have not been characterized, while in most of the characterized TCSs, the signaling molecules have not been identified yet (Sivaneson & Mikkelsen, 2011). Major virulence- and antibiotic resistance-associated, classical-type response regulators and sensor histidine kinases in *P. aeruginosa* include PhoB–PhoR involved in phosphate level regulation, WspR–WspE in biofilm and cyclic-di-GMP level regulation, PhoP–PhoQ in Low Mg^{2+} signal, CzcR–CzcS in metal and imipenem resistance, GacA–GacS system, FleS/FleR in flagellar motility, PilS/PilR in T4P fimbriae expression, CopS/CopR in metal resistance, PmrB/PmrA in Polymyxin B and antimicrobial peptide resistance , AlgZ/AlgR in alginate biosynthesis, and LadS/RetS system (Gooderham & Hancock, 2009). These systems trigger the expression of virulence factors or lead to the transition between chronic and acute infections. Moreover, the development of novel antimicrobials requires the identification of how these two component systems work and the signals they respond to.

In several pathogens, this process triggers the production of virulence factors and/or a switch in bacterial lifestyle that is a major determining factor in the outcome and severity of the infection. Understanding how bacteria control these signaling systems is crucial to the

development of novel antimicrobial agents capable of reducing virulence while allowing the immune system of the host to clear bacterial infection, an approach likely to reduce the selective pressures for development of resistance. We provide here an up-to-date overview of the molecular basis and physiological implications of cell-to-cell signaling systems in Gram-negative bacteria, focusing on the well-studied bacterium *Pseudomonas aeruginosa*.

1.3.2.5 Other Factors

Virulence factor regulator (Vfr) is a cAMP-dependent DNA-binding protein. It functions as global regulator of virulence gene expression in *P. aeruginosa.* Vfr can regulate the expression of the *P. aeruginosa* T3SS by directly activating ExsA transcription through binding to the second promoter (PexsA) upstream of ExsA (Marsden et al., 2016).

PsrA, a stationary phase regulator and RpoS (a sigma factor and stress response regulator) activator, is a transcriptional regulator of T3SS. PsrA binds to the *exsCEBA* promoter region and regulates T3SS gene expression (Shen & Filopon, 2006). In addition, nucleoside diphosphate kinase (Ndk), a host-responsive protein, modulates *P. aeruginosa* virulence during acute infection (Yu et al., 2016). Another global regulator, Crc, an RNA-binding protein mediates T3SS through ExsA and the Cbr/Crc signaling pathway to control virulence and response to environmental stress (Dong, Zhang, & Zhang, 2013). DeaD RNA helicase activity is also necessary for the expression of T3SS genes (Tan et al., 2016). Moreover, PA1611 and RetS interaction exert a role on T3SS regulation and expression (Bhagirath et al., 2017; W. Kong et al., 2013). Additionally, Gac system and c-di-GMP signaling are linked, where high levels of c-di-GMP alters RsmY and RsmZ to modulate T3SS (Moscoso & Mikkelsen, 2011) **(Figure 1.3)**.

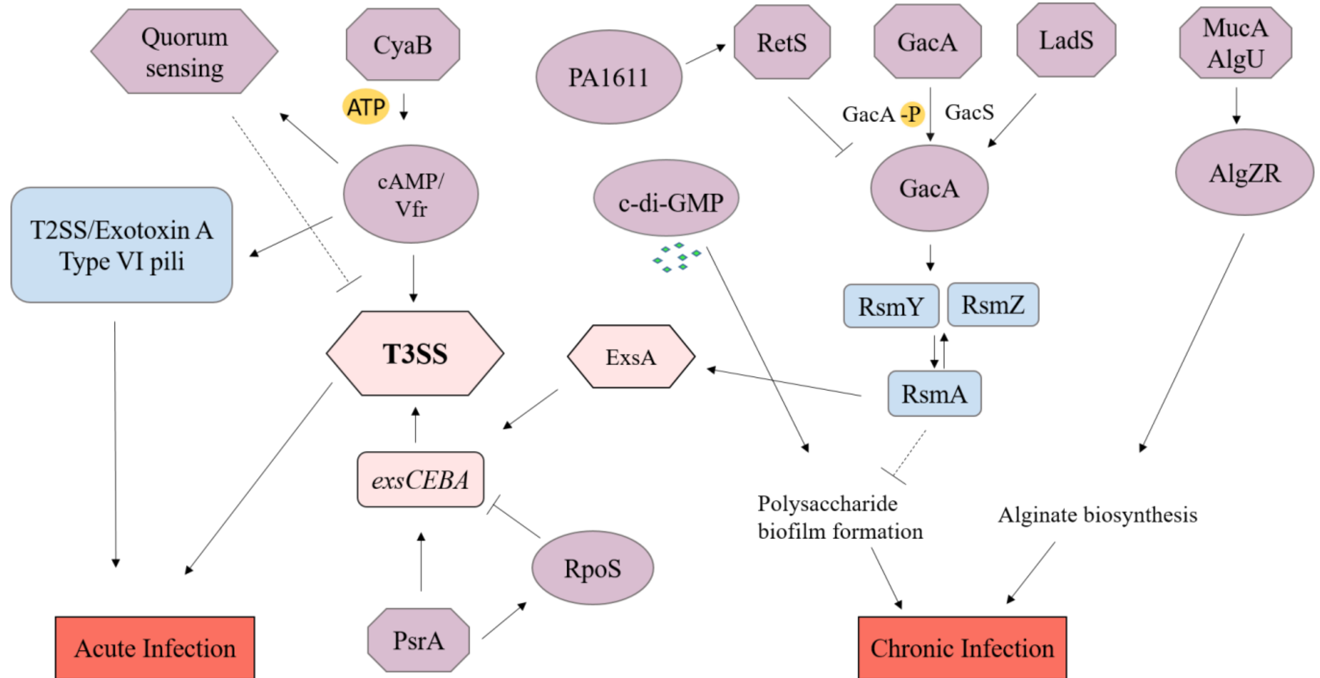


Figure 1.3 T3SS regulatory network

Solid lines indicate experimentally confirmed regulatory connections. Dashed lines indicate hypothetical regulatory connections. The figure was modified from Yahr and Wolfgang & Anantharajah (Anantharajah, Mingeot-Leclercq, & Van Bambeke, 2016; Yahr & Wolfgang, 2006).

1.4 Type Six Secretion System (T6SS)

T6SS is a secretion system only recently recognized in 2006 in *P. aeruginosa* and *Vibrio cholerae* (Pukatzki et al., 2006). It is a one-step secretion system, which functions as a defense mechanism against other bacteria (antibacterial activity) in a polymicrobial environments, and eukaryotic cells as well. T6SS along with T3SS are important for cell-cell interaction and pathogenicity. T6SS targets both eukaryotic and prokaryotic cells. Its expression is known to be controlled by PH, QS, temperature change, two component regulatory systems, and concentrations of iron, magnesium and phosphate.

1.4.1 Characteristics and Regulation of T6SS

There are three T6SS loci in *P. aeruginosa*, namely H1-, H2-, and H3-T6SS. The structure of T6SS consists of a phage like tail with several sub-complexes that secrets toxins into host cell or other bacteria in a one-step manner. T6SS gene cluster contains 13 essential genes. These proteins encoded are named from TssA to TssM. Immunity proteins are encoded close to antibacterial effectors in the T6SS gene cluster that protect the secreting cell from the effects of its own toxins. TssJ, TssL, and TssM make up the membrane anchoring complex, where the lipoprotein TssJ is anchored to the outer membrane and TssM anchored in the inner membrane with a C-terminal region interacting with TssJ. TssL is anchored in the inner membrane through a transmembrane helix, and interacts with TssM. The inner tube of T6SS is composed of Hcp, which is a protein related to gp19 in phage T4 (Miyata, Bachmann, & Pukatzki, 2013). T6SS sheath consists of two contractile proteins, TssB and TssC, forming tubular structures. VgrG a trimeric protein works on stacking Hcp hexamers, and the assembly of TssBC sheath. VgrG also shares similar structure with gp27/gp5 that form the tail spike in T4. PAAR (proline-alanine-

alanine-arginine) -repeat-containing proteins bind to VgrG protein. They particularly bind to the end of VrgG to form a sharp tip of Hcp–VgrG. Lastly, TssE protein forms the base structure of T6SS, and functions as a platform for the bacteriophage like structure. It shares a homology with gp25 in T4, and is needed for the assembly of TssBC (Leiman et al., 2009). Moreover, TssAFGK proteins are suggested to form essential core components of the base (Cianfanelli, Monlezun, & Coulthurst, 2016). A cytoplasmic AAA+ ATPase protein (ClpV) recycles the machinery after contraction by stimulating the disassembly of TssBC (Basler & Pilhofer, 2012). The whole mechanism of function consists of extension or assembly, and contraction-disassembly of the TssBC sheath. Some data suggest that the T6SS machinery is only active in the presence of aggressive competitors **(Figure 1.4)**.

T6SS is subject to complicated regulatory pathways in response to environmental stresses. Gac/Rsm signaling is engaged in the activation of the T6SS system upon cell damage. RetS/LadS/GacS pathway regulates the expression of H1-T6SS in *P. aeruginosa* (Silverman & Brunet, 2012), so do quorum sensing (Hense et al., 2007), iron uptake regulator Fur (Sana et al., 2012), δ^{54}-dependent activators (Bonemann & Pietrosiuk, 2009), and a threonine phosphorylation pathway (TPP) (Schlenker & Surawicz, 2009).

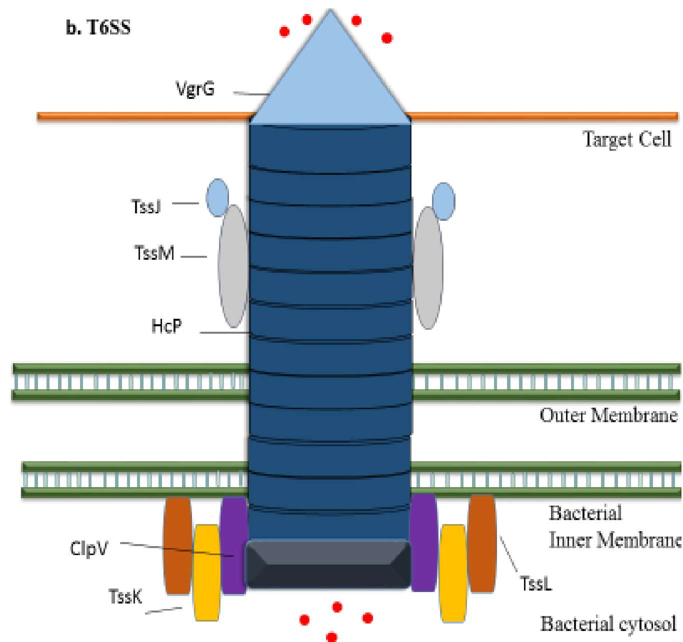


Figure 1.4 Structural organization of T6SS in Gram-negative bacteria

1.4.2 The Second T6SS (H2-T6SS)

In *P. aeruginosa*, H2 and H3 T6SS are proposed to be responsible for eukaryotic epithelial cell invasion, while H1-T6SS targets prokaryotic cells (Sana, Berni, & Bleves, 2016). To evade host innate immunity, invasive bacteria have the tendency to employ tactics such as internalization. Internalization of *P. aeruginosa* in epithelial cells was characterized as H2-T6SS related phenotype (Sana et al., 2012). PldA (phospholipase D enzymes) and VgrG2b toxins of the H2-T6SS promote entry into host cells through actin or microtubules. The VgrG2b effector of H2-T6SS facilitates the uptake of *P. aeruginosa* by epithelial cells by a specific interaction with microtubule-nucleating center γ-tubulin ring complex (γTuRC) (Sana et al., 2015). Another newly discovered H2-T6SS dependent phospholipase effector TplE, a transkingdom effector, has a eukaryotic PGAP1-like domain to facilitate an autophagy response and cytotoxicity. TplE

phospholipase activity confers toxic effect on neighboring bacteria and toxicity towards eukaryotic cells (Jiang et al., 2016).

H2-T6SS is positively regulated by QS, MvfR and LasR. Whereas Fur and iron ion repress the transcription of H2-T6SS (Sana et al., 2012). Unlike H1-T6SS, RetS/LadS does not influence H2-T6SS regulation (Mougous et al., 2006).

1.5 The Function of T3SS and T6SS

In T3SS, four effector proteins are secreted in *P. aeruginosa*, ExoS, ExoT, ExoY and ExoU. ExoS and ExoT possess GTPase activating protein (GAP) activity and ADP-ribosyltransferase activity. ExoY is an adenylate cyclase. ExoY, ExoS, and ExoT exert a cytolytic activity and inhibit phagocytosis. ExoY also affects intracellular cAMP levels and cytoskeleton reorganization. Moreover, ExoS can disrupt endothelial cell junction by cell rounding to modify the cytoskeleton. ExoS can also inhibit DNA synthesis, cell to cell adhesion, and interact with T cells and monocytes resulting in T cell apoptosis. On the other hand, ExoT inhibits cell migration, and delay wound healing. Lastly, ExoU causes rapid necrotic cell death, and attacks neutrophils to increase the susceptibility of host immune system for secondary infections (Woods, 1987). However, studies indicated that T3SS alone without the secretion of effector proteins is able to trigger the activation of caspase-1 by the inflammasome via NLRC4 (NLR family CARD domain-containing protein 4 in humans) to cause cell death (Franchi & Eigenbrod, 2009).

H1-T6SS is responsible for bacterial competition. It play a large role in chronic infections in rat models of chronic respiratory infections, and potentially in CF patients (Mougous et al., 2006). Studies have also demonstrated that T6SS can attack eukaryotic cells. H2- and H3-T6SS

play a dual role in interacting with prokaryotic and eukaryotic cells (He et al., 2004). H2-T6SS deletion mutants showed attenuated infections in a mouse model (Lesic & Starkey, 2009). H3-T6SS is upregulated in the presence of epithelial cells. H2 interacts with bacteria present in the environment through phospholipase D (PLD) PldA, whereas, H3-T6SS-dependent PldB interacts with eukaryotic cells. These two effector proteins have also shown to degrade membrane phospholipids leading to its antibacterial activity, and promoting internalization into epithelia cells in humans (Sana et al., 2015). PldA and PldB activate PI3K-AKT signaling pathways to aid in invasion of epithelial cells (Jiang & Waterfield, 2014).

The effector proteins of T6SS have been identified through bioinformatics, genetics and proteomics. H1-T6SS secrets six effector proteins. First one to be identified was the cell wall targeting enzyme Tae (peptidoglycan amidase) as well as a Tge (peptidoglycan glycoside hydrolase). Another effector is Tle with proposed lipase/esterase activity. There are other effector proteins that remain either unidentified or their specific functions are unknown. Toxins are released into the target cell by the contraction of the sheath-like structure that spikes towards the target cell.

1.6 *P. aeruginosa* and environmental signals

Heavy metals, such as zinc, nickel, copper and cobalt are known to bind to sulphide groups. These metals are also called trace elements, as they play important roles for cell functions at very low concentrations. Metal ions are essential for microorganisms' survival in the environment and in the host during infection. They are required for biological processes, and bacteria must insure homeostasis of these metal ions. Iron, zinc, manganese and copper contribute to pathogenesis in many pathogenic bacteria due to their importance in metabolism. Therefore, the bacteria employ a tight transcriptional regulation for the uptake and export of these metal ions.

1.6.1 Iron Fe^{2+}

Iron is an essential and the most abundant metal in the host. It is engaged in many biological reactions, such as DNA synthesis, gene regulation, respiration, and tricarboxylic acid (TCA) cycle. Free ferrous iron (Fe^{2+}) is rarely available. The host sequesters iron and prevents it from being available to pathogenic bacteria through extracellular metal restriction mechanisms, such as the release of chelator proteins, ferritin, and hemoglobin or transferrin. Hepcidin decreases iron release in the blood circulation. Ferritin is important for intracellular iron storage, while haptoglobin binds free haemoglobin (Parrow, Fleming, & Minnick, 2013). To fight this host defense mechanism, *P. aeruginosa* employ many mechanisms to acquire iron such as the production of siderophores, high affinity iron chelating molecules. Response regulators tune the expression of iron acquisition genes according to iron levels. In *P. aeruginosa*, a global ferric uptake regulator Fur is the response regulator for iron acquisition and homeostasis. The regulation of iron genes by Fur is conserved in Gram-negative and Gram-positive bacteria

(Porcheron & Dozois, 2015). Fur also regulates acid resistance, oxidative stress, genes related with virulence, and required for survival in host during infection. Thus, it is believed that iron limitation in the environment promote the activation of virulence factors through Fur (Payne et al., 2006). Under iron rich conditions, Fur binds to a target sequence on the promoter of iron uptake genes to repress their expression. *In vitro*, Fur activation takes place once iron levels surpass 10^{-6} M, repressing iron acquisition transporters and genes (Waldron & Robinson, 2009). Acquisition of iron involves several mechanisms: 1)Transportation through ATP- energized inner membrane transporters, 2)TonB-ExbB-ExbD ferric-siderophores transport, 3) specific outer membrane receptors for transferrins, haem-bound proteins (Hood & Skaar, 2012).

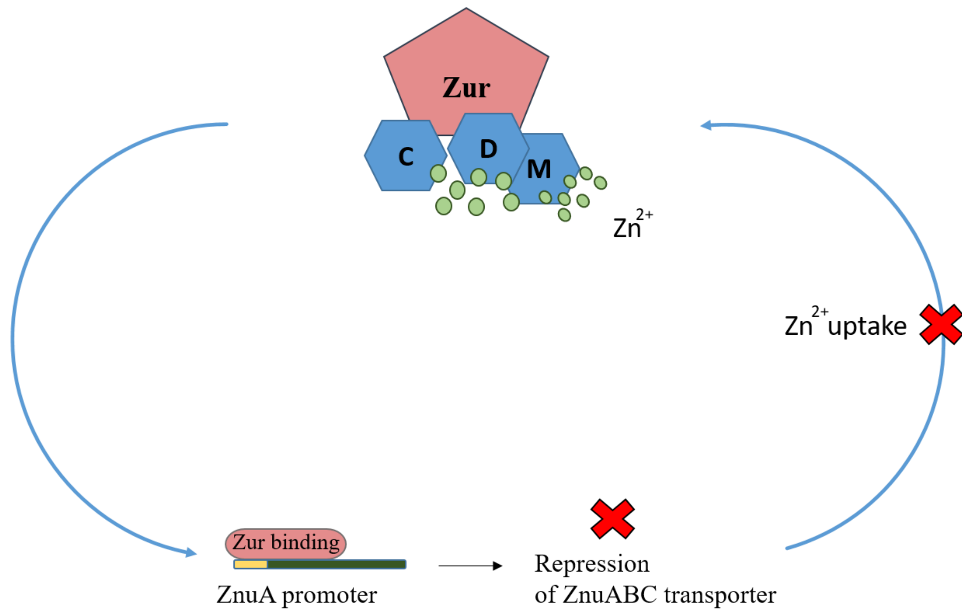
1.6.2 Zinc Transportation and homeostasis in *P. aeruginosa*

Zinc is another essential ion in bacteria due to its role in physiological processes. It functions as a cofactor for enzymes, is a basic component of ribosomal proteins, and plays a role in protein folding. Moreover, it is fundamental for transcriptional regulation, and shield the cell from free radicals. Zinc is found in about 5-6% of the proteins in bacteria (Ellison & Farrow, 2013). Yet, excess zinc can lead to toxicity and misfolded proteins, in addition to the dysfunction of physiological processes. Therefore, bacteria have to possess means of acquiring and mediating zinc in the cell to achieve zinc homeostasis.

Microorganisms utilize two mechanisms, avoidance and sequestration, to shelter themselves from metal toxicity. Transporters facilitate the entry of metal ions into the cell through specific slow and non-specific fast transporters. Zinc transporters span the cellular membrane and transport zinc into and out of cells. Six types of zinc transporters have been identified, and three of the six, the RND transporters, the CorA, and the ABC transporters have

been identified in Gram-negative bacteria (Eide, 2006). In order to balance cellular zinc uptake, microorganisms react to high zinc levels through mediating the expression of genes controlling the release of excess zinc, while downregulating genes in charge of zinc uptake. This tight zinc regulation works through metalloregulatory proteins to insure zinc homeostasis. These proteins are able to specifically sense cellular metal ions, allowing them to control translational or transcriptional feedback. A zinc specific metalloregulatory protein, Zur (Np20 and PA5499) is associated with the ferric uptake repressor (Fur) family of regulators (Andreini & Banci, 2006). It forms an operon with three genes encoding an ATP-binding cassette (ABC). The zinc uptake system is composed of permease ZnuA (PA5498), ZnuB (PA5501), and ZnuC (PA5500). ZnuA is a solute-binding protein (SBP), ZnuB is the transmembrane protein, and ZnuC has a nucleotide-binding domain.

Aside from the main zinc transporter ZnuABC, HmtA low affinity zinc permease can uptake zinc in low zinc conditions (Pederick & Eijkelkamp, 2015). Zur contains two binding sites for zinc ions, site C in charge of structural stabilization, and site M/D in charge of controlling DNA binding. Under high zinc concentrations, interaction between zinc ions and site M/D result in the binding of Zur with ZnuA promoter. This action in return, represses the transcription of ZnuA. On the other hand, low concentrations of zinc lead to a vacant M/D binding sites and initiation of the ZnuA transcription. The transportation of zinc through the cytoplasmic membrane to ZnuBC is then permitted **(Figure 1.5)**.


