# *Pseudomonas aeruginosa* Type III Secretion System: Regulation and Potential Role in Interspecies Interaction

By

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## ABSTRACT

*Pseudomonas aeruginosa* causes various infections in humans, animals and plants. Type III secretion system (T3SS) is one of the essential virulence factors used by *P. aeruginosa*. In this study, a previously uncharacterized gene PA0466 and its role in T3SS regulation have been examined. The results indicate that PA0466 is a novel T3SS regulator. It regulates T3SS directly through an unknown pathway, and has a minor effect on the GacA-RsmA pathway. Besides the role in the interaction between the pathogen and the host, T3SS may also play a role in the interspecies interaction. A real-time PCR based Competitive Index (CI) assay was used to compare the wild type and T3SS mutant with and without the presence of *Staphylococcus* spp.. The results indicate that PAO1 was more competitive than *exsA* mutant and the difference was even bigger in the presence of *Staphylococcus*, suggesting T3SS may play a significant role in bacterium-bacterium interaction.



**Key words:** *Pseudomonas aeruginosa*, type III secretion system, PA0466 over-expression, interspecies interaction

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## ABBREVIATIONS

| | |
|---|---|
| ADPRT | ADP ribosyltransferase |
| AHL | Acyl homoserine lactone |
| AHQ | 2-alkyl-4(1H)-quinolone |
| AI | Autoinducer |
| Amp | Ampicillin |
| cAMP | Cyclic adenosine monophosphate |
| Cb | Carbenicillin |
| CDC42 | Cell division cycle 42 |
| CF | Cystic fibrosis |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| CFU | Colonies forming unit |
| CI | Competitive index |
| Cm | Chloramphenicol |
| cps | Counts per second |
| $C_q$ | Quantification cycle |
| CRK | CT10 regulator of kinase |
| CTD | Carboxy-terminal domain |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxyribonucleotides |
| dsDNA | Double-strand DNA |

| | |
|---|---|
| EDTA | Ethylenediaminetetraacetic acid |
| EGTA | Ethylene glycol bis (2-aminoethyl) tetraacetic acid |
| FAS | Factor Activating ExoS |
| GAP | GTPase activating protein |
| Gm | Gentamicin |
| HEPES | *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid |
| HSL | Homoserine lactone |
| Kn | Kanamycin |
| LB | Luria-Bertani |
| LPS | Lipopolysaccharide |
| MCS | Multiple cloning site |
| NTD | Amino-terminal domain |
| OD | Optical Density |
| OF | Oropharyngeal flora |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PIA | Pseudomonas isolation agar |
| PQS | Pseudomonas quinolone signal |
| qPCR | Quantitative PCR |
| QS | Quorum sensing |
| SCV | Small colony variant |
| SDS | Sodium dodecyl sulfate |

| | |
|---|---|
| SOD | Superoxide dismutase |
| RNA | Ribonucleic acid |
| sRNA | Small RNA |
| Tc | Tetracycline |
| TCA | Trichloroacetic acid |
| TCS | Two-component system |
| $T_m$ | Melting temperature |
| Tmp | Trimethoprim |
| T3SS | Type III Secretion System |
| Vfr | Virulence factor regulator |

# CHAPTER I. INTRODUCTION

*Pseudomonas aeruginosa* causes a broad spectrum of infections in humans, animals and plants (Kerr and Snelling 2009). It is ubiquitous due to its capacity to adapt to various environmental niches and ability to exploit numerous compounds as nutrient sources (Lyczak, Cannon *et al.* 2000). Due to its high intrinsic resistance to antibiotics, arising from the low permeability of its outer membrane, the presence of multi-drug efflux pumps and capability to cause enzymatic antibiotic modifications, it is included in the group of so-called "superbugs". It is clear that in-depth investigations of the mechanism of *P. aeruginosa* infections are needed (Breidenstein, de la Fuente-Núñez *et al.* 2011).

## 1.1 *Pseudomonas aeruginosa* Pathogenesis

*P. aeruginosa* is a rod-shaped, gram-negative bacterium, which has widespread occurrence in the environment. Having 6.3 million base pairs, *P. aeruginosa* genome is one of the largest among the known sequenced bacterial genomes (Stover, Pham *et al.* 2000). By carrying a broad repertoire of genes encoding various virulence determinants, this organism is able to occupy diverse niches. *P. aeruginosa* is aerobic, however, it also can adapt to microaerophilic and anaerobic environments (Williams, Zlosnik *et al.* 2006).

*P. aeruginosa* is an opportunistic pathogen that causes infections in individuals with

immunodeficiency, severe burns, or the most notable, cystic fibrosis (Richards, Edwards *et al.* 2000). It is notable for causing either acute or chronic infections. High virulence free-swimming planktonic pathogens usually trigger acute infections. However, less virulent and dawdling growing bacteria trigger chronic infections which are characterized by biofilms formation (Kuchma, Connolly *et al.* 2005, Kipnis, Sawa *et al.* 2006).

The motile surface appendages of *P. aeruginosa*, specifically flagella and pili, are associated with the irreversible epithelial cell adhesion, which is the initial key step for colonization. Lipopolysaccharide (LPS) has similar function. Upon cell contact, *P. aeruginosa* launches the type III secretion system (T3SS) by injecting toxic effector proteins into the host cell. Other virulence factors are also secreted into the extracellular space to facilitate pathogen intrusion and cytotoxicity, such as elastase, alkaline phosphates, exotoxin A and phospholipase C (Kipnis, Sawa *et al.* 2006).

These virulence factors are coordinated by a cell-density-dependent regulatory system, namely quorum sensing (Winzer and Williams 2001). Quorum sensing (QS) is a global gene regulatory mechanism that enables individual bacterium to communicate and coordinate their behaviors in populations via extracellular signals (Cámara, Hardman *et al.* 2002). Bacteria release a diffusible signal called autoinducer (AI). When this signal accumulates and reaches a critical threshold, the bacteria are subjected to an auto-induction feedback, by functioning through targeting and

modulating their QS genes (Duan and Surette 2007).

In total, three intertwined QS systems have been identified to date: the two acyl homoserine lactone (AHL) mediated systems and the 2-alkyl-4(1H)-quinolone (AHQ) signal-based system. The two AHL mediated systems termed Las and Rhl systems, each possesses a homoserine lactone synthase (LasI or RhlI) and a response regulator (LasR or RhlR). Their cognate signals are *N*-(3-oxododecanoyl)-L-homoserine lactone ($C_{12}$-HSL) and *N*-butanoyl-L-homoserine lactone ($C_4$-HSL), respectively (Pearson, Pesci *et al.* 1997, Hogardt, Roeder *et al.* 2004).

The third player in *P. aeruginosa* quorum sensing system is the AHQ signal-based system (Pesci, Milbank *et al.* 1999), which includes a 2-heptyl-3-hydroxy-4-quinolone based signaling system called Pseudomonas quinolone signal (PQS). The PQS synthesis is carried out by the gene clusters *pqsABCDE*, *phnAB* and *pqsH*. The transcriptional regulator PqsR (Mvfr) is also required for PQS synthesis (Cao, Krishnan *et al.* 2001, Gallagher, McKnight *et al.* 2002, Déziel, Lépine *et al.* 2004).

Arranging in a cascade, the AHLs are released in early exponential phase, while PQS occurs later on and acts as the terminal signal in the *P. aeruginosa* QS system (Lépine, Déziel *et al.* 2003, Dietrich, Price-Whelan *et al.* 2006).

Phenazine compounds are among the virulence factors controlled by PQS signaling (Déziel, Lépine *et al.* 2004). These compounds inhibit ciliary function of the respiratory epithelial cells, and alter the host's immune and inflammatory response (Wilson, Pitt *et al.* 1987, Laursen and Nielsen 2004, Allen, Dockrell *et al.* 2005). *P. aeruginosa* carries two homologous operons for phenazine synthesis: *phzA1B1C1D1G1* (*phzA1*) and *phzA2B2C2D2G2* (*phzA2*) (Mavrodi, Bonsall *et al.* 2001). Both *rhl* system and PQS activate *phzA1* expression, which accounts for the majority of phenazine production (Whiteley, Lee *et al.* 1999, Diggle, Winzer *et al.* 2003).