High Concentrations of Zinc
Zur
C
D
M
Zn2+
Zn2+ uptake
Zur binding
ZnuA promoter
Repression
of ZnuABC transporter

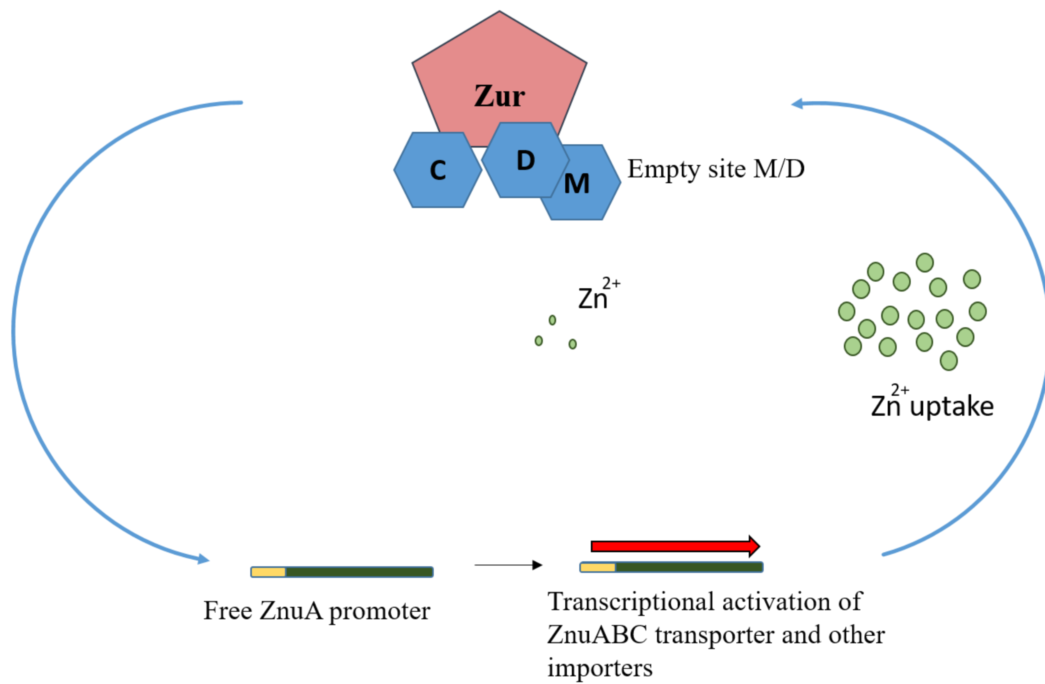

Low Concentrations of Zinc
Zur
C
D
M
Empty site M/D
Zn2+
Zn2+ uptake
Free ZnuA promoter
Transcriptional activation of
ZnuABC transporter and other
importers

Figure 1.5 Zinc intracellular regulation by Zur

Under high zinc concentrations, interaction between zinc ions and site M/D result in the binding of Zur with ZnuA promoter. This action in return, represses the transcription of ZnuA. Low concentrations of zinc lead to leaving the M/D binding sites vacant, initiating the transcription of ZnuA, and allow the transportation of zinc through the cytoplasmic membrane to ZnuBC.

1.7 Hypothesis and Objectives

1.7.1 Objective I (T3SS)

Rationale:

The T3SS in *P. aeruginosa* is a critical virulence factor. Its regulation is associated with both acute infection and disease progression. A transposon-insertion-mutant library of *P. aeruginosa* PAO1 was constructed previously in our lab, and mutants that demonstrated altered promoter activity of the T3SS toxin *exoS* were identified (Zhu, Zhao, & Liang, 2016). ExoS was used as a T3SS reporter to screen for potential regulators of T3SS and investigate the regulatory pathways. A chromosomal integrated reporter of *exoS,* CTX- *exoS lux* transcriptional reporter, was integrated in the chromosome of the wild type strain PAO1. The sites of transposon insertion in the selected mutants were determined by semi-arbitrary PCR and subsequent sequencing of the PCR products. Among the mutants acquired, insertions in three genes demonstrated increased *exoS* expression, while twelve genes had decreased levels of *exoS* expression. From this library, an insertion in *fimV* (PA3115) caused eleven-fold decrease in *exoS* expression, and PA3284 a six-fold increase in *exoS* expression **(Figure 1.6 - 1.7)**. I hypothesis that operon PA3284-3281 and *fimV* are involved in the regulation of T3SS and possibly other virulence factors. Thus, these two

genes were selected for further study in this project and their influence on T3SS regulatory network was investigated. Previously, PA3284-81 operon of four genes PA3281, PA3282, PA3283 and PA3284, was shown in microarray and transcriptome studies to be possibly influenced by zinc concentrations in the environment, and stressors such as peracetic acid (Pederick & Eijkelkamp, 2015).

Although many factors seem to influence T3SS gene expression, the mechanisms by which they exert control over T3SS gene expression are poorly understood. Further studies are needed to understand the regulatory pathways, and to identify the signals to which they respond to. In this study, the roles of operon PA3284-81 and *fimV* in the regulation of T3SS and other virulence factors were investigated. In addition, the environmental factors that activate PA3284-81 are also studied. Specific aims of this objective are I) Characterization of the regulatory function and pathway of *fimV*; and II) Characterization of the regulatory pathway and mechanism of PA3284-81.

Figure 1.6 The location of *fimV* in *P. aeruginosa* genome

The gene *fimV* located on negative strand at 3495446 - 3498205. This figure was drawn per information in the Pseudomonas Genome Database (Winsor et al., 2011)

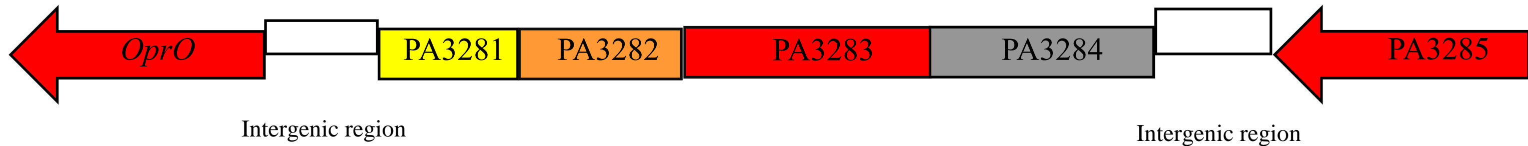


Figure 1.7 The location of PA3284-81 in *P. aeruginosa* genome

The gene PA3284-81 located on negative strand at 3674570 - 3677081. This figure was drawn per information in the Pseudomonas Genome Database (Winsor et al., 2011)

1.7.2 Objective II (T6SS, H2-T6SS)

The type six secretion system T6SS is one of the secretion systems recognized recently in *P. aeruginosa* and *Vibrio cholerae* (Pukatzki et al., 2006). T6SS promotes commensal or mutualistic relationships between bacteria and the eukaryotic host, and mediates cooperation or competition in interbacterial interactions. There are three T6SS loci: H1, H2, H3- T6SS in *P. aeruginosa*. H2- and H3-T6SS target both prokaryotic and eukaryotic cells by using the PldA, VgrG2b effectors. H2-T6SS contributes to the virulence of *P. aeruginosa* in animal and plant infection models, and plays a role in epithelial cell invasion (Jiang & Waterfield, 2014).

This system is essential for *P. aeruginosa* virulence and could be a potential target for the development of antibacterial therapy to limit *P. aeruginosa* pathogenesis. There is significant lack of information about the function and regulation of H2-T6SS, and the specific signals for its activation. The specific aim of this objective was to construct a transposon mutant library in *P. aeruginosa* (PAO1) and screen for *H2-T6SS* regulators to understand the T6SS regulatory pathways.

CHAPTER II. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are described in **Table 2.1**. The oligonucleotide primers used in this study are listed in **Table 2.2**

Table 2.1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics/sequence	Source
E.coli		
DH5α	F^- φ80*lac*ZΔM15 Δ(*lacZYA*-*argF*) U169 *recA*1 *endA*1 *hsdR*17 (r_k^-, m_k^+) phoA supE44 λ^- *thi*$^-$1 *gyrA*96 *relA*1	Invitrogen
SM10-λ *pir*	Mobilizing strain, RP4 integrated in the chromosome; Kn^r	(Simon,Priefer *et al,*1983)
P. aeruginosa		
PAO1	Wild type, lab strain	This lab
Δ*fimV*	*fimV* replacement mutant of PAO1	This study
ΔPA3282	PA3282 replacement mutant of PAO1	This study
ΔPA0961	PA0961 replacement mutant of PAO1	This study
Δ*norC*	*norC* replacement mutant of PAO1	This study

Plasmids		
pBT20	Mini-TnM delivery vector; Gm	This lab
pMS402	Expression reporter plasmid carrying the promoterless *luxCDABE*; Km^r Tmp^r	(1)
CTX-6.1	Integration plasmid origins of plasmid mini-CTX-*lux*; Tc^r	This lab
pRK2013	Broad-host-range helper vector; Tra^+, Km^r	(2)
pEX18Tc	*oriT*$^+$ *sacB*$^+$ gene replacement vector with multiple-cloning site from pUC18; Tc^r	(3)
pAK1900	*E. coli*-*P. aeruginosa* shuttle cloning vector, Amp^r	(5)
pAK3284-81	pAK1900 with a 1024 bp fragment of PA3284-81 between BamHI and HindIII; Amp^r , Cb^r	This study
pKD3284-81	pMS402 containing PA3284-81 promoter region; Knr, Tmpr	This study
pEX18Tc-*fimV*$_{up}$	pEX18Tc carrying the upstream fragment of *fimV*	This study
pEX18Tc-*fimV*	pEX18Tc carrying the upstream and downstream fragment of *fimV*	This study
pEX18Tc-PA3282$_{up}$	pEX18Tc carrying the upstream fragment of PA3282	This study
pEX18Tc-PA3282	pEX18Tc carrying the upstream and downstream fragment of PA3282	This study
pEX18Tc-PA0961$_{up}$	pEX18Tc carrying the upstream fragment of PA0961	This study
pEX18Tc-PA0961	pEX18Tc carrying the upstream and downstream fragment of PA0961	This study

pEX18Tc-*norC*$_{up}$	pEX18Tc carrying the upstream fragment of *norC*	This study
pEX18Tc-*norC*	pEX18Tc carrying the upstream and downstream fragment of *norC*	This study
CTX-*exoS*	Integration plasmid, CTX6.1 with a fragment of pKD-*exoS* containing *exoS* promoter region and *luxCDABE* gene; Km^r , Tmp^r , Tc^r	This lab
CTX-*rhlA*	Integration plasmid, CTX6.1 with a fragment of pKD-*rhlA* containing *rhlA* promoter region and *luxCDABE* gene; Km^r , Tmp^r , Tc^r	This lab
CTX-*algD*	Integration plasmid, CTX6.1 with a fragment of pKD-*algD* containing *algD* promoter region and *luxCDABE* gene; Km^r , Tmp^r , Tc^r	This lab
CTX-*phzA1*	Integration plasmid, CTX6.1 with a fragment of pKD-*phzA1* containing *phzA1* promoter region and *luxCDABE* gene; Km^r , Tmp^r , Tc^r	This lab
CTX-*phzA2*	Integration plasmid, CTX6.1 with a fragment of pKD-*phzA2* containing *phzA2* promoter region and *luxCDABE* gene; Km^r , Tmp^r , Tc^r	This lab
CTX-*rsmA*	Integration plasmid, CTX6.1 with a fragment of pKD-*rsmA* containing *rsmA* promoter region and *luxCDABE* gene; Km^r , Tmp^r , Tc^r	This lab
CTX-*rsmY*	Integration plasmid, CTX6.1 with a fragment of pKD-*rsmY* containing *rsmY* promoter region and *luxCDABE* gene; Km^r , Tmp^r , Tc^r	This lab
CTX-*rsmZ*	Integration plasmid, CTX6.1 with a fragment of pKD-*rsmZ* containing *rsmZ* promoter region and *luxCDABE* gene; Km^r , Tmp^r , Tc^r	This lab

CTX-*rhlR*	Integration plasmid, CTX6.1 with a fragment of pKD-*rhlR* containing *rhlR* promoter region and *luxCDABE* gene; Km^r , Tmp^r , Tc^r	This lab
CTX-*MexAB*	Integration plasmid, CTX6.1 with a fragment of pKD-*MexAB* containing *MexAB* promoter region and *luxCDABE* gene; Km^r , Tmp^r , Tc^r	This lab
CTX-*pqsA*	Integration plasmid, CTX6.1 with a fragment of pKD- *pqsA* containing pqsA promoter region and luxCDABE gene; Kn^r, Tmp^r, Tc^r	This lab
CTX-*PslA*	Integration plasmid, CTX6.1 with a fragment of pKD- *PslA* containing *PslA* promoter region and luxCDABE gene; Kn^r, Tmp^r, Tc^r	This lab
CTX-*H2-T6SS*	Integration plasmid, CTX6.1 with a fragment of pKD- *H2-T6SS* containing *H*2 promoter region and luxCDABE gene; Kn^r, Tmp^r, Tc^r	This lab

Note: The following abbreviations have been used: Ap^r = ampicillin resistance; Cb^r = carbenicillin resistance; Gm^r = gentamycin resistance; Kn^r = kanamycin resistance; Tc^r = tetracycline resistance; Tmp^r = trimethoprim resistance; MCS: multiple-cloning site.

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2. Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. *Proc Natl Acad Sci U S A*, **77**, 7347-7351.
3. Hoang, T.T., Karkhoff-Schweizer, R.R., Kutchma, A.J. and Schweizer, H.P. (1998) A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked Pseudomonas aeruginosa mutants. *Gene*, **212**, 77-86.
4. Sharp, R., Jansons, I.S., Gertman, E. and Kropinski, A.M. (1996) Genetic and sequence analysis of the cos region of the temperate Pseudomonas aeruginosa bacteriophage, D3. *Gene*, **177**, 47-53.

Table 2.2 Primers used in this study

Primer	Sequence (5'→3') [a]	Restriction site
fimV-UP-S	TAAgaattcCAGACCGCCGAACTGCTCA	*EcoRI*
fimV-UP-AS	CAAggatccAGTGTACGAAGCCGAACCAT	*Bam*HI
fimV-DW-S	TAAggatccGCCGAAGGTAATGACAGCCA	*Bam*HI
fimV-DW-AS	GTAaagcttGGTTCCAGGTGACCTCCTC	*HindIII*
PA3282-UP-S	ACAggtaccGCCGACAAGAAGAAGTCGTA	*KpnI*
PA3282-UP-AS	TAAggatccAAGGTCGTGTGGTTCATGTG	*Bam*HI
PA3282-DW-S	ATAggatccCGCTCTGGAGTGATGATGAA	*Bam*HI
PA3282-DW-AS	GGCaagcttCGAGCAACATCACCAGGTAG	*Hin*dIII
arb1	GGCCACGCGTCGACTAGTACNNNNNN NNNNGATAT	
arb2	GGCCACGCGTCGACTAGTAC	
P7-1	CTAACAATTCGTTCAAGCCG	
P7-2	GGATGCGTCTAAA AGCCTGC	
norC-UP-S	TACgaattcTGTTATACGCCGCAGGCTTG	*EcoRI*
norC-UP-AS	AAGggatccGCAGGATGAAGAACACACTC	*Bam*HI
norC-DW-S	AAGggatccATCACCATCGACAAGGAGG	*Bam*HI
norC-DW-AS	TCTaagcttGCGAAATCAGCATCCTCGAC	*Hin*dIII
PA0961-UP-S	TTGgaattcGCCTTTGACATAGACCAAGAG	*EcoRI*
PA0961-UP-AS	TACggatccTACTCAAATCAAGCGGTTGG	*Bam*HI
PA0961-DW-S	TATggatccTGCTAAGCCTGGTGCTGGC	*Bam*HI
PA0961-DW-AS	TAGaagcttACGTGGTGACGACGCCTGG	*Hin*dIII
PA3284 Promoter-S	TAActcgagAGGAGTGTGGCTGGGCTTG	*Xho*I
PA3284 -Promoter-AS	TTAggatccGACGACGGAAGAAGCGAG	*Bam*HI

PA3284-81-S	ATGtctagaGAGGAGTGTGGCTGGGCTTG	*XbaI*
PA3284-81-AS	GGCggatccTGTTTCATTACAGAGGAACTT	*Bam*HI

Note: Restriction endonuclease sites introduced into the primer are indicated as lowercases.

2.1.2 Culture Media and Chemicals

2.1.2.1 Luria-Bertani (LB) Medium

For 1L, 10 g tryptone, 5 g yeast extract and 10 g NaCl was added to 950 ml of deionized H_2O. The solutes were mixed by shaking until dissolved. The volume of the solution was adjusted to 1 L with deionized H_2O. The medium was then sterilized by autoclaving in 121 °C for 20 minutes.

2.1.2.2 Pseudomonas Isolation Agar (PIA) Medium

For 1L, 45 g of PIA agar powder, and 20 ml glycerol were added to 950 ml deionized H_2O. The solutes were mixed by shaking until dissolved. The volume of the solution was adjusted to 1 L with deionized H_2O. The medium was sterilized by autoclaving for 20 minutes on liquid cycle.

2.1.2.3 SOC Medium

For1 L, 20 g of Tryptone 2%, 5 g Yeast extract 0.5%, and 0.5 g of NaCl 0.05% were added to deionized H_2O. The solutes were mixed by shaking until dissolved. 10 ml of a 250 mM solution of KCl was added. (This solution was made by dissolving 1.86 g of KCl in 100 ml of deionized H_2O.) The pH of the medium was adjusted to 7.0 with 5 N NaOH (~0.2 ml). The volume of the solution was adjusted to 1 L with deionized H_2O. The medium was sterilized by autoclaving for 20 minutes on liquid cycle. 20 ml of a sterile solution of 1 M glucose and 5 ml of a sterile solution of 2 M $MgCl_2$ were added before use of the medium.

2.1.2.4 Phosphate-Buffered Saline (PBS) Buffer

For 1 L, 8 g of NaCl 137 mM, 0.2 g of KCl 2.7 mM, 1.44 g of Na_2HPO_4 10mM, and 0.24 g of KH_2PO_4 2 mM were added to 950 ml of deionized H_2O. In 800 ml of distilled H_2O, the pH was adjusted to 7.4 with HCl. The volume of the solution was adjusted to 1 L with deionized H_2O. The solution was sterilized by autoclaving for 20 minutes on liquid cycle. The buffer was stored at room temperature.

2.1.2.5 TAE 50×

242 g of Tris base, 57.1 ml of Glacial acetic acid, and 100 ml of EDTA 0.5 M (pH 8.0). The volume of the solution was adjusted to 1 L with deionized H_2O.

2.1.2.6 Twitching Motility Medium

Media was prepared as follow: % LB- agar (1%LB):4g Tryptone, 2g yeast extract, 2g NaCl, 4g agar, 400 mL water.

2.1.2.7 Swimming Motility Medium

Media for this assay consisted of per 1L liter: 5 g peptone and 3.0 g yeast extract, and 3 g Difco Bacto-Agar.

2.1.2.8 Swarming Medium

For 1 L, 5 g of Ager 0.5%, 8 g of Nutrient Broth, and 5 g glucose were added to 950 ml of deionized H_2O. The solutes were mixed with shaking until dissolved. The volume of the solution was adjusted to 1 L with deionized H_2O.The medium was sterilized by autoclaving for 15 minutes at 110°C.

2.1.3 Equipment

PCR machine: LifePro Thermal Cycler purchased from Bioer Serves Life (Tokyo, Japan); Real-time PCR machine: Real-time PCR System purchased from MBI Lab Equipment (Montreal, Canada); Imaging system: Fusion Fx purchased from MBI Lab Equipment (Montreal, Canada); Centrifuge: Optima XE-90 Ultracentrifuge purchased from Beckman Coulter (Hebron, US) and Sigma 1-14 purchased from MBI Lab Equipment (Montreal, Canada); Electrophoresis system: FB300 purchased from Fisher Science (Ottawa, Canada); Autoclave system: LV 250 Laboratory Steam Sterilizer purchased from Steris (Mississauga, Canada); Bio-safety Hood: 1300 Series A2 purchased from Thermo Scientific (Ottawa, Canada); and Electroporator: Personal Electroporation Pak purchased from BTX Harvard Apparatus (Holliston, US).

2.2 Methods

2.2.1 Bacterial Growth Conditions

P. aeruginosa and *E. coli* were routinely grown on Luria-Bertani (LB) agar or in LB broth at 37°C unless otherwise specified. LB was used as a T3SS non-inducing medium and LB supplemented with 5 mM EGTA and 2 M MgCl2 was used as a T3SS inducing medium (calcium-depletion condition). For zinc depletion 20µM of TPEN was added to the medium. 10mM Zinc concentrations added in medium for zinc experiments Antibiotics were used at the following conditions and concentrations: For *P. aeruginosa*, gentamycin (Gm) at 50 µg ml^{-1} in LB or 150 µg ml^{-1} in Pseudomonas isolation agar (PIA), tetracycline (Tc) at 70 µg ml^{-1} in LB or 300 µg ml^{-1} in PIA, carbenicillin (Cb) at 250 µg ml^{-1} in LB and trimethoprim (Tmp) at 300 µg ml^{-1} in LB, ampicillin (Amp) at 100 µg ml^{-1}. For *E. coli*, kanamycin (Kn) at 50 µg ml^{-1}, ampicillin (Amp) at 100 µg ml^{-1}, Tc at 15 µg ml^{-1} and Gm at 15 µg ml^{-1} in LB.

2.2.2 Genomic DNA Isolation

P. aeruginosa (PAO1) was inoculated in LB broth and incubated at 37°C with agitation at 225 rpm overnight. A 3 ml culture was centrifuged at 4,000×*g* for 10 minutes at room temperature. The media was aspirated and discarded. 100 µl TE buffer was added and the pellet was re-suspended completely via vortex, followed by the addition of 10 µl Lysozyme and incubation at 37°C for 10 minutes. After that, 100 µl BTL buffer and 20 µl Proteinase K Solution were added and mixed thoroughly, then incubated at 55°C in a shaking water bath. 5 µl RNase was added and the tube was inverted several times to mix. The tube was incubated at room temperature for 5 minutes, then centrifuged at 10,000×*g* for 2 minutes to pellet any undigested material. 220 µl BDL buffer was added after the supernatant was transferred to a new 1.5 ml microcentrifuge tube, to distribute the pellet. Followed by incubation at 65°C for 10 minutes, 220 µl 100% ethanol was added and vortexed for 20 seconds at maximum speed.

A HiBind DNA Mini Column was inserted into a 2 ml collection tube, and the entire sample was transferred to the column, followed by a centrifugation at 10,000×*g* for 1 minute. The column was then inserted in a new 2 ml collection tube and 500 µl of HBC buffer was added. After centrifugation, 700 µl DNA wash buffer was added and then centrifuged again. The wash step was repeated twice. The empty column was centrifuged a t a maximum speed for 10 minutes to dry the column. The DNA was eluted by adding pre-warmed Elution buffer 50 µl, and stored at -20°C.

2.2.3 Plasmid Isolation

Presto^{TM} Mini Plasmid Kit (Geneaid) was used for plasmid isolation. Cultures were grown overnight in media supplemented with the appropriate antibiotic. 1.5 ml of the overnight culture was transferred into a centrifuge tube at room temperature, and then re-suspended in 200 µl PD1 buffer, then 200 µl of lysis buffer PD2 was added, and the tube was gently mixed by inverting 10 times. 300 µl neutralization buffers PD3 were added within 5 minutes, and the solution was mixed by gently inverting10 times, followed by a 10 minutes centrifuge at 12,000×*g* to clarify the lysate. An PDH column was placed in a 2ml Collection Tube, and the clarified supernatant was transferred to the column followed by centrifugation at 12,000×*g* for 1 minute. 600 µl of wash buffer was added into the column and centrifuged at 12,000×*g* for 1 minute. This step was repeated twice. After that, the filtrate was discarded and the column was centrifuged to dry for 10min. 30-50 µl pre-warmed elution buffer was added to the column to purify the plasmid DNA followed by centrifugation to collect the eluted plasmid DNA. Plasmid was stored at -20°C.

2.2.4 Preparation of *P. aeruginosa* Competent Cells

P. aeruginosa cells were grown overnight in LB media at 37°C. The bacteria were collected and re-suspended in 1 ml 0.3 M sucrose and then centrifuged at 8000 rpm for 3 minutes at room temperature. The supernatant was discarded and the wash step was repeated two times. Then, the cells were subjected to another wash with 1 ml 10% glycerol. The amount of 10% glycerol added was based on the concentration of competent cells, and 1 ml aliquots of the resulting solution were stored at -80°C.

2.2.5 Preparation of *E. coli* Competent Cells For Chemical transformation

For chemical transformation, *E. coli* cells subjected to calcium cation induces competence and allows the cells to uptake DNA from the environment. A DH5α colony was transferred to a tube with 3 ml of LB medium. The culture was incubated for 12 hours at 37°C with agitation at 220 rpm. After that, the culture was transferred into 50 ml of LB broth. The cells were then grown until optical density (OD) of 0.5. The cells were cooled for 10 minutes on ice, then centrifuged at 5000 rpm at 4°C for 10 minutes, and the pellet was gently re-suspended in 20 ml ice cold 100 mM $CaCl_2$. The suspension was kept on ice for 10 minutes and centrifuged one more time. The pellet was gently re-suspended in 4 ml 100 mM $CaCl_2$ and kept on ice for 30 minutes. After the addition of 1 ml 80% glycerol, aliquots of 100 µl were transferred into 1.5 ml centrifuge tubes. The aliquots were frozen and stored at -80°C.

2.2.6 Preparation of *E. coli* Competent Cells For Electroporation

E. coli cells were grown in LB broth at 37°C overnight with agitation at 225 rpm. 100 ml of fresh LB broth was inoculated with 1 ml of inoculum from the overnight culture, and incubated for 3 additional hours with agitation. 50 ml of the 3-hour culture was transferred to falcon tubes, and centrifuged at 4500 rpm for 7 minutes at 4°C. The cells were re-suspended in 25 ml 10 mM HEPES and centrifuged again. The supernatant was discarded and the process is repeated one more time. Then, the cells were washed again with 1 ml 10% glycerol. The amount of 10% glycerol added was based on the concentration of competent cells. 100 µl aliquots were stored at -80°C.

2.2.7 Transformation

5 µl of ligation mix was added to DH5α competent cells thawed on ice and gently mixed. After incubation on ice for 30 minutes, the mixture was heat-shocked for 20 seconds in a 42°C water bath, and then tubes were placed on ice for 2 minutes for cold shocking. 950 µl of pre-warmed SOC medium was added into the tubes and the cells were cultured at 37°C for 1 hour with agitation at 225 rpm. 200-300 µl of transformation was spread on selective media and the plates were incubated overnight at 37°C.

2.2.8 Electroporation

As described previously by (Filloux & Ramos, 2014) for *P. aeruginosa* transformation. Electroporation cuvettes and centrifuge tubes were pre-chilled on ice. 100 µl of electroporation competent cells were thawed on ice and suspended well by gently flicking the tubes. 2-3 µl plasmid was added into the competent cells, mixed well gently, and the entire solution was transferred to the cuvette gently avoiding bubbles. Then, electroporation was carried out immediately by setting the electroporator to 2000 V. After that, 500 µl of 37°C pre-warmed SOC media were added into the cuvette and mixed well gently, then transferred to a centrifuge tube. The cells were cultured for about 1 hour at 37°C, and were spread on selective media plates and cultured overnight. The remaining cells were stored at -80°C.

2.2.9 PCR

Polymerase chain reaction (PCR) is a molecular biology technique that utilize a thermo stable DNA polymerase enzyme to amplify a specific DNA sequence of interest. All PCR reactions were carried out in LifePro Thermal Cycler (Bioer Serves Life), with either *P.*

aeruginosa genomic DNA or plasmid DNA as the template. The template denaturation was at 95°C, while annealing occurred at a ranged 55-65°C. Lastly, elongation was carried out at 72°C for 30 seconds per every 1 Kb of product. Ready PCR mix kit from AMRESCO was used to perform standard PCR and colony screening. Supplied as a 2× mixture of reaction buffer, *Taq* polymerase blend, dNTPs, electrophoresis tracking dye and a non-mutagenic EZ-Vision visualization dye was used.

The size of the PCR products was analyzed and confirmed by agarose gel electrophoresis, which was used to separate the fragments from the template DNA, unincorporated nucleotides, polymerase and buffer salts as well. After PCR amplification, 1-2 µl of 10× DNA loading buffer was added to the reaction mixtures containing the amplified fragments, and loaded on to the pre-made gel with 3 µl molecular weight marker (GeneRular™ 1Kb DNA ladder, Fermentas). The gel was run at 70 V for 70 minutes. Results were performed using imagine machine Fusion FX7 (Montreal Biotech Inc.).

2.2.9.1 Standard PCR Reactions

Primers, template DNA, and Ready Master Mix were thawed on ice; then they were all assembled on ice per **Table 2.3**. Standard PCR amplification was performed per **Table 2.4**.

Table 2.3 Standard regular PCR reaction mix

Table 2.4 Standard regular PCR system settings

Components	Volume (20 µl Reaction)
Ready PCR Mix, 2×	10 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
DNA Template	0.5-4 µl
Nuclease-Free Water	As needed

Steps	Time (minutes)	Temperature (°C)
A	2:00	95
B	0:30	95
C	0:30	55-65
D	1:00	72
Repeat Steps B-D 30× cycles		
E	10:00	72
F	Hold	4

2.2.9.2 Colony Screening

Bacteria from a single colony are directly used as a template for PCR, due to disruption of the bacteria cell walls during the initial denaturing step from the heat, which makes the DNA

reachable for the amplification process. Primers, template DNA, and Ready Master Mix were thawed on ice; then assembled on ice per **Table 2.5**. Single colony bacteria were added directly into 10 µl of UltraPure™ DNase/RNase-Free Distilled Water (Thermo Scientific) and subjected to lysis in PCR at 95C° for 15 min. Then, 2-3 µl of the lysis product was used as templet for the PCR. The PCR mixture and the standard PCR amplification was performed per **Table 2.6**.

Table 2.5 Standard PCR mix for colony screening

Components	Volume (20 µl Reaction)
Ready PCR Mix, 2×	10 µl
forward primer	0.5 µl
reverse primer	0.5 µl
Nuclease-Free Water	9 µl

Table 2.6 Standard PCR system setting for colony screening

Steps	Time (minutes)	Temperature (°C)
A	5:00	95
B	0:30	95
C	0:30	55-65
D	1:00	72
Repeat Steps B-D 30× cycles		
E	10:00	72
F	Hold	4

2.2.10 Digestion

Restriction endonucleases or restriction endonucleases are bacterial enzymes which cleave dsDNA at specific sites by recognizing specific short sequences about four to six nucleotides, resulting in restriction fragments. The target DNA is excised from the chromosome or plasmid by two restriction enzymes, creating sticky overhangs of the PCR products which can be used for ligation process. Double digestion for chromosome or plasmid occurred at 37°C for 4 hours, and for PCR products it was carried out at 37°C overnight. Restriction enzymes and buffers used are listed in the **Table 2.7**.

2.2.11 Ligation

It is performed by T4 DNA ligase enzyme. DNA ligase is capable of catalyzing the structure of covalent phosphodiester linkages, by transferring an adenyl residue to the phosphate located at the 5' terminus of one DNA molecule. Thus, conducting by hydroxyl groups on the closer 3' terminus of neighboring DNA molecules. Thus, permanently joining the nucleotides together, and the resulted DNA is joined to the backbone and the plasmid can be transformed into bacterial cells for further generation. The T4 DNA ligase (Thermo Scientific) from bacteriophage T4 have been used in a range of applications laboratory research. It is capable of ligating cohesive ends of DNA, oligonucleotides, RNA and RNA-DNA hybrids as well as blunt-ended DNA.

The reaction mixtures are assembled and incubated at room temperature for 3hours then at 4°C overnight. The required amount of inserted DNA and vector DNA was calculated per 3:1

the recommended molecular ratio and based on DNA concentrations measured with NanoDrop (Thermo Scientific). Reaction mix was assembled per **Table2.8**.

Table 2.7 Restriction enzymes and buffers used for digesting plasmids, PAO1 chromosome and PCR products.

DNA fragments	Restriction Enzymes	Buffers
pEX18Tc	*Bam*HI, *Eco*RI	*Bam*HI Buffer 1
fimV upstream fragment	*Bam*HI, *Eco*RI	*Bam*HI Buffer 1
fimV downstream fragment	*Bam*HI, *Hin*dIII	*Bam*HI Buffer 1
PA3282 upstream fragment	*BamHI, KpnI*	*BamHI* Buffer 1
PA3282 downstream fragment	*BamHI, HindIII*	*BamHI* Buffer 1
PA0961 upstream fragment	*Bam*HI, *Eco*RI	*BamHI* Buffer 1
PA0961 downstream fragment	*BamHI, HindIII*	*BamHI* Buffer 1
norC upstream fragment	*Bam*HI, *Eco*RI	*BamHI* Buffer 1
pKD 3284-81	*BamHI, XhoI*	*BamHI* Buffer 1
pAK 3284-81	*BamHI, XbaI*	*BamHI Buffer 1*

Table 2.8 Standard ligation reaction

Components	Volume (20 µl Reaction)
Linear vector	20-100 ng
DNA Insert DNA	3:1 molar ratio over vector
10× T4 DNA ligase	1 µl
Buffer T4 DNA ligase	2 ul
Nuclease-free water	

2.2.12 DNA Purification

After PCR reaction or agarose gel electrophoresis, amplified fragments were purified by Gel/PCR DNA Fragments Extraction Kit (Geneaid). As for fragments acquired from the gel, a scalpel was used to cut out the fragments with the right size while subjected to UV light. Then, to the tube with the cut-out fragment, polymerase enzyme was denatured with the addition of DF buffer and placed in a water bath at 60 ºC for 10-15 minutes. After that, the dissolved gel and DNA fragment is transferred to a DF column, allowing the DNA to bind to the column. The fragments were eluted with elution buffer after a washing step.

2.2.13 Swarming and swimming Motility Test

Motility assays were carried out in petri dishes (polystyrene, diameter of 82 mm). Plates were kept at 25 °C for 24 h upside down after preparation. The assay was performed after the plates were left to dry for 9 h, by placing 2 µL of optimized cultures (bacterial broth culture representing approximately 10^8 CFU/ml) directly into the center of the agar to evaluate motility within the semisolid agar.

Swarming assay was carried out by preparing plates containing a semisolid medium on the day of use. Plates were then inoculated with 1.5 µL culture volume with bacteria on the surface of the agar in the center of the plate enabling visualization of motility across the agar surface. After incubation at 25° C for 48 h, and the motility (migration of the bacteria through the agar) diameter was measured from the center towards the periphery of the plate. The diameters of the swarming and swimming motility zones were measured after incubation (37°C) for the indicated times.

2.2.14 Twitching Motility Test

For twitching motility, a colony was stab-inoculated through 1% Luria Bertani (LB) agar layer, to the bottom of the Petri dish then incubated at 37˚C. This method allows the cells to move in the interstitial space between the Petri dish and the surface of the agar. After incubation, a hazy area of bacterial growth is observed between the petri dish and the surface of the agar representing a positive twitching assay. While, negative controls only grow in the point of entry in the agar. To visualize the twitching motility diameter, the agar was carefully removed with tweezers and the plates were stained with 0.1% (wt/vol) crystal violet (1 min) before rinsing. The motility at this interface was subsequently measured.

2.2.15 Pyocyanin Production Measurement

To measure the pyocyanin production, first 3ml of chloroform was added to 5 ml of culture supernatant after centrifugation. Once extracted, the chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 M HCl then centrifuged and the top layer of (0.2 M HCl) was removed by pipetting. The absorption at 520 nm was measured. Acquired concentrations are

represented as micrograms of pyocyanin produced per milliliter of culture supernatant, and calculated using an extinction coefficient of 17.072 at 520 nm.

2.2.16 Biofilm Assay

Biofilm formation was quantified as described previously by O'Toole and Kolter (1998) with minor modifications. Cells from overnight cultures were inoculated at 1:100 dilutions into LB medium in 96-well polystyrene microtiter plates (Costar) and grown at 37°C for 24 h. The supernatant was gently removed by pipetting, and the wells were washed gently with PBS then left to dry for 10minutes. Volume of 100 ul 1% crystal violet was added to each well and staining was allowed for 20min. Then, the stain is removed by aspiration and wells are rinsed three times with distilled water. Remaining crystal violet was dissolved in 200 ul of 95% ethanol. 100 µl was transferred to a new polystyrene microtiter plate, and the absorbance was measured at 600 nm.

2.2.17 Antibiotic Susceptibility Test by Disc Diffusion

Bacterial cultures were added to 20 ml of LB broth containing the appropriate antibiotic for the strain. Filter paper discs inoculated with 2 µL antibiotics were placed on the surface of the LB plates and incubated overnight at 37°C. Cultured bacteria with halos were considered susceptible to the antibiotic tested. After the incubation, zone of inhibition around the discs were measured and compared with PAO1.

2.2.18 H2-T6SS Transposon Mutagenesis Library

Transposons are mobile transposable genetic elements identified in several organisms. They are classified into two groups depending on the mechanism of transposition. Class I transposon (retrotransposons) transpose themselves through copy and paste method by transcription into RNA molecules then reveres transcribe into DNA reverse transcriptase at the integral site. Class II transposons are known as DNA transposons; they transpose from one position to another by the cut and paste mechanism (Skipper & Andersen, 2013). A third class of transposons is Miniature Inverted-repeat Transposable Elements(IMTEs), they are short repeats of 400 base pairs that are flanked by 15 base pairs inverted repeats (Hayes, 2003). The mechanism of transposition of this class is still not clear.

Transposon mutagenesis utilizes foreign DNA, which is randomly insert into a chromosome to disrupt genetic elements, leading to the disruption of gene expression. Consequently, they can alter expression of not just a particular gene, but also the surrounding genes (Ruvkun & Ausubel, 1981). Transposons are characterized by their ability to carry antibacterial resistance facilitates mutant selection, mutation site detection through arbitrary PCR, and the fact that they are delivered through suicide vectors guarantee that only the initial transposition will occur within the same chromosome. The techniques used in transposon mutagenesis are considered simple and easy. Initially, transposon mutagenesis is implemented to create a library or a pool of insertion mutants through multiple disruption sites of genomic loci (Jacobs et al., 2003). Afterwards, transposon insertion mutants are grown in selective conditions to allow for the attenuated mutants to be surpassed by mutants with higher growth and survival rate. Then transposon junctions are quantified by high-throughput sequencing. These randomly pooled transposon libraries are usually produced by mariner-based transposons, or Tn5-based

vectors (Rubin et al., 1999). Mariner-based transposons target thymine–adenine (TA) dinucleotides, and characterized for their insertion in non biased manner in both essential and non essential loci. Moreover, they can generate highly saturated libraries by integrating into fixed numbers of insertion sites. On the other hand, Tn5 transposons are inserted at random sites without the need of target sequence for insertion. Also, Himar1 mariner transposon from horn fly, shares a similar mechanism with Tn5. A hyperactive C9 transposase from Himar1 mariner consist of two mutations on the vectors pBT20, pBTK30, and pBAM1(Liberati et al., 2006). Transposon insertion sequencing (TIS) is the most recent transposon based genomic analyses, which permit wide range of studies on bacterial species under different conditions.

PAO1 containing the CTX-*H2-lux* reporter fusion was subjected to transposon mutagenesis using the mariner transposon vector pBT20 as previously described with some modifications (Kulasekara et al., 2005). Briefly, the donor strain (*E. coli* SM10) harboring pBT20, and the recipient strain PAO1 were scraped from overnight incubated plates. The bacterial suspensions were adjusted to OD_{600} of 40 for the donors and an OD_{600} of 20 for the recipient. 20 µL of the donor and recipient each were mixed together, and spotted on LB agar plate then incubated at 37°C. After 3 h incubation, the mixed cultures were scraped and diluted and spread on PIA plates containing Gm at 150 µg/ml. A transposon mutant library was constructed by screening and picking 30,000 colonies grown on the selective plates and 96 well plates. To screen for genes involved in *H2- T6SS* expression, colonies were incubated overnight with appropriate antibiotics in LB. The next day, the library was inoculated into 96 well plate containing LB and Gm at 50 µg/ml. luminescence and absorbance values were measured at 37°C for 24h. Colonies that showed changes in expression by at least one fold to two or more were identified and collected. Six additional rescreens were performed to eliminate false-positive

clones. Confirmed mutants with altered *H2- T6SS* expression profiles were selected and further characterized.

The transposon insertion sites of the selected mutants were determined by an arbitrary primed polymerase chain reaction (PCR) and subsequent sequencing of the PCR products (Liang, Li, & Duan, 2008). Arbitrary primed PCR was performed in two steps with minor modifications. For the first-round the reaction was assayed under the following conditions: 95°C for 5 min followed by six cycles of 95°C for 30 s, 30°C for 30 s, and 72°C for 1.5 min; 30 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 2 min; and 72°C for 5 min. For PCR template, chromosomal DNA from the transposon mutants was used, together with the two primers, P7-1 (5′-CTAACAATTCGTTCAAGCCG-3′), reading out from one end of the transposon, and the arbitrary primer arb1 (5′GGCCACGCGTCGACTAGTACNNNNNN NNNNGATAT-3′). In the second-round, using the primers P7-2 (5′-GGATGCGTCTAAA AGCCTGC-3′), second transposon primer downstream the first primer, and arb2 (5′-GGCCACGCGTCGACTAGTAC-3′) analogous to the constant region of the original arbitrary primer. Thus, the second reaction amplifies the product of the first PCR reaction. 2 μl of the first-round PCR product was used as a template and the PCR was carried out as follows: 95°C for 1 min followed by 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 2 min; 72°C for 5 min. After that, PCR products were subjected to DNA purification by Gel/PCR Extraction Kit (Geneaid). PCR products were sequenced at the Sequencing Core Facility of Research Institute in Oncology and Hematology (RIOH), Manitoba. A BLAST query was performed on the sequences to identify the transposon insertion sites (http://www.pseudomonas.com).

2.2.19 Construction of Deletion Mutants (Δ*fimV*, ΔPA3282, ΔPA0961, Δ*norC)* in PAO1

An allelic exchange strategy using a suicide vector pEX18Tc and sucrose counter-selection system was employed for the construction of deletion mutants (Schweizer and Hoang 1995). pEX18Tc contains a counter-selectable marker *sacB*, which allow for positive selection of precise mutants amon g more frequently occurring mero-diploids on 10% sucrose LB plates (Hoang, Karkhoff-Schweizer *et al.* 1998) Upstream and downstream fragments of the genes (*fimV*, *norC*, PA3282, PA0961) were PCR – amplified using the primers in Table 2. Then, the upstream fragments and downstream fragments for each of these gene were cloned into pEX18Tc using the specified digestion enzymes indicated in **Table2.7** to obtain the plasmids pEX18Tc-*fimV*, pEX18Tc-*norC,* pEX18Tc-3282, and pEX18Tc-0961. The mutants were acquired by triparental mating. Donor, helper and recipient strains were grown in LB broth supplemented with the appropriate antibiotic at 37°C with agitation at 225 rpm. Overnight cultures of donor strain E. coli containing the plasmid pEX18Tc-*fimV,* pEX18Tc-*norC,* pEX18Tc-3282, pEX18Tc-0961, and the helper strain containing pRK2013, plus the recipient PAO1 were collected and resuspended in PBS. The supernatant was discarded and the bacterial pellets were re-suspended completely using 1 ml PBS buffer. The wash step was repeated three times. Next, cells were mixed in a ratio of 2:2:1 spotted onto LB agar plates and cultured overnight. Then, bacterial growth was scraped off the plate on the following day, and collected in 1ml SOC. Subsequently, strains that had undergone a second crossover event were selected by plating on LB supplemented with 10% sucrose. For PCR verification, forward primers from the upstream sequence and the reverse primers from the downstream sequence were used, and the accurate of the correct PCR product compared with the control and confirmed. The resultant mutants were designated as Δ*fimV,* Δ*norC,* ΔPA3282, ΔPA0961. Each deletion allele carries complete removal

of a particular open reading frame except the translation initiation and the translational stop codons.

2.2.20 Construction of Reporter Promoter Strain pKD3284 -3281

Promoter reporter strain was constructed as described previously (Liang et al., 2008). The reporter strain was constructed using the plasmid pMS402 carrying the promoter-less *luxCDABE* reporter gene cluster. Briefly, the promoter region of PA3284 -81 were amplified by PCR and the primers were synthesized based on *P. aeruginosa* PAO1 strain genome database. The fragment digested and cloned upstream the *lux* genes in pMS402 plasmid. Then, the resultant plasmid was transformed into *P. aeruginosa* by electroporation to generate the construct pKD3284 -81 (Huang, Kutchma, & Schweizer, 1998). Then, to generate chromosome integrated reporter, the plasmid pKD3284 -81 and CTX6.1 plasmid was digested with *Pac* I. The CTX6.1 is an integrated plasmid from the mini-CTX-*luxCDABE.* This plasmid carries the origin of replication, and a resistance marker (Tc resistance). After digestion, the fragments are run on electrophoresis gel and the large fragment is isolated by gel extraction. After that, the fragment is ligated with CTX6.1 plasmid and transformed into *E. coli* SM10-λ pir. Next, construct is integrated on PAO1 chromosome by bi-parental mating.

2.2.21 Construction of Over-expression strain PA3284-3281

PA3284-81 whole fragment was amplified by PCR from PAO1 genomic DNA using the primers specified in **Table 2.2**. The fragment was digested with *XbaI* and *BamHI* and cloned into the multi copy number E. coli-*P aeruginosa* shuttle vector pAK1900 (Poole et al., 1993) under *lac* promoter. The plasmid was then transferred into *P. aeruginosa* by electroporation.

2.2.22 Protein secretion measurement

Bacteria were grown in T3SS-inducing, calcium-depleted conditions (LB with 10 mM EGTA and 2 M $MgCl_2$) for 6 hours at 37°C. Bacteria were removed by centrifugation at 14,000×*g*, proteins were precipitated from the supernatant by adding an equal volume of 100% TCA, washed with acetone, and pelleted (Goodman, Kulasekara *et al.* 2004). The proteins were re-suspended in sample buffer, then separated by SDS-PAGE and visualized with Coomassie blue stain.

2.2.23 SDS-PAGE

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed as previously described by Laemmli (Laemmli 1970). The SDS-PAGE resolving gels (12%) were prepared freshly. Protein samples were boiled in addition of the loading buffer for 10 minutes prior to loading in a 12% resolving gel. The 2× SDS-PAGE gel loading buffer (100 mM pH 6.8 Tris-Cl, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 200 mM 1 M dithiothreitol) was added. A pre-stained molecular weight marker was used, and electrophoresis was carried out at 100 V. Gels were then stained in Coomassie Brilliant Blue R-250 (0.05% w/v Coomassie Brilliant Blue R-250, 10% v/v acetic acid, 30% v/v ethanol) for 1 hour and de-stained for 45 minutes. After that, the gel was submerged in fresh de-stain solution overnight (7.5% acetic acid, 20% methanol). About 50-100 µg protein samples were analyzed on the gels. The gel components are shown in **Table 2.9**.

Table 2.9 SDS-PAGE Gel

Components	SDS-PAGE Resolving Gel (12%) Per 10 ml
Sterile deionized H_2O	4.0 ml
Acrylamide mix 30%	3.3 ml
Tris 1.5 M (pH 8.8)	2.5 ml
SDS 10%	0.1 ml
Ammonium persulfate 10%	0.1 ml
TEMED	0.004 ml

2.2.24 Triparental Mating

P. aeruginosa PAO1 recipient cells were grown overnight in LB broth. Donor and helper cells carrying the mobilizing plasmid pRK2013 (Figurski and Helinski 1979) were grown in LB broth supplemented with the appropriate antibiotic at 37°C with agitation at 225 rpm. Cell cultures were collected and centrifuged at 8000 rpm for 3 minutes. The supernatant was discarded and the bacterial pellets were re-suspended completely using 1 ml PBS buffer. Typically, 1 ml of the recipient culture, the donor culture and helper culture were added to separate centrifuge tubes, and centrifuged at 8000 rpm for 3 minutes. The supernatants were discarded and cells were washed with PBS two times. Weights of the cell pellets were measured and volumes of SOC media added were calculated per the pellets weights for each of the strains and mixed well. Typically, the final concentration of *P. aeruginosa* is 250 µg/µl and 500 µg/µl for *E. coli.* Equal volumes of cell suspensions were mixed thoroughly (20-40 µl of the mixtures) were plated on LB plates and cultured overnight. Then, bacterial growth was scraped of the plate and collected in 300 µl SOC

media and spread on PIA (Gm150) plus 10% sucrose for selection. Colonies grown were confirmed by single PCR.

2.2.25 Biparental Mating

A mobilizing strain SM10 containing the plasmids CTX-*exoS* and other chromosome integrated virulence reporters were grown in LB (Tc15) (Simon, Priefer *et al.* 1983). Overexpression strain pAK3284-81 and mutants Δ*fimV,* Δ*PA3282,* Δ*norC,* Δ*PA0961* were grown in LB overnight at 37°C with agitation at 225 rpm. The inoculums were collected into falcon tubes and centrifuged at 8000 rpm for 3 minutes. The supernatant was discarded and the bacterial pellets were thoroughly re-suspended in 1 ml PBS buffer. 1 ml of each of the recipient culture, the donor cultures were added to centrifuge tubes for centrifugation at 8000 rpm for 3 minutes, and the supernatant was discarded. Cells wash was repeated twice and the weight of the tubes was measured. Volumes of SOC media were added per the calculated weigh for each of the strain. Cell suspensions were mixed well with equal volumes (20-40 µl of the mixtures) and plated on LB plates then cultured overnight. The next day, grown pellets were scraped and collected in 500 µl SOC media then spread on LB plates with appropriate antibiotics (Tc).

2.2.26 Gene Expression Detection

The over-expression vector pAK 3284-81 and the control pAK1900 were transformed into the reporter strains CTX-*rhlA*, CTX-*algD*, CTX-*phzA1*, CTX-*phzA2*, CTX-*rsmA*, CTX-*rsmY*, CTX-r*smZ*, CTX-*rhlR*, CTX-*MexAB*, CTX-*pqsA*, CTX *PslA*, CTX- *exoS* by electroporation. Additionally, the mutants Δ*fimV*, ΔPA3282, Δ*norC* were transferred into CTX-*exoS* through biparental mating. Gene expression was measured through the *lux*-based reporters

by measuring the light production in counts per second (cps). Cells were grown overnight in liquid cultures. Overnight cultures of the reporter strains and diluted to (OD_{600}) of 0.2 then cultured for additional 3 hours. After culturing, cells were used as inoculant into a white 96 well plate with a transparent bottom. Briefly, 5 µl of the fresh cultures was inoculated into wells including 95 µl medium with appropriate antibiotics plus other specific conditions based on the experiments. 60 µl mineral oil was added to the wells to prevent evaporation of the assay during the read. Promoter activity was measured every 30min for 24h as well as the bacterial growth by measuring OD_{600} in a microplate reader by Synergy 2 Multimode Microplate Reader (Bio Tek). To assess the reporters expression on LB agar plates, imagine machine Fusion FX7 (Montreal Biotech Inc.) was used.

CHAPTER III. RESULTS

Characterization of potential T3SS regulators

A transposon-insertion-mutant library of *P. aeruginosa* PAO1 was constructed previously in our lab, using a CTX- *exoS lux* transcriptional reporter integrated on the chromosome of PAO1 (Zhu, Zhao, & Liang, 2016). Mutants with altered promoter activity of the T3SS effector gene *exoS* were identified. The sites of transposon insertion in the selected mutants were determined by semi-arbitrary PCR and subsequent sequencing of the PCR products. Among the mutants acquired, insertions in two genes, *fimV* (PA3115) displayed eleven fold decrease, and PA3284 with a six-fold increase in *exoS* expression, these two genes were both selected to further study, their influence on the T3SS regulatory network and *P. aeruginosa* virulence.

3.1 Characterization of Regulatory Function and Phenotypic Characteristics of *fimV*

3.1.1 Decreased T3SS expression in *fimV* deletion mutant

FimV is located upstream of the *truA* gene. To verify if *fimV* was responsible for the eleven-fold decrease in *exoS* expression in the mutant library, a *fimV* deletion mutant was constructed by employing the allelic exchange method with pEX18Tc sucrose counter-selection system (*sacB* strategy) (Hmelo et al., 2015). The resulting mutant PAO1(Δ*fimV)* was confirmed by PCR using the forward primer from the upstream fragment and the reverse primer from the downstream fragment. To test *exoS* expression in PAO1(Δ*fimV*), the CTX-*exoS* promoter reporter was integrated on the chromosome of the PAO1(Δ*fimV*) by biparental mating. The reporter was integrated on PAO1 chromosome as well for control, yielding CTX-*exoS* in PAO1(Δ*fimV)* and CTX-*exoS* in PAO1. We then measured gene expressions for CTX-*exoS* in PAO1 as well as

CTX-*exoS* in PAO1 ((Δ*fimV*). Based on the lux reporters, gene expression was measured as counts per second (CPS) of light production in a Microplate Reader every 30 min for 24 h. The bacterial growth was monitored at the same time by measuring the OD600 (absorbance or optical density based on the concentration of bacterial cells in the cultures at a wavelength of 600 nm). In agreement with the transposon insertion library, *fimV* displayed decreased expression in *exoS*, although it was an about a two-fold decrease (at 5-6 hour time point) in comparison with the drastic decrease of eleven folds (at 5-6 hour time point) (data not shown) observed in the transposon mutant **(Figure 3.1)**. This indicates *fimV* involvement in T3SS regulatory pathway.

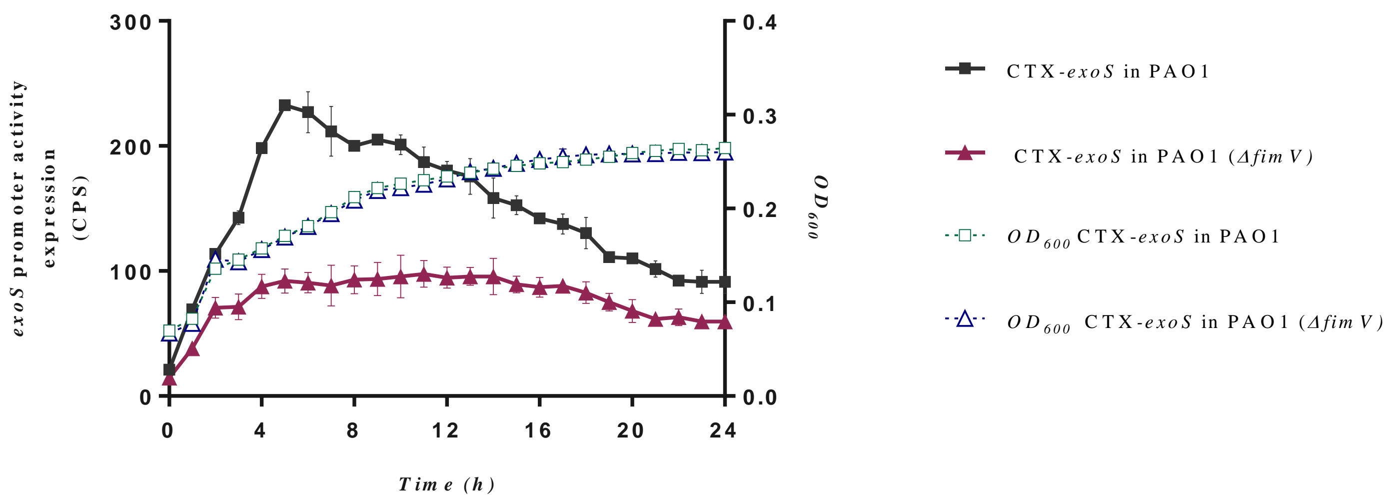


Figure 3.1 Decreased *exoS* expression in PAO1(Δ*fimV*)

CTX-*exoS* promoter reporter fusion was integrated on the chromosome of PAO1(Δ*fimV*) and PAO1 **respectively** and used to compare expression levels of *exoS*. The result represents the average of triplicate experiments and the error bars indicate standard deviations.

3.1.2 Phenotypic changes in *fimV* mutant

It is known that most T3SS regulators in *P. aeruginosa* also control other virulence factors, for example, the GacA/GacS system regulates not only T3SS, but also biofilm formation and motility (Parkins, Ceri, & Storey, 2001). To assess the effect of *fimV* on the other virulence factors and its potential connection with other regulatory pathways, the phenotypic characteristics of PAO1 (Δ*fimV*) mutant and PAO1 were compared. Each of the following virulence phenotypes were assessed in PAO1 (Δ*fimV*) mutant and compared with PAO1: swimming, swarming and twitching motility, antibiotic resistance pyocyanin production. *P. aeruginosa* utilizes polar flagellum for swimming motility with both clockwise and counterclockwise rotation of the flagellum, to facilitate bacterial movement in liquid and vicious environments. As for swarming motility, it occurs on semisolid surfaces based on two rhamnolipids and 3-hydroxyalkanoic acids (HAA) in addition to the flagellum. Twitching motility is a form of surface translocation that mediates attachment to host cells. Mechanism of translocation and motility involves a repeated series of fimbrial retraction and extension along the surface (Hazlett & Moon, 1991). Twitching motility has been shown to be a substantial virulence factor for infection and possibly biofilm formation (Potera, 1999). Twitching motility test was carried out by stab-inoculating a colony of either the wild type PAO1 or PAO1 (ΔfimV) through 1% Luria Bertani (LB) agar layer, to the bottom of the Petri dish then incubated at 37˚C. After incubation, a hazy area of bacterial growth is observed between the petri dish and the surface of the agar to visualize the twitching motility diameter, the agar was carefully removed and the plate was stained with 0.1% (wt/vol) crystal violet (1 min) before rinsing. The diameter of the motility interface was then measured. Interestingly, PAO1 (Δ*fimV*) displayed significant

decrease in twitching motility in comparison with the wildtype **(Figure 3.2)**, although, it was not completely defective in twitching motility.

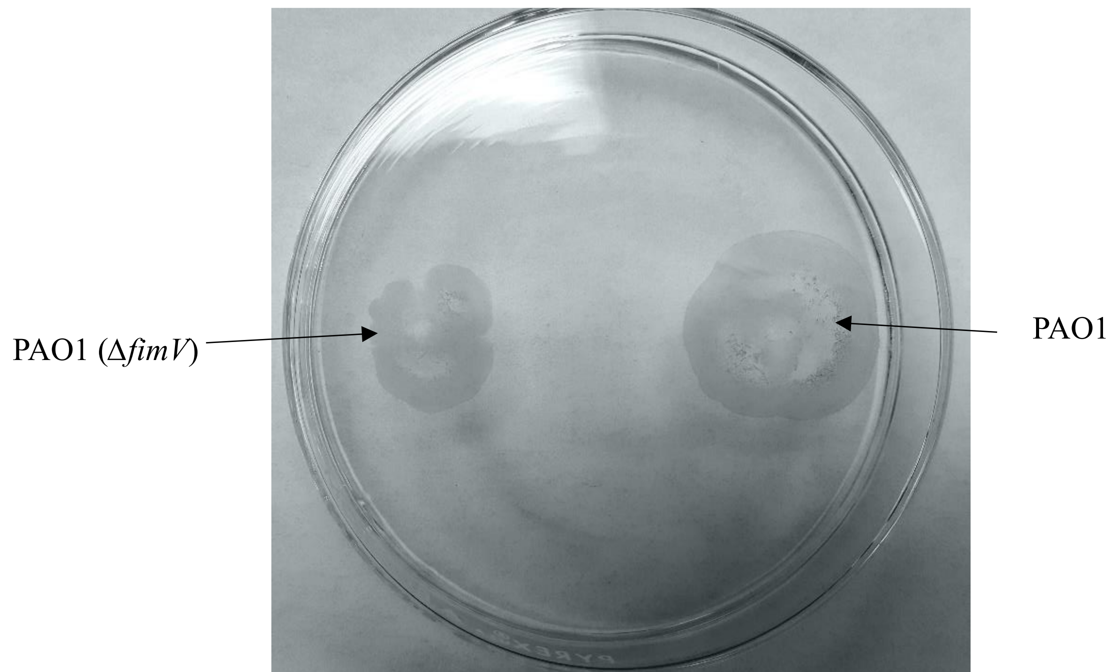


Figure 3.2 Defective twitching motility of PAO1 (Δ*fimV*)

A colony was stab-inoculated through 1% Luria Bertani (LB) agar layer, to the bottom of the Petri dish then incubated at 37˚C. After incubation, a hazy area of bacterial growth is observed between the petri dish and the surface of the agar to visualize the twitching motility diameter, the agar was carefully removed and the plate was stained with 0.1% (wt/vol) crystal violet (1 min) before rinsing. The motility at this interface was subsequently measured. Assays were performed at least in triplicate.

A previous study reported a correlation between twitching motility and antibiotic resistance (Yang, Chen, & Duan, 2011). Accordingly, we evaluated antibiotic susceptibility of PAO1 (Δ*fimV*) for tetracycline, carbenicillin and polymyxin B. Antibiotic susceptibility test by disc diffusion was performed for PAO1 (Δ*fimV*) and the wild type PAO1, where bacterial cultures were added to 20 ml of LB broth containing the appropriate antibiotic for the strain. Filter paper discs inoculated with 2 µL antibiotics were placed on the surface of the LB plates and incubated overnight at 37°C. Cultured bacteria with halos were considered susceptible to the antibiotic tested. After the incubation, zone of inhibition around the discs were measured and compared with PAO1. As shown in **Figure 3.3**, PAO1 (Δ*fimV*) displayed increased sensitivity to tetracycline, whereas the sensitivity for carbenicillin and polymyxin B was not different.

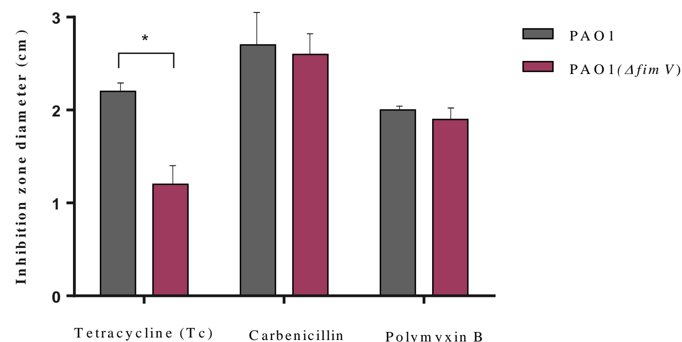


Figure 3.3 Increased antibiotic susceptibility of PAO1 (Δ*fimV*)

Bacterial cultures were added to 20 ml of LB broth containing the appropriate antibiotic for the strain. Filter paper discs inoculated with 2 µL antibiotics were placed on the surface of the LB plates and incubated overnight at 37°C. Cultured bacteria with halos were considered susceptible to the antibiotic tested. After the incubation, zone of inhibition around the discs were measured and compared with PAO1. Assays were performed at least in triplicate. Statistical significance is

determined using Student's *t*- test (* P ≤ 0.05).

Phenazine is an intermediate metabolic product for pyocyanin and is one of the major virulence factors in *P. aeruginosa*. Pyocyanin production in the bacterial culture medium of PAO1(Δ*fimV*) was determined. For pyocyanin production measurement, first 3ml of chloroform was added to 5 ml of culture supernatant after centrifugation. Once extracted, the chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 M HCl then centrifuged and the top layer of (0.2 M HCl) was removed by pipetting. The absorption at 520 nm was measured. Acquired concentrations are represented as micrograms of pyocyanin produced per milliliter of culture supernatant, and calculated using an extinction coefficient of 17.072 at 520 nm. A significant decrease in pyocyanin production was observed in PAO1(Δ*fimV*) when compared with the wild type PAO1 **(Figure 3.4)**.

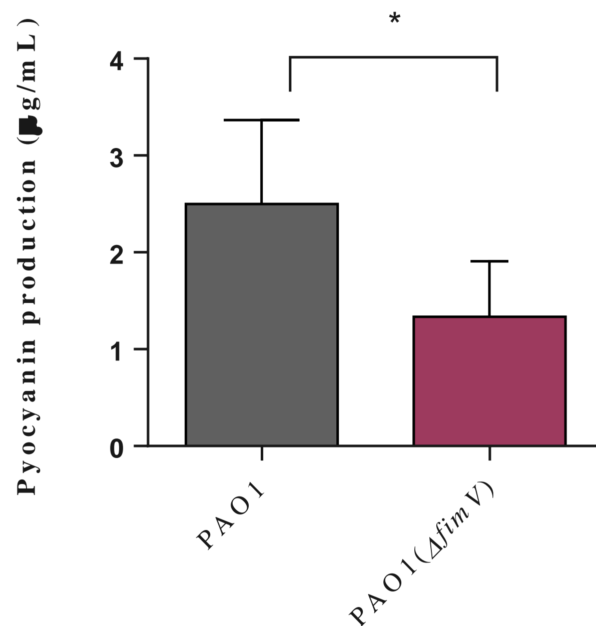


Figure 3.4 Decreased pyocyanin production in *fimV* mutant

The absorption at 520 nm was measured after treatment with chloroform and HCL. Acquired concentrations are represented as micrograms of pyocyanin produced per milliliter of culture supernatant, and calculated using an extinction coefficient of 17.072 at 520 nm. Statistical significance determined using Student's *t*- test (* $P \leqslant 0.05$). The result represents the average of triplicate experiments and the error bars indicate standard deviations.

3.1.3 Summary of *fimV* Results

In this specific aim my intent was to identify and confirm *fimV* as a potential regulator of T3SS. As shown in my results, *fimV* influenced *exoS* expression, implying a possible role of *fimV* in T3SS regulation. Since T3SS regulators modulate virulence factors, we examined *fimV* effect on virulence factors, through phenotypic characteristics, including twitching motility, pyocyanin production, and antibiotic susceptibility. *fimV* is required for T4P pili assembly and twitching motility, thus PAO1 (Δ*fimV*) was shown to be defective in twitching motility. Whereas, *fimV* mutation did not seem to have an influence on swimming and swarming motility as no differences were observed (results not shown). Additionally, PAO1 (Δ*fimV*) showed decreased pyocyanin production and tetracycline sensitivity, suggesting a role of *fimV* in virulence and antibiotic resistance.

3.2 Characterization of Regulatory Function of PA3284-81

In addition to *fimV,* PA3284 was identified from the previously mentioned transposon-insertion-mutant library (Zhu, Zhao, & Liang, 2016). The transposon mutant of PA3284 exhibited a six-fold increase in *exoS* expression compared with the wild type. In the transposon mutagenesis, a transposon has a strong promoter and when its inserted in a gene, it either destroys the gene function in the point of insertion or overexpresses the downstream genes. Therefore, to confirm the result from the transposon library, and to verify if the observed effect on *exoS* expression from the library is due to gene dysfunction in the gene PA3284 the insertion site, a PAO1(ΔPA3284) deletion mutant was constructed previously in our lab, where its effect on *exoS* expression was tested, but no difference in *exoS* expression was observed compared to wild type. Therefore, deletion mutants of the other genes in the operon PAO1(ΔPA3281) and PAO1(ΔPA3283) were previously constructed individually, and their effect on *exoS* was measured. It showed no change to little change in *exoS* activity (data not shown). Thus, for this project we first constructed a deletion mutant of PAO1(ΔPA3282) since it is the last gene in the operon left to study its effect on *exoS* expression. The expression was measured for both PAO1(ΔPA3282) and the control pKD- *exoS* in PAO1, where pKD- *exoS* reporter strain was constructed using the plasmid pMS402 carrying the promoter-less *luxCDABE* reporter gene cluster and *exoS* promoter.

The result indicates that *exoS* activity decreased in the deletion mutant PAO1(ΔPA3282) **(Figure 3.5)**. This suggests the involvement of PA3282 in T3SS regulation.

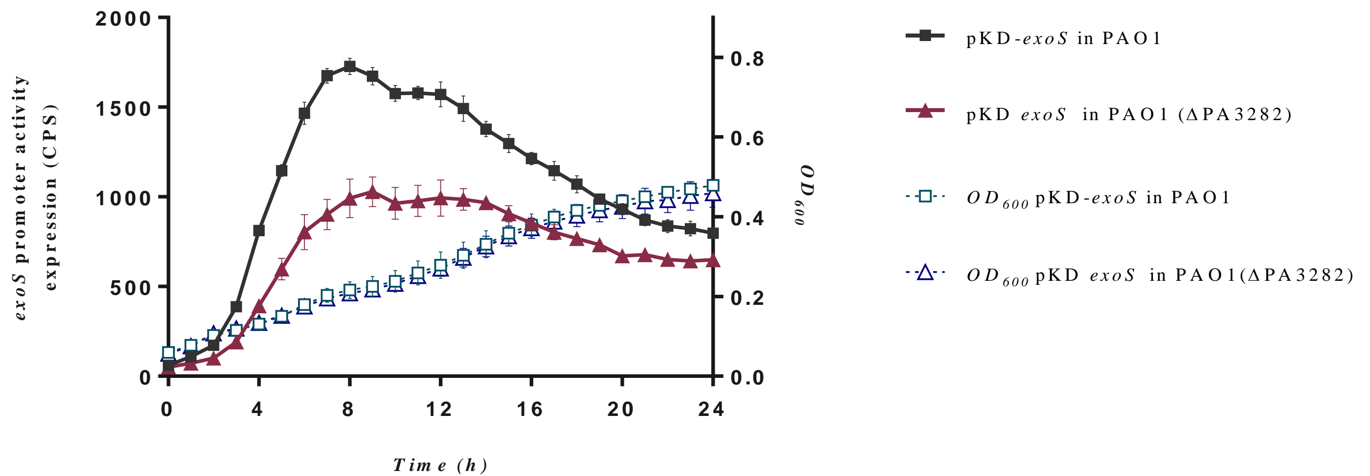


Figure 3.5 Decreased *exoS* expression in PAO1(ΔPA3282)

pKD- *exoS* reporter fusion promoter activity was used to measure *exoS* expression levels in PAO1(ΔPA3282). The result represents the average of triplicate experiments and the error bars indicate standard deviations.

One explanation for the discrepancy between the result in **Figure 3.5** and the transposon screen is that the increased *exoS* expression in the transposon mutant was possibly due to the overexpression of the operon caused by the promoter on the inserted transposon, instead of the disruption of the gene. Therefore, to investigate the cause of the observed effect on *exoS* expression in the transposon mutant, an overexpression strain of the operon PA3284-81was constructed. To generate the overexpression strain, the whole fragment of PA3284-PA3281 was amplified by PCR using PAO1 genomic DNA as template and primers specified in **Table 2.2**. The fragment was digested with *Xba*I and *Bam*HI and cloned into the multi copy number *E. coli-P aeruginosa* shuttle vector pAK1900 under *lac* promoter to generate the overexpression strain pAK 3284-81(Poole et al., 1993). The plasmid was then transferred into *P. aeruginosa*, and then integrated into the chromosome of the promoter reporter CTX-*exoS* by transformation.

Consistent with the observation from the transposon mutant, *exoS* promoter activity increased six folds (at 12hour time point) in the PA3284-81 overexpression strain **(Figure 3.6)**. Confirming that increased *exoS* in the transposon mutant was due to overexpression of the operon rather than the disruption of the gene PA3284.

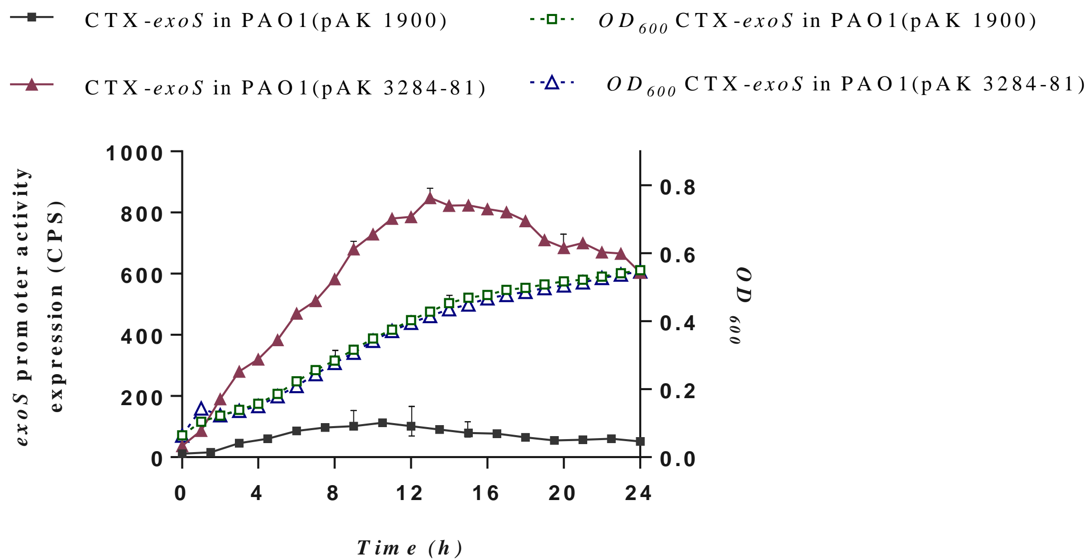


Figure 3.6 Increased *exoS* promoter activity under overexpression of PA3284-81

CTX-*exoS* reporter fusion integrated on the chromosome was used to measure expression levels. The result represents the average of triplicate experiments and the error bars indicate standard deviations.

To confirm this increase in *exoS* expression, the effector proteins produced in the PA3284-3281 overexpression strain was determined and compared with the control. The culture supernatants of pAK3284-81 and the control strains containing the pAK1900 were incubated for 6 hours in T3SS inducing media. The Ca^{2+} - chelator, EGTA, was added in the media to achieve a T3SS inducing condition of low calcium level (Chuang et al., 2013). Then, proteins in the

supernatants were concentrated with TCA, and analyzed by SDS-PAGE, followed by staining with Coomassie blue. pAK3284-81 showed a clear band of ExoS in comparison with the control confirming ExoS increase under pAK3284-81 overexpression **(Figures 3.7 and 3.8)**.

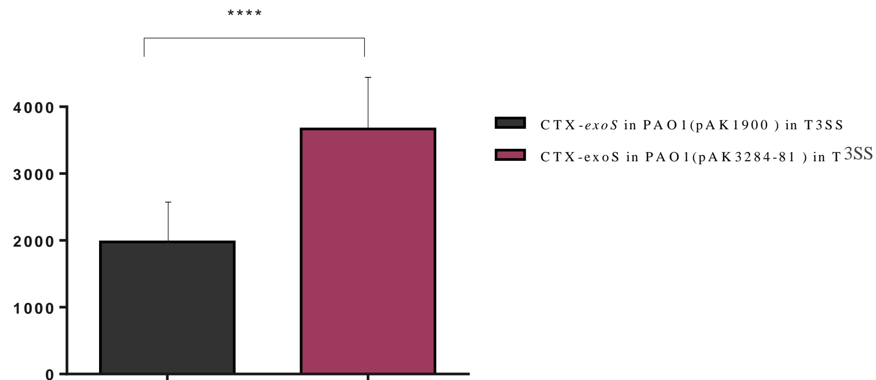


Figure 3.7 Increased *exoS* promoter activity under overexpression of PA3284-81 in T3SS inducing condition

Measurement of *exoS* reporter fusion under overexpression of pAK 3284-81 with growth in T3SS inducing condition. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Statistical significance determined using Student's *t*- test (**** P ⩽ 0.0001).

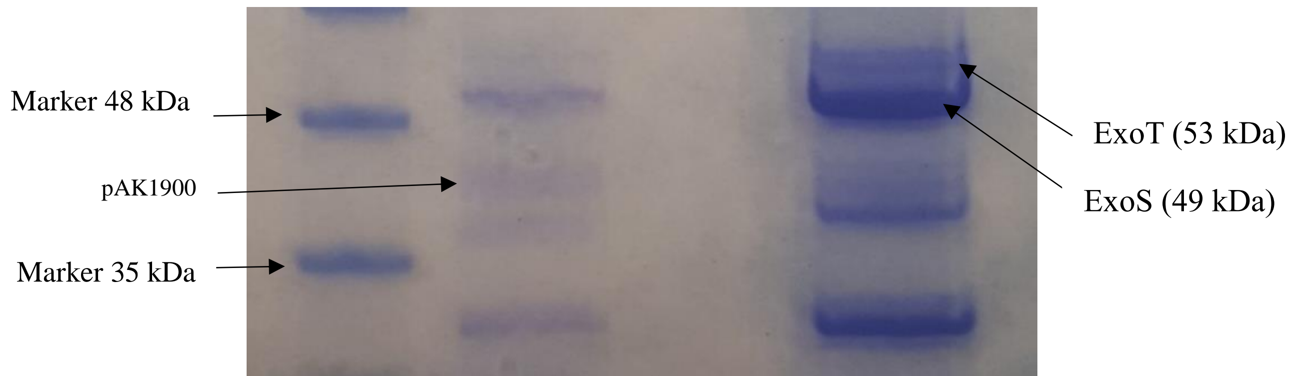


Figure 3.8 Enhanced ExoS secretion under PA3284-81 overexpression

Culture supernatants of pAK3284-81 and the control pAK1900 strains were incubated for 6 hours in T3SS inducing condition. Ca^{2+} -specific chelator, EGTA, was added in the media to achieve T3SS inducing condition of low calcium level. Then, concentrated with TCA and analyzed by SDS-PAGE (12%) gel, followed by staining with Coomassie blue. ExoS corresponding band is indicated by the arrows. A representative gel is shown.

3.2.1 The relationship between the PA3284-81 regulation and the Gac-RsmA pathway

Since T3SS expression and activity are under the control of RsmAYZ regulatory pathway. It was worthwhile to investigate whether if the increased expression of T3SS under PA3284-81 overexpression condition was connected to the Gac-RsmA pathway. Accordingly, CTX chromosomal reporter fusions of *rsmA, rsmY* and *rsmZ* promoter regions were cloned into Mini-CTX-*luxCDABE* then integrated into the PAO1 *att* site on the chromosome. This resulted in the reporters CTX- *rsmA*, CTX- *rsmY*, and CTX -*rsmZ*. pAK 3284-81 and the control pAK1900 were transformed into these reporters to measure their promoter activity by the

intensity of luminescence emission detected in a Microplate reader. The pAK 3284-81overexpression strain showed a decreased promoter activity of *rsmY* and *rsmZ* compared to the control. While *rsmA* did not exhibit a difference in expression compared to pAK1900 **(Figure 3.9)**. As previously described, once GacA upregulates the small regulatory RNAs, RsmZ and RsmY, they sequester RNA-binding regulatory protein RsmA limiting its availability, therefore repressing exsA and the T3SS. On the other hand, if we have low levels of RsmY and RsmZ, we have higher levels of free RsmA, thus ExsA transcription and activation of T3SS. This indicates that PA3284-81 possibly affects T3SS through RsmAYZ pathway.

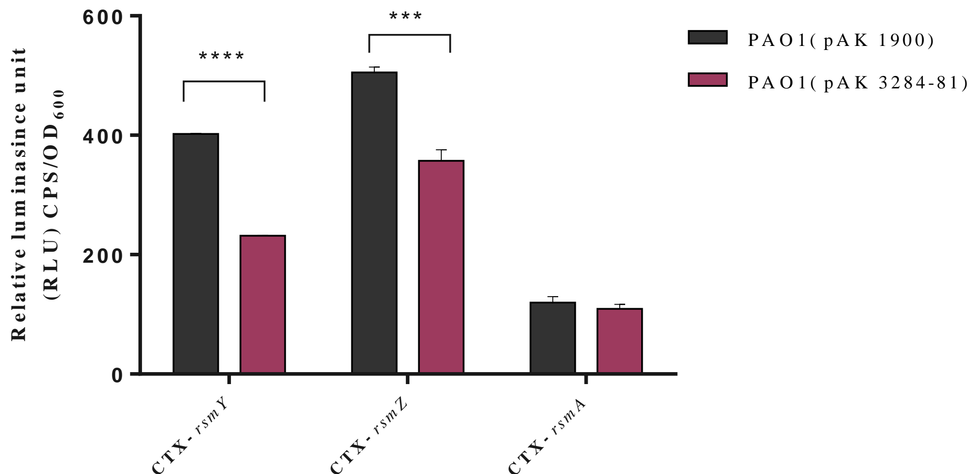


Figure 3.9 Influence of pAK 3284-81 on *rsmA, rsmY and rsmZ* promoter activities

CTX-*rsmA*, CTX-*rsmY* and CTX-*rsmZ* reporter fusions integrated on the chromosome were used to measure the promoter activity of *rsmA, rsmY* and *rsmZ* genes. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Statistical significance determined using Student's *t*- test (*** $P \leq 0.001$), (**** $P \leq 0.0001$).

3.2.2 Measurement of different virulence factors upon overexpression of PA3284-81

To test if other virulence factors are influenced by the overexpression of PA3284-81, and if the regulation of this operon is connected with other regulatory pathways in *P. aeruginosa*, we measured the expression and effect of PA3284-81 under thirteen virulence related reporters by transformation of PA3284-81 overexpression strain and pAK1900 to these reporters , CTX- *PqsA*, CTX- *lasA*, CTX- *lasR*, CTX- Δ*RetS*, CTX- *rhIA*, CTX- *xcpR*, CTX- *rpoS*, CTX- *rnr*, CTX- *algD*, CTX- *MexAB*, CTX- *phzA1,* CTX- *phzA2,* CTX- *rhIR* **Table 3.1**. Out of the thirteen reporters, CTX- *phzA2,* CTX- *rhIR* demonstrated reduced promoter activity in comparison with the control pAK1900, while CTX- *phzA1* displayed increased promoter activity **(Figure 3.10)**.

Table 3.1 Reporters tested under PA3284-3281 overexpression

Gene	Reporters in PAO1	Gene/Protein Function	Promoter Activity Under pAK3284-3281	Figure
exoS	CTX-*exoS-luxCDABE*	T3SS effector (toxin)	Increased	3.6
rsmY	CTX-*rsmY-luxCDABE*	Small RNA	Decreased	3.9
rsmZ	CTX-*rsmZ-luxCDABE*	Small RNA	Decreased	3.9
rsmA	CTX-*rsmA-luxCDABE*	Small RNA-binding regulatory protein	No difference	3.9
PqsA	CTX- *PqsA-luxCDABE*	PQS synthesis	No difference	Not shown
lasA	CTX- *lasA-luxCDABE*	Protease precursor	No difference	Not shown
lasR	CTX- *lasR-luxCDABE*	Quorum sensing regulator protein	No difference	Not shown
Δ*RetS*	CTX- Δ*RetS-luxCDABE*	Regulator of Exopolysaccharide and T3	No difference	Not shown
rhIA	CTX- *rhIA-luxCDABE*	Rhamnosyltransferase chain A	No difference	Not shown
xcpR	CTX- *xcpR-luxCDABE*	General secretion pathway protein E	No difference	Not shown
rpoS	CTX- *rpoS-luxCDABE*	Sigma factor RpoS	No difference	Not shown
rnr	CTX- *rnr-luxCDABE*	Exoribonuclease RNase R	No difference	Not shown
algD	CTX- *algD-luxCDABE*	Alginic acid biosynthetic process	No difference	Not shown
MexAB	CTX- *MexAB -luxCDABE*	Efflux Pump	No difference	Not shown
phzA1	CTX- *phzA1-luxCDABE*	Phenazine synthesis	Increased	3.10
phzA2	CTX- *phzA2-luxCDABE*	Phenazine synthesis	Decreased	3.10
rhIR	CTX- *rhIR-luxCDABE*	Quorum sensing regulator protein	Decreased	3.10

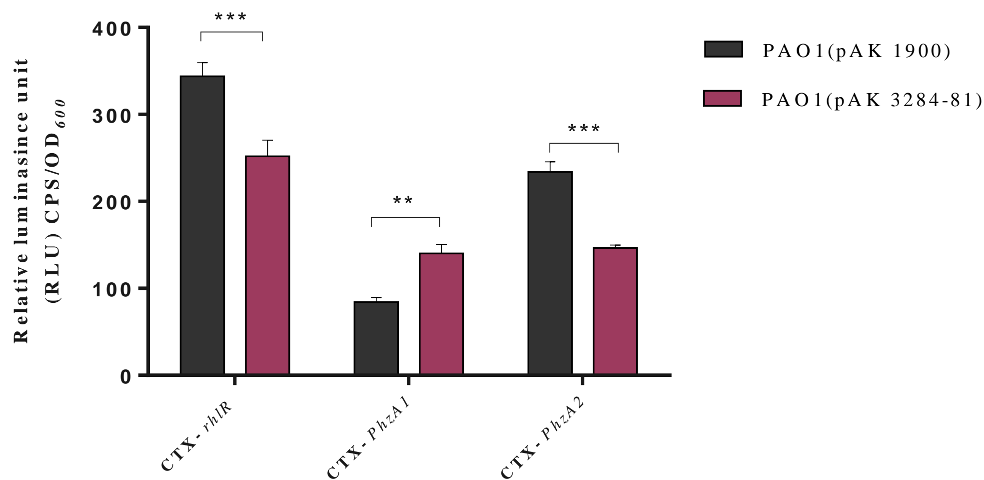


Figure 3.10 Effect of PA 3284-81 overexpression on promoter activities of virulence factors

CTX-*phzA1*, CTX-*phzA2*, and CTX-*rhIR* reporter fusions integrated on the chromosome were used to measure the promoter activity of *phzA1, phzA2* and *rhIR* genes. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Statistical significance determined using Student's *t*- test (** $P \leqslant 0.01$), (*** $P \leq 0.001$).

3.2.3 Phenotypic changes caused by PA3284-3281 overexpression

Since T3SS regulators in *P. aeruginosa* also control phenotypes not just virulence factors, phenotypes related to pyocyanin production were tested due to the difference observed at the transcriptional level (promoter activities). For pyocyanin production measurement, 3ml of chloroform was added to 5 ml of culture supernatant after centrifugation. Once extracted, the chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 M HCl then centrifuged and the top layer of (0.2 M HCl) was removed by pipetting. The absorption at 520 nm was measured. Acquired concentrations are represented as micrograms of pyocyanin produced per milliliter of culture supernatant, and calculated using an extinction coefficient of 17.072 at 520 nm. PAO1(ΔPA3282) exhibited no difference in pyocyanin production compared

to the wild type **(Figure 3.11)**. In contrast, the overexpression strain showed increased pyocyanin production in comparison with pAK1900. This indicates a potential role for PA3284-81 in pyocyanin production. We also measured pyocyanin production in the transposon mutant PA3284, and a similar increase in pyocyanin production between PA3284 transposon mutant and our overexpression strain of PA3284-81 was observed. Thus, once again asserting that the result of the increased *exoS* expression in the transposon library was due to the overexpression of the whole operon caused by the transposon strong promoter and not the disruption of PA3284 gene.

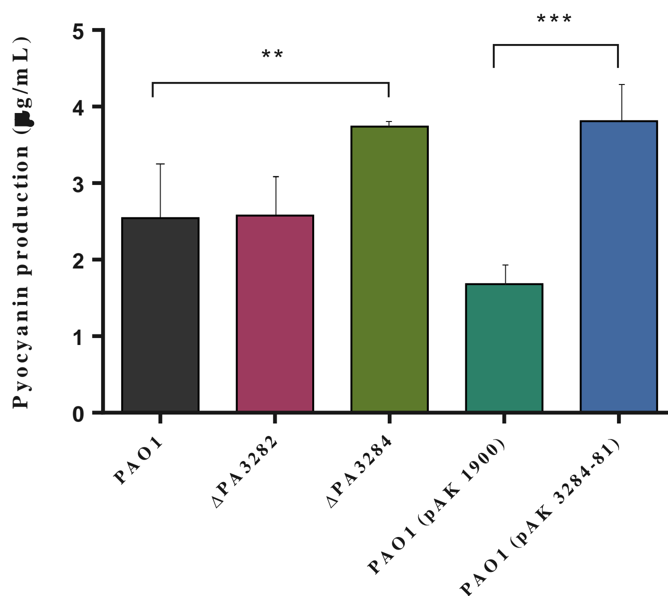


Figure 3.11 Pyocyanin production assay for PAO1(ΔPA3282), pAK 3284-81, and PA3284 transposon mutant

The absorption at 520 nm was measured for pyocyanin production. Acquired concentrations are represented as micrograms of pyocyanin produced per milliliter of culture supernatant, and calculated using an extinction coefficient of 17.072 at 520 nm. The result represents the average

of triplicate experiments and the error bars indicate standard deviations. Statistical significance determined using Student's *t*- test (** $P \leqslant 0.01$), (*** $P \leq 0.001$).

Biofilm formation was assessed in both the mutant strain PAO1(ΔPA3282) and the pAK3284-81overexpression strain. Biofilm formation was quantified as described previously by O'Toole and Kolter (1998) with minor modifications for both PAO1(ΔPA3282) and the pAK3284-81overexpression strain. Cells from overnight cultures were inoculated at 1:100 dilutions into LB medium in 96-well polystyrene microtiter plates (Costar) and grown at 37°C for 24 h. The supernatant was gently removed by pipetting, and the wells were washed gently with PBS then left to dry for 10minutes. Volume of 100 ul 1% crystal violet was added to each well and staining was allowed for 20min. Then, the stain is removed by aspiration and wells are rinsed three times with distilled water. Remaining crystal violet was dissolved in 200 ul of 95% ethanol. 100 µl was transferred to a new polystyrene microtiter plate, and the absorbance was measured at 600 nm.

PAO1(ΔPA3282) had a decreased biofilm formation compared to PAO1, whereas pAK3284-81 displayed increased biofilm production compared to pAK1900 **(Figure 3.12)**. Thus, PA3284-81 is involved in biofilm formation. In addition to these phenotypes, motility (twitching, swarming, swimming), and antibiotic resistance were examined as well in strain containing pAK3284-81 and the mutant PAO1(ΔPA3282), though no significant difference was observed (data not shown).

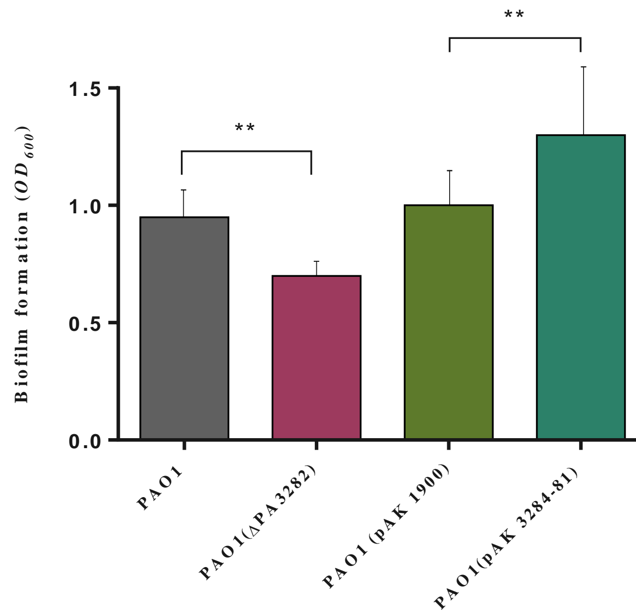


Figure 3.12 Biofilm formation assay for PAO1(ΔPA3282) and pAK 3284-81

Cells from overnight cultures were inoculated at 1:100 dilutions into LB medium in 96-well polystyrene microtiter plates and grown at 37°C for 24 h. The supernatant was gently removed and the wells were washed then left to dry. Volume of 100 ml 1% crystal violet was added to each well and staining was allowed for 20min. Then, the stain is removed and wells are rinsed with distilled water. Remaining crystal violet was dissolved in 200 ml of 95% ethanol. 100 μl was transferred to a new polystyrene microtiter plate, and the absorbance was measured at 600 nm. The result represents the average of triplicate experiments. Statistical significance determined using Student's *t*- test (** $P \leqslant 0.01$).

3.3 Regulation of T3SS by environmental signals potentially via PA3284-3281

3.3.1 Increased expression of reporter under zinc depletion and repression of pKD3284-81 with zinc supplementations

To investigate how and to what signals PA3284-81 respond to that can affect *P. aeruginosa* expression of virulence factors. Also, to define what is affecting the expression of this gene, and which environmental factors it responds to. Furthermore, to verify the previous reports of a probable link between PA3284-81 and zinc regulation in zinc depletion conditions, I first constructed the promoter reporter strain pKD3284-1 (Pederick & Eijkelkamp, 2015). This reporter was constructed by PCR amplification of the promoter region of PA3284 -81. The fragment was digested and cloned upstream of the *lux* genes in pMS402 plasmid. Then, the resultant plasmid was transformed into *P. aeruginosa* by electroporation to generate the construct pKD3284 -81 (Huang, Kutchma, & Schweizer, 1998).

Initially, I wanted to confirm the upregulation of PA3284-81 in zinc depleted condition. To pursue this, I measured the promoter activity of pKD3284-81 in the presence of TPEN (N, N, N, N Tetrakis(2-pyridylmethyl) ethylenediamine in the bacterial culture at the concentrations of 20 µM, 100 µM, 200 µM. TPEN is a membrane-permeable zinc chelator causing a decrease in the intracellular level of zinc and induced apoptosis. In **Figure 3.13**, pKD3284-81 demonstrated an increase of PA384-81 expression at the concentration of 100 µM of TPEN. Next, I tested with addition of zinc minimal medium at the concentrations of 10 µM, 20 µM, 50 µM, 100 µM, 200 µM. **Figure 3.14**, displays a repression effect of zinc on the transcription of pKD3284-81.

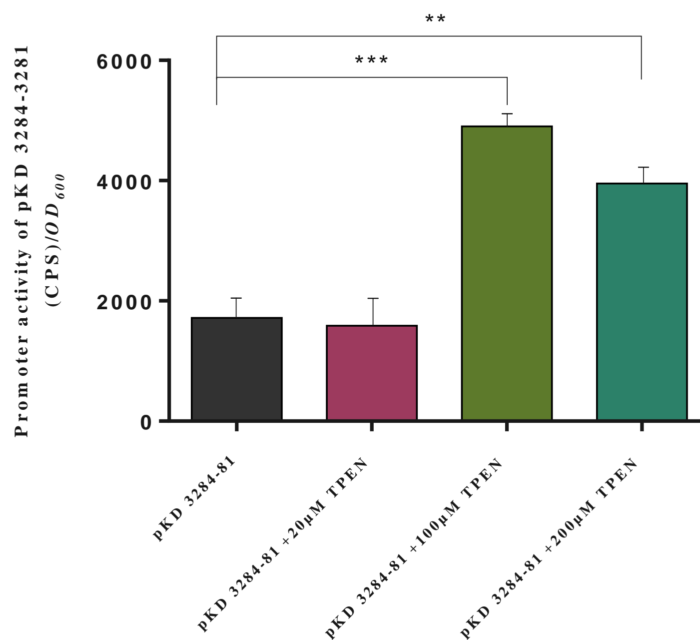


Figure 3.13 Increased pKD3284-81 promoter activity under zinc depletion

Promoter activity of pKD3284-81 in zinc depletion at the concentrations of 20 µM, 100 µM, 200 µM of TPEN. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Data normalized (CPS)/OD_{600}. Statistical significance determined using Student's *t*- test (** $P \leqslant 0.01$), (*** $P \leq 0.001$).

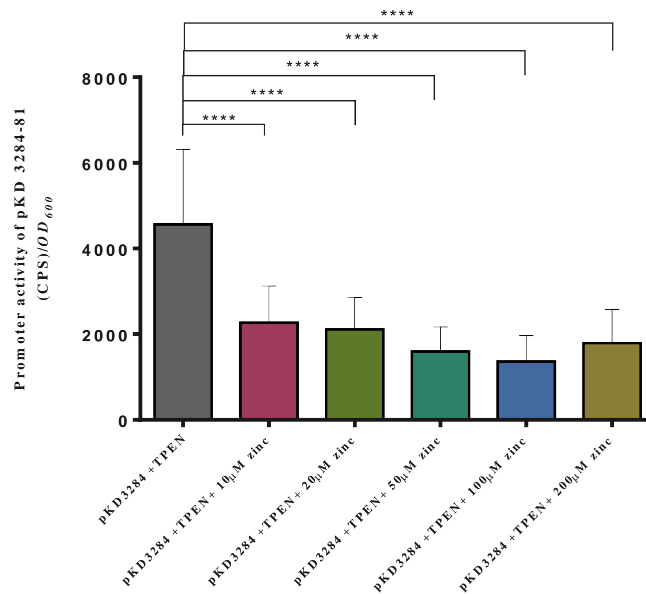


Figure 3.14 Repression of pKD3284-81 promoter activity with zinc supplementation

Promoter activity of pKD3284-81 in zinc deplete condition with added zinc at 10 µM,20 µM,50 µM,100 µM, 200 µM. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Data normalized (CPS)/OD_{600}. Statistical significance determined using Student's *t*- test (**** $P \leq 0.0001$).

3.3.2 Increased expression of *exoS* with zinc supplementation

As the effect of zinc on PA3284-81 was confirmed and PA3284-81 was previously shown to induce *exoS* expression, we tested *exoS* expression with varied concentrations of zinc and TPEN. The chromosome integrated promoter reporter fusion CTX-*exoS* was utilized to measure the effect of zinc on T3SS with the presence of TPEN as zinc chelator in Luria-Bertani (LB) medium. *exoS* expression was measured with the addition of 100 µM TPEN as well as zinc concentrations of 5 µM,10 µM, 20 µM, 50 µM, 100 µM, 200 µM, 500 µM. *exoS* expression was

found to decrease with the addition of TPEN, and then gradually increase where it reached the highest at 50 µM. **(Figure 3.15)**. Thus, we could conclude *exoS* promoter activity is influenced by zinc concentrations in the environment.

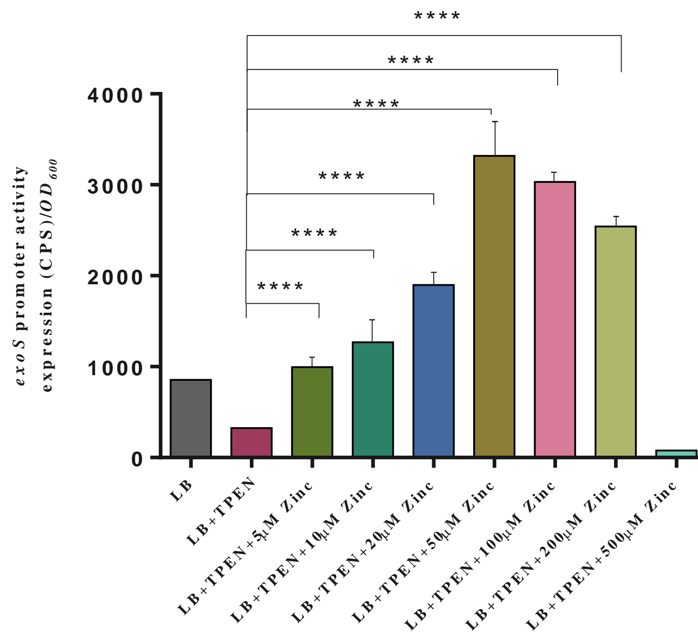


Figure 3.15 Increased expression of *exoS* with zinc supplementation

Promoter activity of CTX- *exoS* with zinc supplementation at 10 µM,20 µM,50 µM,100 µM, 200 µM,500 µM of zinc. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Data normalized (CPS)/OD_{600}. Statistical significance determined using Student's *t*- test (**** $P \leq 0.0001$).

3.3.3 Confirmation of TPEN depletion for zinc ions

TPEN has high affinity for zinc, yet it can chelate iron with lower affinity. Thus, it was possible that the effect of TPEN was affecting iron. To elucidate that the elevated expression CTX- *exoS* in presence of zinc is solely due to zinc ions, we measured *exoS* promoter activity under the same conditions as previously stated with zinc, except this time zinc was replaced with varying concentrations of iron **(Figure 3.16)**. *exoS* promoter activity displayed no change in expression with added iron. Indicating that the increased expression of *exoS* promoter activity in presence of zinc is solely due to zinc ions.

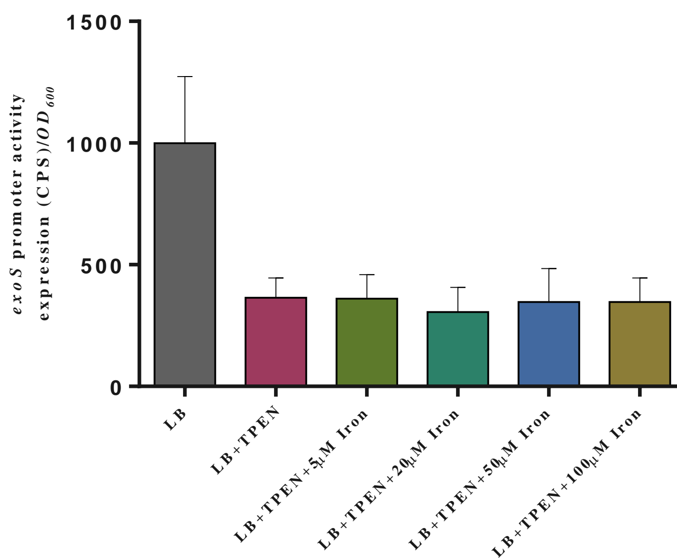


Figure 3.16 No effect of iron on *exoS* promoter activity with TPEN as a chelator

Promoter activity of CTX- *exoS* with added iron at 5 µM, 20 µM,50 µM,100 µM in the presence of TPEN as a chelator. The result represents the average of triplicate experiments and the error bars indicate standard deviation. Data normalized (CPS)/OD_{600}.

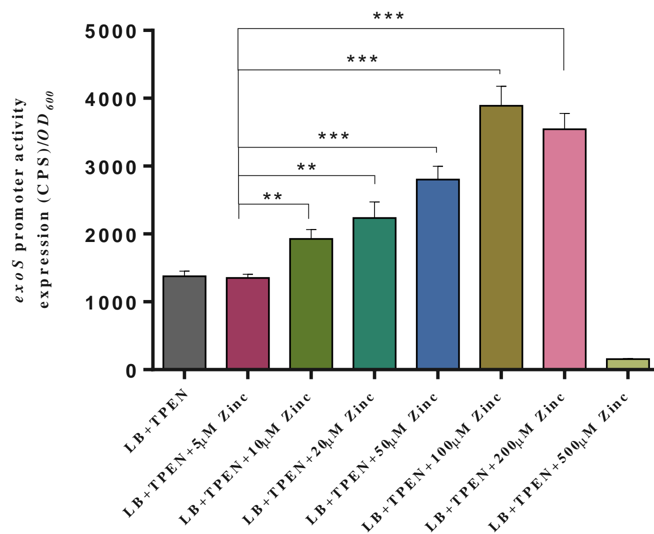
3.3.4 Increased expression of *exoS* under pAK3284-81 and in PAO1(ΔPA3282) in zinc depletion condition with zinc supplementation

Next, to verify if there is an interconnection between *exoS* and PA3284-81through zinc, we measured *exoS* expression under PA3284-81 overexpression, and under PA3282 mutation in the presence of zinc and TPEN. Similar to CTX- *exoS* results with zinc, *exoS* promoter activity was increased under pAK3284-81 overexpression and PA3282 mutant with zinc, where the highest was noticed at 100 µM and 20 µM respectively **(Figure 3.17**).

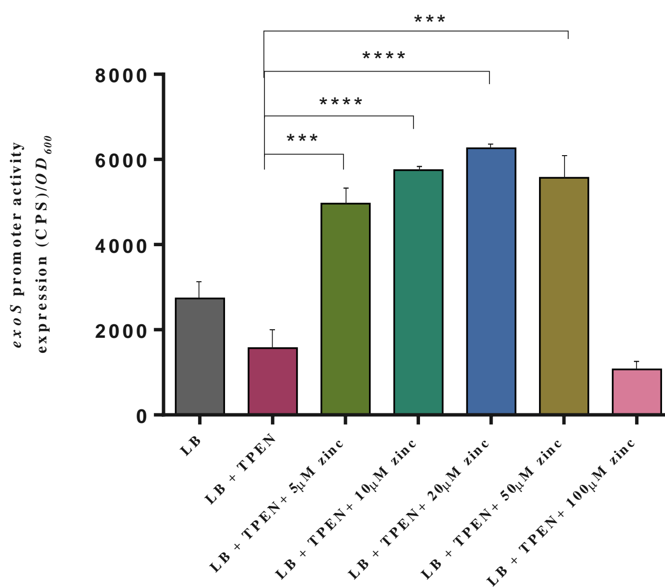
The increase in *exoS* expression is observed with addition of zinc in zinc depletion condition. This increase in *exoS* promoter activity was also noticed under overexpression of PA3284-81 and in PA3282 mutant. Therefore, zinc affects *exoS* not through PA3284-81, but from another unknown pathway.

Figure 3.17 *exoS* promoter activity in zinc depletion with added zinc under pAK 3284-81 and in PAO1(ΔPA3282)

A) *exoS* increased expression under pAK 3284-81with zinc supplementation under zinc depletion



B) ***exoS*** **increased expression in PAO1(ΔPA3282) with zinc supplementation under zinc depletion**



A) Promoter activity of CTX- *exoS* in zinc depletion condition with zinc supplementation under zinc depletion under overexpression of PA3284-81. **B)** Promoter activity of pKD-*exoS* with added zinc supplementation in zinc depletion condition in PA3282 mutant. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Data normalized (CPS)/OD_{600}. Statistical significance determined using Student's *t*- test (** $P \leqslant 0.01$), (*** $P \leqslant 0.001$), (**** $P \leq 0.0001$).

3.3.5 Increased expression of pKD 3284-81 and *exoS* reporter under peracetic acid (PAA) exposure

Our results of the effect of zinc on PA3284-81 prompted us to test the response of this operon with other ions and environmental stressors. We tested three more conditions, with peracetic acid, iron limited condition and under low calcium conditions (T3SS inducing conditions).

Peracetic acid (CH3CO3H) is a peroxide of acetic acid made from the combination of reacting acetic acid and hydrogen peroxide in addition to sulfuric acid catalyst. It is hypothesized that it is capable of denaturing proteins and enzymes, plus increasing cell wall permeability by breaking sulfhydryl and disulfide bonds in the cell wall. Chang et al, (Chang & Small, 2005) analyzed the effect of peracetic acid on *P. aeruginosa,* through GenChip. Among the genes they identified through their investigation, operon PA3281-3284 was upregulated from 26 to 48 folds (Chang & Small, 2005). In this study, pKD3284-81 promoter activity was measured with the addition of PAA at the following concentration: 1 mM, 1.5 mM, 2 mM, 5 mM to the cultures in a 96 well plate before measuring the promoter activity in the microplate reader. Promoter activity was significantly increased with PAA exposure as shown in **Figure 3.18**, this encouraged us to

examine *exoS* response under PAA. Thus, we measured CTX-*exoS* expression under exposure of PAA. *exoS* expression was slightly increased at 1mM of PAA **(Figure 3.19).** It is suggested that *P. aeruginosa* upregulates *exoS* and virulence associated genes as a protective measure to protect itself from phagocytes and oxidative stress in the host environment. Thus, suggesting a possible involvement of PA3284-81 in responding to environmental stress.

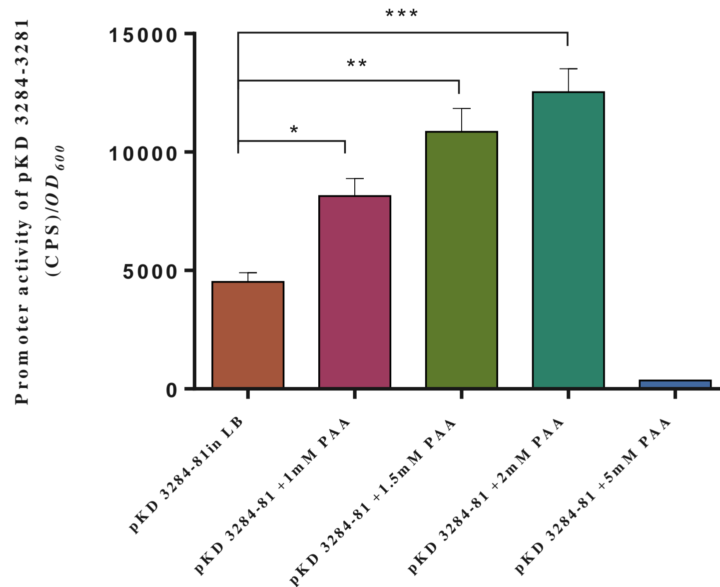


Figure 3.18 Increased pKD3284-81 promoter activity under exposure to PAA

Promoter activity of pKD3284-81in the presence of PAA was detected at concentrations of 1 mM, 1.5 mM, 2 mM, 3 mM, 4 mM, 5 mM. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Data normalized (CPS)/OD_{600}. Statistical significance determined using Student's *t*- test (* $P \leqslant 0.05$), (** $P \leqslant 0.01$), (*** $P \leqslant 0.001$).

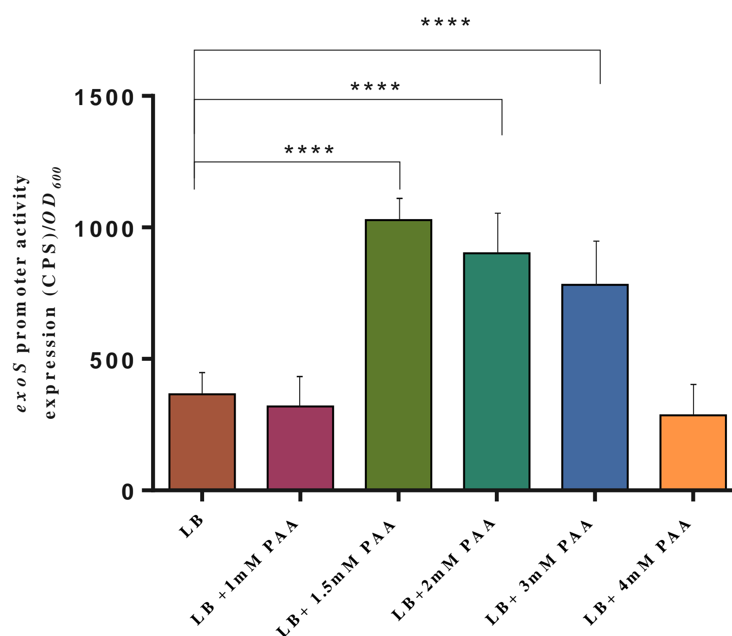


Figure 3.19 Increased *exoS* expression under PAA exposure

Promoter activity of *exoS* the presence of PAA was detected at concentrations of 1 mM, 2 mM, 3 mM, 4 mM. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Data normalized (CPS)/OD_{600}. Statistical significance determined using Student's *t*- test (**** P $\leqslant$ 0.0001).

3.3.6 Low Ca^{2+} condition and iron deficiency influence on PA3284-81 overexpression

Then since PA3284-81 was influenced by zinc ions and peracetic acid as shown previously, we wanted to verify the response of PA3284-81 under other conditions such as iron and calcium limited conditions. We examined pKD3284-81 promoter activity in iron limited condition with CAS medium and with the addition of iron at the concentration of 50µM. There was a significant increase with the addition of iron to the culture in comparison with the untreated control **(Figure 3.20)**, implying PA3284-81 might be influenced by iron. In addition,

we measured PA3284-81 expression in T3SS inducing condition of limited calcium by chelating with EGTA, and it showed decreased expression of PA3284-81 promoter activity **(Figure 3.21)**. This can be explained as that low calcium impact on *exoS* is more dominant than that of PA3284-81 effect on *exoS*.

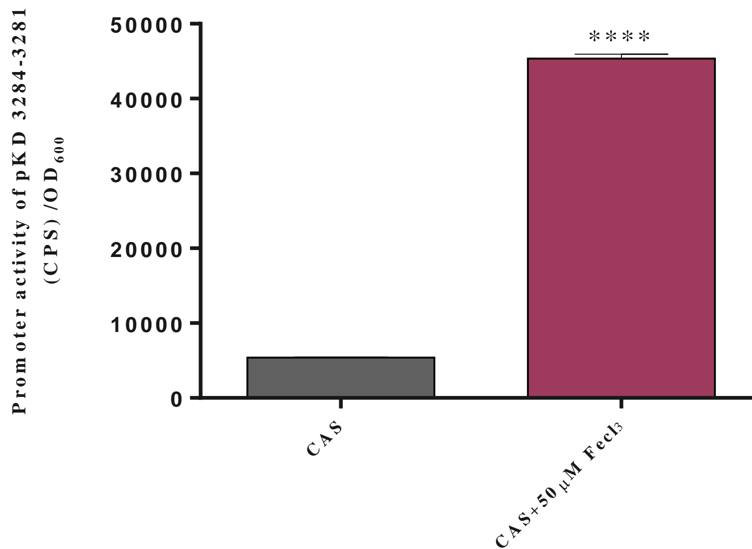


Figure 3.20 Increased pKD3284-81 expression in iron limited condition with added iron

Promoter activity of pKD3284-81 with added iron at 50 µM in the culture medium in iron limited environment. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Data normalized (CPS)/OD_{600}. Statistical significance determined using Student's *t*- test (**** $P \leqslant 0.0001$).

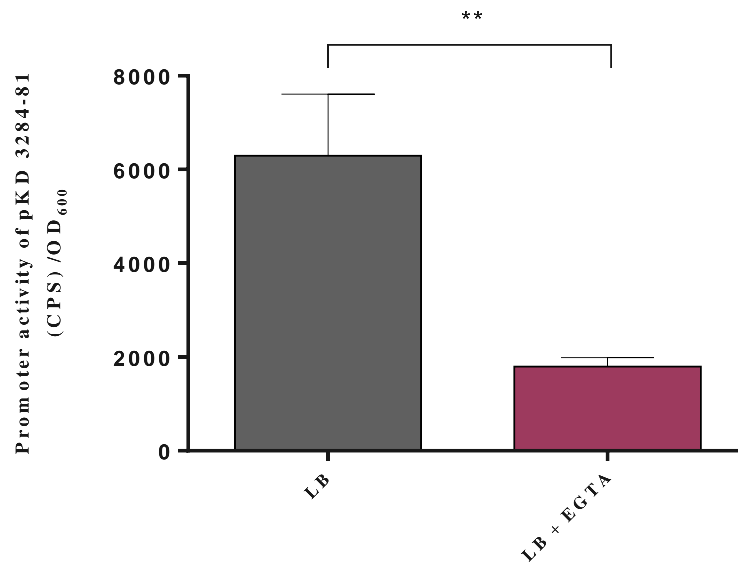


Figure 3.21 Repression of pKD3284-81 promoter activity under T3SS inducing condition

Promoter activity of pKD3284-81 in calcium limited condition with the addition of EGTA chelator n bacterial culture. The result represents the average of triplicate experiments and the error bars indicate standard deviations. Data normalized (CPS)/OD_{600}. Statistical significance determined using Student's *t*- test (** $P \leqslant 0.01$).

3.4 H2-T6SS Transposon Library

3.4.1 Screening of T6SS regulators

T6SS is important for competition with other bacterial species and competitors as well as a virulence factor against eukaryotic cells, as H2-T6SS has been shown to play a role in *P. aeruginosa* virulence and epithelial cell invasion. Much is still unknown about H2-T6SS regulation and the signals it responds to. Therefore, to understand the regulatory pathway of T6SS a transposon mutant library was constructed to screen for H2-T6SS regulators as previously described. PAO1 containing the CTX-*H2-lux* reporter fusion was subjected to transposon mutagenesis using the mariner transposon vector pBT20 as previously described with some modifications (Kulasekara et al., 2005). The transposon mutant library was constructed by screening and picking 30,000 colonies grown on selective plates containing GM. To screen for genes involved in *H2- T6SS* expression, colonies were incubated overnight with appropriate antibiotics in LB. The next day, the library was inoculated into 96 well plate containing LB and Gm. Colonies with altered expression of CTX- *H2* were identified and analyzed. The screen was performed six times for the identified colonies to eliminate false-positive clones. Confirmed mutants with altered *H2- T6SS* expression profiles were selected for arbitrary PCR and sequencing. Sequenced junctions were aligned to PAO1 in the NCBI DNA and protein database. From this library, eight genes were identified as potential regulators of *H2-T6SS* **Table3.2**. Out of these gene, two genes were selected for farther characterization, PA0523 (*norC*) and PA0961. Deletion mutants PAO1(Δ*norC*) and PAO1(ΔPA096) were constructed by utilizing marker-less allelic exchange method described previously (Hmelo & Borlee, 2015).

Table3.3 Potential regulators of H2-T6SS (Transposon mutants)

Gene name or number	Insertion site	Protein description	Max fold[a]
PA4467	4997059	Hypothetical protein	-2
PA3798	4257397	Probable aminotransferase	-2.5
PA0525 (norD)	585588	Probable denitrification protein	-2.8
PA2929	3285258	Hypothetical protein	-2
PA0523(norC)	582172	Nitric oxide reductase subunit C	-2.8
PA1689	1840252	Conserved hypothetical protein	+3
PA0961-0962	1046969	Probable cold-shock protein	+3
PA0337(ptsP)	380328	Phosphoenolpyruvate-protein phosphotransferase ptsp	+2.5

a Max fold, Maximal ratio of expression between the mutant and the wild-type

3.4.2 Confirmation/ of H2-T6SS reporter influence on PAO1(Δ*norC*) and PAO1(ΔPA0961)

For confirmation of the effect observed in the transposon mutants of *norC* and PA0961, both PAO1(Δ*norC*) and PAO1(ΔPA0961) constructed deletion mutants were integrated into CTX-*H2* chromosomal reporter by biparental mating to measure *H2*-T6SS promoter activity. Briefly, A mobilizing strain SM10 containing the plasmids CTX-*H2-T6SS,* and the deletion mutant PAO1(Δ*norC*) were both grown in LB overnight at 37°C with agitation at 225 rpm (Simon, Priefer *et al.* 1983). The inoculums were collected into falcon tubes and centrifuged at 8000 rpm for 3 minutes. The supernatant was discarded and the bacterial pellets were thoroughly re-suspended in 1 ml PBS buffer. 1 ml of each of the recipient culture, the donor cultures were added to centrifuge tubes for centrifugation at 8000 rpm for 3 minutes, and the supernatant was discarded. Cells wash was repeated twice and the weight of the tubes was measured. Volumes of SOC media were added per the calculated weigh for each of the strain. Cell suspensions were mixed well with equal volumes (20-40 µl of the mixtures) and plated on LB plates then cultured overnight. The next day, grown pellets were scraped and collected in 500 µl SOC media then spread on LB plates with appropriate antibiotics.

In **Figure 3.23** PAO1(Δ*norC*) demonstrated decreased expression of the *H2* promoter replicating the effects of the transposon mutant in our library. On the contrary, PAO1(ΔPA0961) showed the opposite effect on the expression of *H2*-T6SS promoter activity in comparison with the transposon mutant with a decrease in H2 promoter activity instead of an increase **(Figure3.23-3.25)**.

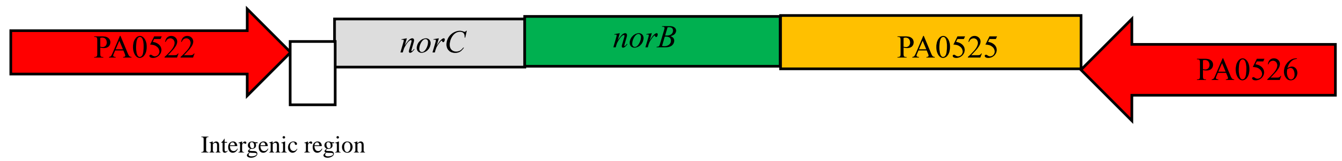


Figure 3.22 The location of *norC* in *P. aeruginosa* genome

The gene *norC* located on positive strand at 582015 - 582455. This figure was drawn per information in the Pseudomonas Genome Database (Winsor et al., 2011)

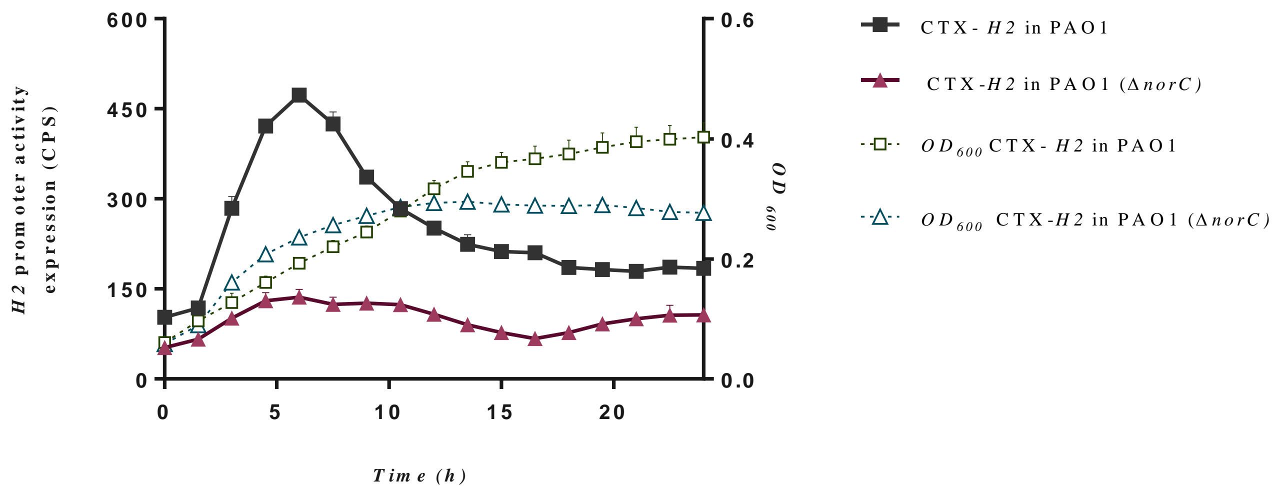


Figure 3.23 Decreased expression of *H2* reporter under *norC* mutation

CTX-*H2-T6SS* reporter fusion integrated on the chromosome was used to measure the promoter activity of *H2-T6SS* gene in PAO1(Δ*norC*). The result represents the average of triplicate experiments and the error bars indicate standard deviations.

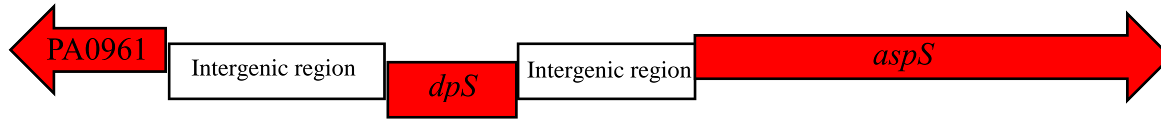


Figure 3.24 The location of PA0961 in *P. aeruginosa* genome

The gene PA0961 located on negative strand at 1046720 - 1046911. This figure was drawn per information in the Pseudomonas Genome Database (Winsor et al., 2011)

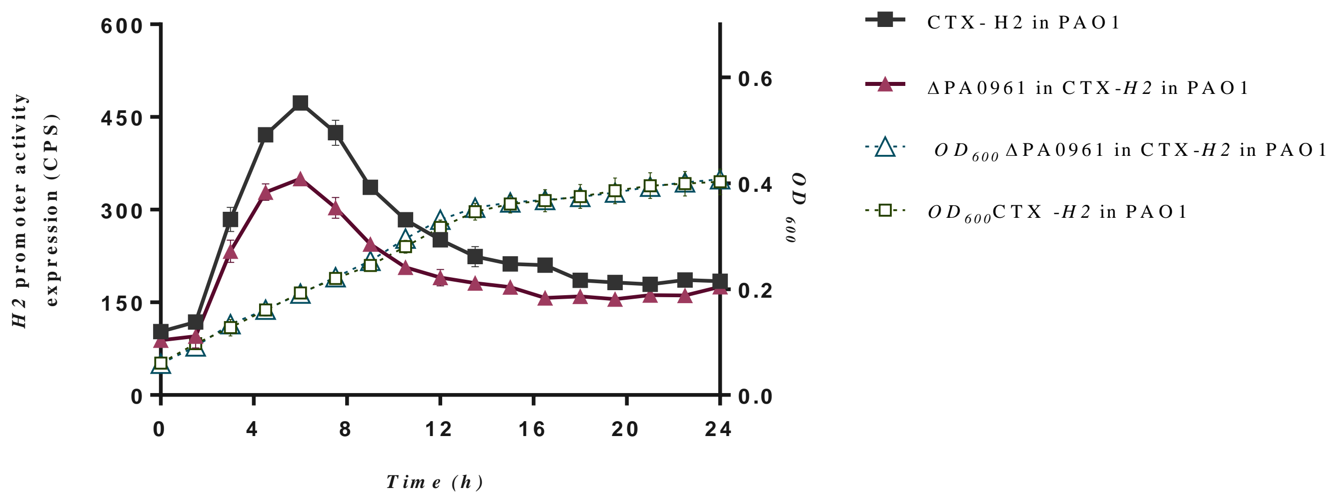


Figure 3.25 Decreased expression of *H2* reporter under PA0961 mutation

CTX-*H2-T6SS* reporter fusion integrated on the chromosome was used to measure the promoter activity of *H2-T6SS* gene in PAO1(ΔPA0961). The result represents the average of triplicate experiments and the error bars indicate standard deviations.

3.4.3 Decreased *exoS* expression in PAO1(Δ*norC*)

A previous study has identified that T3SS genes are required for amoeba killing, and both *norC* and *norB* were upregulated during co-culture with the amoeba *Acanthamoeba castellanii* (Matz et al., 2008). This led us to address the possibility of *norC* involvement not only in H2-T6SS, but also in T3SS, making it a potential novel link between T3SS and T6SS. Therefore, we measured pKD-*exoS* promoter activity in PAO1(Δ*norC*), and it displayed a significant decrease in *exoS* expression providing an evidence for potential novel target that connects the two-complex virulence associated secretion systems in *P. aeruginosa (***Figure 3.26)**.

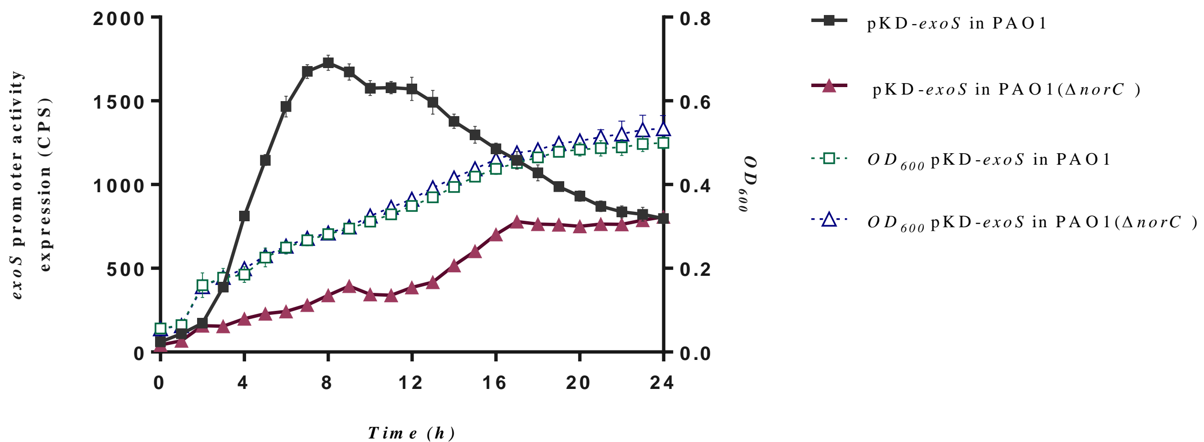


Figure 3.26 Decrease promoter activity of pKD-*exoS* in PAO1(Δ*norC*)

pKD-*exoS* reporter fusion was used to measure the promoter activity of *exoS* gene in PAO1(Δ*norC*). The result represents the average of triplicate experiments and the error bars indicate standard deviations.

CHAPTER IV. DISCUSSION

4.1 Putative T3SS Regulator (*fimV*)

P. aeruginosa is one of the major pathogens causing health-care associated infections such as cystic fibrosis, burn wound infections and pneumonia. T3SS is a crucial virulence factor for *P. aeruginosa* pathogeneses and establishment of infection. T3SS translocate four effector proteins (toxins) directly into the cytoplasm of target host cells to evade immune system, and inhibit cellular functions to facilitate bacterial survival. These toxins including ExoS, ExoU, ExoT and ExoY, contribute to epithelial cell and macrophage damage *in vitro*, in animal models of disease, and in human infections (Hauser, 2009) The regulation of T3SS is associated with acute infection manifestation and disease progression. To investigate the regulatory pathway of T3SS, a transposon-insertion-mutant library of *P. aeruginosa* PAO1 was constructed previously in our lab. Mutants with altered promoter activity of the T3SS effector gene *exoS* were identified (Zhu, Zhao, Kang, Kong, & Liang, 2016). Among the mutants acquired, *fimV* and PA3284-81 were chosen for further study. In this project, the roles of operon PA3284-81 and *fimV* in the regulation of T3SS and possibly other virulence factors were investigated.

FimV gene was initially identified by transposon mutagenesis where *P. aeruginosa* mutants defective in twitching motility were screened for (Semmler & Whitchurch, 2000). It plays a role in twitching motility, and Type 4 Pilli (T4P) components. FimV is a large protein (97 kDa) with a periplasmic domain bearing a peptidoglycan binding LysM motif. LysM motif is essential for the formation of PilQ and motility possibly by allowing movement through the peptidoglycan layer. The structure and function of T4aP is regulated by the Chp chemotaxis like system through CyaB adenylate cyclase. The second messenger molecule cyclic adenosine

monophosphate (cAMP) is bound to Vfr (virulence factor regulator) and regulates virulence related genes as well as the T4aP components (Wolfgang, Lee, Gilmore, & Lory, 2003). CyaA and CyaB synthesize cAMP while CyaB is more importance than CyaA for controlling the cAMP cellular levels. FimL, PilG and PilH modulate CyaB activity consequently cAMP, Vfr expression and PilMNOPQ. Additionally, it seems that the Pil-Chp system modulate T4Ap through a cAMP independent pathway (Burrows, 2012). It had been recently identified that FimV positively regulates CyaB function through its predicted tetratricopeptide repeat (TPR) motifs at the cytoplasmic C terminus domain. Consequently, modulating cAMP levels and thus Vfr-dependent transcription (Buensuceso et al., 2016).

FimV/FimL/PilG together play a major role in T4P virulence regulation and activation. T4P modulates surface gene expression through Chp system-cAMP/Vfr depending on PilA, PilJ, ChpA, CyaB, and Vfr for its activity as a mechanosensor (Persat & Inclan, 2015). Altogether, FimV/FimL/PilG complex plays a fundamental role in T4P-Chp-CyaB signaling tempering the cAMP production and Vfr-dependent transcription.

Moreover, FimV transposon mutant was identified to be defective in HeLa cell lifting (Ahn, Ha, Jia, Wu, & Jin, 2004). A deletion of *fimV* in the PAK background exhibited defective twitching motility as well as defective expression of the T3SS. Later it was confirmed that the effect on the T3SS was a result of a polar effect on the other gene in the same operon upstream *fimV* gene*, truA (Hauser, 2009).* Hence, *fimV* complementation restored twitching motility, but not the T3SS defect. On the contrary, a complementation of *truA* mutant could complement T3SS defect, but not the twitching defect. Therefore, it was concluded that *fimV* is required for the twitching motility, whereas *truA* is required for T3SS regulation and expression through modulating tRNAs.

Data from our work and recent studies together, suggest that defective twitching motility and decreased pyocyanin production in *fimV* mutant is associated with FimV/FimL/PilG complex. This complex modulates cAMP/Vfr regulation and virulence, through the T4P-Chp-CyaB signaling pathway. Vfr is a global virulence regulator that can control many virulence associated genes in *P. aeruginosa.* Previous work showed that Vfr suppresses the expression of the efflux pump MexAB/OprM in *Pseudomonas syringae* mediating tolerance to antibiotics (Taguchi & Ichinose, 2013). Therefore, it is possible that Vfr affects antibiotic resistance in *P. aeruginosa* through modulating efflux pumps expression. Moreover, a probable correlation between a potential role of MuxABC-OpmB efflux pump and twitching motility in antibiotic resistance was reported, although the mechanism is unknown (Yang et al., 2011). This can explain *fimV* mutant susceptibility to tetracyclic, carbenicillin and polymyxin B in our results. Both the decreased pyocyanin production and antibiotic resistance susceptibility of *fimV* mutant haven't been reported in the literature. This indicates the significance of FimV for *P. aeruginosa* virulence and persistence, even though truA effect on T3SS is more prevalent than that of FimV on T3SS.

4.2 Putative T3SS Regulator PA3284-3281

Cystic fibrosis is a result of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *P. aeruginosa* infection in CF lungs occurs in adolescent. *P. aeruginosa* undergo several alterations in different stages of infection. Accumulation of mutations in key virulence factors including motility and T3SS is mostly seen in chronic phase of infection (Goldberg & Pler, 1996). Early stage of infection or acute infection is marked with elevated expression of T3SS and other acute virulence factors, with decreased alginate production. *P.*

aeruginosa adapts to CF lung airways by switching between acute and chronic state through tightly controlled regulatory pathways. RetS/LadS/GacS/GacA pathway is considered the switch between chronic and acute infection, although the exact mechanism and signals are not clear. RetS activates T3SS through increasing levels of RsmA, a global post-transcriptional regulator. In addition to T3SS, RsmA exerts its role in virulence by elevating cytotoxicity levels and influencing other virulence related genes. RsmY and RsmZ are two small RNAs, which sequester free RsmA promoting a chronic phenotype of infection.

Our result demonstrated that overexpression of PA3284-81 upregulates *exoS* expression and T3SS. This was confirmed at protein level through SDS-PAGE, exhibiting a strong secretion of ExoS under overexpression of PA3284-81. Additionally, our data showed both RsmY and RsmZ were downregulated in PA3284-81 overexpression strain. Even though there is no difference in RsmA at transcriptional level, the difference could be at post-transcriptional or even post-translational level. The results obtained in this study suggest that the observed effect of PA3284-81 on T3SS likely takes place through the RetS/GacAS/RsmAYZ pathway.

It is established that RetS/GacS signalling cascade is responsible for switching between acute and chronic infection. RetS mutant is characterized with increased RsmYZ, and thus inhibition of motility and T3SS leading to a chronic type of infection. Both the RetS/GacS and the c-di-GMP pathways have been linked previously, where RsmYZ were shown to be required for c-di-GMP dependent response. PA3284-3281 overexpression increased biofilm and pyocyanin production, while decreasing RsmYZ as previously shown in our results. Thus, indicating that PA3284-81 is potentially involved in the regulatory pathway controlling the transition between acute and chronic infections in *P. aeruginosa*.

Recently, Pederick et al (Pederick et al., 2015) applied bioinformatics analysis of *P. aeruginosa* whole genome, characterized ZnuABC, and analyzed their transcription under zinc regulator Zur. Along with his colleagues, they constructed and used PAO1(Δ *znuA*) mutant strain and PAO1 wild type to study zinc pathways under zinc depletion conditions. Total of 88 genes were identified by transcriptomes and RNA sequencing. They showed either upregulated or downregulated genes between 2-4 folds. Among these genes, operon PA3284-81 was upregulated between four to five folds in the PAO1(Δ *znuA*) under zinc_depletion. PA3284-81 seems to be regulated by the zinc regulator Zur. In high zinc condition, zur binds to ZnuA promoter preventing its activation and zinc uptake. Whereas in zinc low condition, ZnuA promoter is free to initiate zinc ion uptake through ZnuBC transporter and other zinc transporter genes. To investigate weather PA3284-81 is associated with zinc, we measured the promoter activity of PA3284-81using the construct pKD 3284-81 under zinc depleted condition, and with zinc supplementation. Zinc depletion upregulated the expression of PA3284-81, while its activity was repressed by the addition of zinc, hence confirming the regulation of this operon by zinc, and probably through Zur. The operon has two cytoplasmic membrane proteins PA3282 and PA3281. It also has a transmembrane protein PA3284 in addition to the cytoplasmic membrane PA3283. It is possible that it might function as zinc transporter spanning the outer and inner membrane.

Siderophores are secreted low-molecular weight molecules to chelate Fe^{3+} with a high affinity through specific receptors. There have been reports on non-classical functions of siderophore in antibiotic resistance, oxidative stress, as metallophores and zincophores (Johnstone & Nolan, 2015). There is a lack in the literature surrounding the high-affinity zinc chelators zincophores. One study stated that *Streptomyces coelicolor* a soil bacterium, produced

a siderophore named coelibactin, which functions in iron transportation. Coelibactin levels decreased in presence of zinc and it was designated as a putative zincophore (Hesketh & Kock, 2009). Later another study confirmed the regulation of coelibactin by the high affinity zinc uptake system Zur (Kallifidas & Pascoe, 2010). Our results showed that PA3284-81 is not only affected by zinc, but also by calcium, iron, and peracetic acid. Therefore, another theory for the function of this operon is that it can be a potential siderophore that function as a zincophore. Although, more evidence and experiments are required to investigate and confirm this theory.

As we confirmed the effect of zinc on PA3284-81 and PA3284-81 induced *exoS* expression, we measured CTX- *exoS* expression with zinc supplementations in zinc depleted condition. Zinc was found to influence *exoS* expression positively in this project. Next, to verify if zinc effect on *exoS* is through PA3284-81, we measured *exoS* expression under PA3284-81 overexpression, and under PA3282 mutation in the presence of zinc supplementation in zinc depleted situation. Similar to CTX- *exoS* results with zinc, *exoS* promoter activity increased under pAK3284-81 overexpression and PA3282 mutant. This increase in *exoS* expression confirms that zinc affects *exoS* expression is not through PA3284-81, but through another unknown pathway.

One novel finding of this study is the effect of zinc on *exoS* expression and thus possibly the T3SS. Our data showed a novel response for T3SS with zinc supplementation in zinc depleted condition with upregulation of *exoS* promoter activity and expression. When assessing these data together, we deduct that in zinc limited situation PA3284-81 is upregulated possibly via Zur to obtain zinc ions from the surrounding environment, thus increasing the abundance of zinc. While zinc presence represses PA3284-81 expression. In zinc rich condition, *exoS* expression is increased independently of PA3284-81 operon, while in zinc limited condition

exoS expression is increased dependently on PA3284-81. This data links PA3284-81 with T3SS and virulence through both RetS/GacAS/RsmAYZ pathway, and possibly through zinc regulatory network. Furthermore, in calcium low condition the T3SS inducing condition, PA3284-81 exhibited a downregulation in expression. This can be interpreted as that low calcium impact on *exoS* is more dominant than that of PA3284-81 on *exoS*.

Previous data have reported a role for iron, zinc and copper in pathogenicity. For example, zinc promotes increased biofilm formation, antibiotic resistance, and T2SS (Marguerettaz & Dieppois, 2014). Higher concentrations of metal ions in CF airways are associated with severs disease outcomes, tissue necrosis inflammation (Smith, 2014). Zinc is present in the cells at the concentrations of 100-500μM, where it makes up 10% of the human proteome. Zinc is mostly bound to proteins, and functions as a catalytic component of around 300 enzymes. It undergoes a tight regulation, and free amounts of zinc required for signal transduction pathways (around 0.2% circulates in the plasma) , are the ones subjected to chelation by chelators such as TPEN (Thambiayya & Kaynar, 2012). Dietary supplementation with zinc is normally given to malnourished and zinc deficient individuals. In enteropathogenic *Escherichia coli,* zinc supplementations have been shown to affect T3SS, by causing envelop stress, which result in defects of T3SS integrity and secretion (Mellies & Thomas, 2012). About 30% of young infants with cystic fibrosis suffer from zinc deficiency (Van Biervliet, Van Biervliet, & Robberecht, 2006). Zinc supplementation to patients improved their health conditions in many disorders, yet there is a lack in knowledge about the effect of zinc supplementations for CF patients, and the data available in the literature is contradicting (Swe & Abas, 2013). A couple of pilot studies investigated the effect of zinc supplementations on CF patients. One study indicated that zinc treated patients required less use of antibiotics, while the

other study resulted in no improvement with zinc supplementations (Van Biervliet & Vande Velde, 2008). During infection, neutrophils secret zinc chelators such as calprotectin to sequester zinc form microorganisms and prevent their growth and proliferation. Recently, zinc was shown to play an important role between group A *streptococcus* (GAS) and host interaction, where GAS employs high affinity zinc transporter to combat calprotectin. Additionally, Salmonella *Typhimurium* displayed a similar response by upregulating the high affinity zinc transporter (ZnuABC) during gut infection to combat calprotectin-mediated zinc chelation (Liu & Jellbauer, 2012). These reports provide evidence of metalloregulatory genes involvement in the regulation of virulence, thus modulating the interactions between the host and pathogens (Makthal, Nguyen, & Kumaraswami, 2017). This can be employed as an antibacterial therapeutic mechanism by targeting zinc uptake systems such as ZnuABC. Moreover, Siderophore−Antibiotic Conjugates have been employed as a ‘’Trojan horse’’ strategy for therapeutics, through pairing siderophores to antibiotics to form a complex with iron, and facilitate the delivery of antibiotics to bacteria (Wencewicz & Long, 2013). I assume that the same strategy can be employed with zinc for the treatment of *P. aeruginosa* infections, especially in CF due to the complications associated with host defense and the biofilm formation, which makes it hard to deliver antibiotics to the bacterium.

For future work, it is suggested to evaluate the expression of virulence factors under PA3284-81 overexpression in artificial sputum/M9 medium in addition to zinc and without zinc. Examine the expression at protein levels by measuring mRNA levels at the same conditions tested in this project. Further investigation of structure and function of this operon, and the regulatory pathway connecting zinc with PA3284-81through Zur, controlling zinc and other metal ions. Elucidate other possible virulence regulators such as Vfr, QS, and c-di-GMP on

PA3284-81. Identification of binding site of PA3284-81 on the promoter of potential target genes by mobility shift assay.

In summary, we found PA3284-81 to be important for T3SS expression and for zinc regulation, and we further show that this operon is influenced by different signals such as peracetic acid, iron ions and possibly by other unknown signals. It also plays a role in both biofilm formation and pyocyanin production, making it crucial factor in *P. aeruginosa* pathogenicity. Moreover, through this study a novel effect of zinc on ExoS and T3SS is identified and confirmed to be independent on the operon PA3284-81. *P. aeruginosa* possess substantial ability to respond to a wide range of signals, and stimuli to adapt and disseminate in its niche, and combat other pathogens. Further studies are in need to elucidate the influence of host-pathogen interaction through signals such as essential trace elements, and how these signals are sensed, through which pathways and networks the response is initiated.

4.3 Possible Connection Between T3SS and T6SS Through *norC*

P. aeruginosa utilize T6SS against invasion by other microorganisms at the site of infection. Clinical isolates from CF patients are shown to have active T6SS (Mougous et al., 2006). Nitric oxide detoxification is an essential physiological necessity for bacteria. The process of nitrate reduction to dinitrogen includes four consecutive steps with nitrite (NO2-), nitric oxide (NO) and nitrous oxide (N2O) as intermediates. Both *norC* and *norB* encode for nitric oxide reductase (Nor), to reductively metabolize ˙NO to N2O (Firoved & Wood, 2004). The significance of these nitric oxide detoxification gens for *P. aeruginosa* comes from the fact that in host cells, specifically in CF environment, ˙NO is considered an innate immunity mechanism to fight infection.

Our transposon insertion using CTX-*H2*-T6SS reporter was inserted in two genes of the gene cluster PA0523- PA0525 harboring *norC*, therefore the whole operon was deleted for the construction of PAO1 (Δ*norC*). Nitric oxide reductase gene *norC*, have been shown in the literature in several studies to be important in anaerobic respiration and biofilm formation (Hentzer, Eberl, & Givskov, 2005). Furthermore, amoeba-induced transcriptome analysis identified T3SS genes required for amoeba killing. *norC* and *norB* were upregulated against amoeba killing. It was suggested that the need of detoxification of amoeba-derived reactive nitrogen intermediates was behind the cause for this change in regulation (Matz et al., 2008). This led us to address the possibility of *norC* involvement not only in H2-T6SS as discovered in our transposon library, but also in T3SS. It could be a novel potential link between T3SS and T6SS. We investigated the expression of *exoS* promoter activity in *norC* mutant. To our surprise, *exoS* activity was significantly reduced in *norC* mutant providing an evidence for potential novel target that connects the two complex virulence associated secretion systems in *P. aeruginosa.* Reinforcing our evidence, *norC* was also found to be downregulated in *mucA* mutant, causing a repression of T3SS expression under inducing and non-inducing condition resulting in a mucoid phenotype (Wu & Badrane, 2004).

In *P. aeruginosa*, anaerobic regulation and adaption is under the control of FNR (fumarate and nitrate reductase regulator) transcriptional regulators Anr and Dnr. Fnr shares 51% amino acid sequence identity to Fnr of Escherichia coli. Nitrite reductase (NIR) and nitric oxide reductase (NOR), have been shown to be under the regulation of Anr (Arai, Kodama, & Igarashi, 1997).Dnr is activated through Anr to regulate the expression of denitrification genes. Thus, in addition to Anr, Dnr was found to be involved in the expression of *norCB* genes as well as *nirS* and *nirQ* (Trunk et al., 2010). Unlike Dnr, Anr is not only essential for denitrification, but also plays a role

in arginine deiminase pathway and cyanide production (Galimand & Gamper, 1991). Our data and previous studies led us to the following speculation. Taking together, the mucus of the cystic fibrosis (CF) lung combined with the biofilm mode of bacterial growth, *P. aeruginosa* is forced to adapt to these conditions by upregulating genes necessary for anaerobic adaption through Anr. Therefore, Anr-Dnr activate *norC* expression in addition to NarL, a response regulator involved in regulating energy metabolism through inducing nitrate metabolism. Once NarL is activated by Anr, it suppresses and modulates the RsmAYZ circuit. NarL affects RsmAYZ transcription by binding to sRNAs RsmY and RsmZ promoters decreasing their expression, subsequently allowing free RsmA protein to positively regulate the T3SS. Similarly, Anr might affect *norC* in the same way, explaining the decreased *exoS* expression in *norC* mutant in our results.

Furthermore, second messenger c-di-GMP controls transition between chronic infection and acute infection. A diguanylate cyclases, *WspR*, and PA2133 a phosphodiesterases, inimical activities oversee intracellular levels of c-di-GMP (Hickman et al., 2005). *WspR* favors the upregulation of genes in control of chronic phenotypes, such as biofilm formation, while PA2133 degrades levels of c-di-GMP. More recently a connection between c-di-GMP signaling network and RetS/GacS regulatory pathway was reported (Moscoso & Mikkelsen, 2011). c-di-GMP controls the transition from acute to chronic and vice versa through inversely altering T3SS and the T6SS expression independently on RetS. In addition to the two sRNAs, RsmY and RsmZ dependently on WspR diguanylate cyclase.

It is believed that in acute infection T3SS is activated, while in chronic infection, T6SS is stimulated and T3SS is repressed or expressed at very low levels. *norC* have been identified in our transposon mutagenesis library of H2-T6SS. H2-T6SS is important for epithelial cell invasion and

cytotoxicity in animal, plant, human hosts, and prokaryotic. These data suggest that *norC* might hold key answers for the regulatory relationship between T3SS and T6SS.

CHAPTER V

Conclusion and Future Prospects

Bacteria deliver a series of effector proteins via different secretion systems to provide a fitness advantage in survival and competing for resource. Over the past few years, advances in structural and molecular biology have considerably improved our understanding of bacterial T3SS and T6SS, and have led to the emergence of new drug-design efforts. The central regulators of each system, such as ExsA master regulator of T3SS, core component of the secretion systems, like clpV in T6SS, and common regulators of these two secretion systems, such as quorum sensing, iron uptake regulator can be used as the targets (Badr, Li, & Duan, 2016).

To find new antimicrobial therapeutic targets against these secretion systems, the effector proteins, structure components, regulatory genes have been considered as well as inhibition of their assembly and action. Yet, there is still more about these systems to be understood. Even though we have made great progress on these two secretion systems, there are many questions still unknown. For example, what detailed environmental signals specifically trigger different secretion system, and how? Which regulatory networks influence the T3SS/T6SS switch? What kind of regulators and signals are responsible for the activation of T3SS/T6SS regulatory system? Answering these questions are needed to fully understand the important secretion systems in *P. aeruginosa* and to use them as potential drug targets.

From our study, we show that overexpression of PA3284-81 upregulates *exoS* expression. This was confirmed by SDS PAGE gel result showing a clear significant increase in ExoS under pAK3284-81 overexpression. This effect possibly occurs through RetS/GacAS/RsmAYZ

pathway, since both *rsmY* and *rsmZ* were decreased under pAK3284-81. Moreover, we show that PA3284-81 promoter activity was repressed in presence of zinc and upregulated under zinc depletion, demonstrating a highly probable role for this operon in zinc regulation possibly under Zur. The observation of increased *exoS* expression with the addition of zinc is independent of PA3284-81. This is a novel finding that links zinc concentrations with T3SS aggression of *P. aeruginosa* and pathogenicity. Many infections and disease cause disruption in metal balance in the host. In CF patients, there have been few contradicting reports on the impact of zinc on health the of CF patients (Van Biervliet & Van Biervliet, 2007). This finding provides a basis for further investigations of the correlation between zinc and virulence, and how it can influence *P. aeruginosa's* interaction with other microbes in polymicrobial diseases such as CF, especially considering some CF patients are at risk of zinc deficiency.

Additionally, our data has shown that *norC* is important not only for T6SS, but possibly for T3SS regulation, making it a potential link between T6SS and T3SS. Further studies are guaranteed to elucidate the mechanisms of the potential regulators of these two systems. For future work, it is suggested to construct potential regulatory pathway following the measurement of virulence associated factors in wild type and the mutant *norC*. Construct expression profiles acquired from promoter-reporter system. Measure single transition c-di-GMP second messenger, other major virulence regulators to understand expression order and gene regulatory pathway. Characterize the interplay of quorum sensing, antibiotic regulation, and interspecies interaction for *norC* mutant.

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