Cystic fibrosis (CF) is a common life-threatening hereditary disease in Caucasians (Gibson, Burns *et al.* 2003). Among all the organs or systems affected by CF, pulmonary disorders determine the length and quality of patients' life and is the most challenging problem (Cohen and Prince 2012). Infection of the lower airway of CF patients has been defined as a polymicrobial infection (Sibley, Rabin *et al.* 2006). *P. aeruginosa* is the predominant pathogen colonized in the lungs of CF patients causing pulmonary failure (Rajan and Saiman 2002). In CF patients' lungs, *P. aeruginosa* exists in biofilms (Singh, Schaefer *et al.* 2000). Biofilms are defined as living and continuously renewing multicellular aggregates (Branda, Vik *et al.* 2005, Mayansky, Chebotar *et al.* 2012). The development of biofilms begins with the attachment of planktonic bacteria to a solid surface, followed by the formation of micro-colonies and surrounding extracellular matrix (O'Toole, Kaplan *et al.* 2000). The extracellular

matrix covers the whole cell community, permitting structural maintenance, as well as providing protection to individual members from being attacked by the host immune responses (Sutherland 2001). Single cells benefit from joining the group, as they can avoid insults and assaults, most importantly (Davey and O'Toole 2000). Living in a biofilm, *P. aeruginosa* becomes more resistant to antibiotics, in comparison to their planktonic counterparts (Whiteley, Bangera *et al.* 2001). As mentioned before, *P. aeruginosa* is able to grow in anaerobic environments, such as in the lungs of CF patients, it has been reported that anaerobiosis promotes biofilm formation (Yoon, Hennigan *et al.* 2002, Palmer, Mashburn *et al.* 2005).

Along with its high virulence, the antibiotic-resistance capacity of *P. aeruginosa* posts an obstacle in infection treatment. Due to the presence of multidrug efflux pumps, low permeability of outer membrane and capability to aggregate in biofilms, eradication of this bacterium is extremely difficult to achieve (Rocchetta, Burrows *et al.* 1999). Long-term use of antibiotics in classical antibiotic therapy often results in the rise of antibiotic-resistant pathogens (Ash 1996). Therefore, alternative approaches such as weakening the virulence of *P. aeruginosa* are urgently required (Keyser, Elofsson *et al.* 2008). T3SS is an attractive target on account of these criteria, which will be introduced in the following texts (Hueck 1998).

## 1.2 Type III Secretion System

### 1.2.1 Characteristics of T3SS

Bacteria can deliver virulence factors directly into the host cells. Such delivery requires penetrating both the bacterial cell envelope and the host cell plasma membrane. The success of this process largely depends on a wide range of protein secretion systems, arising from gram-negative bacteria (Filloux 2011). Six secretion systems are currently described as type I to VI secretion systems (Bleves, Viarre *et al.* 2010). Being made up of more than 20 proteins, the type III secretion system (T3SS) stands out from the others due to its complexity (Galan and Wolf-Watz 2006).

T3SS was first discovered in *Yersinia enterocolitica*, and later proven to exist in *Escherichia coli*, *Pseudomonas*, *Shigella*, *Salmonella* and *Vibrio* strains (Salmond and Reeves 1993, Lee 1997, Hueck 1998, Coburn, Sekirov *et al.* 2007). It plays a key role in *P. aeruginosa* virulence, along with biofilm and quorum sensing (Veesenmeyer, Hauser *et al.* 2009). It has been reported that up to 90% of *P. aeruginosa* isolates from patients diagnosed with acute respiratory infections secreted type III proteins (Roy-Burman, Savel *et al.* 2001).

The T3SS regulon is composed of five operons. The *pscN* to *pscU* and *exsD-pscB* to *pscL* operons encode components of the secretion machinery, the

*popN-pcr1-pcr2-pcr3-pcr4-pcrD-pcrR* operon encodes proteins involved in the control of effector release, the *pcrGVH-popBD* operon encodes the translocators that form a pore in the eukaryotic cell membrane, and finally, the *exsCEBA* operon encodes regulatory components (Soscia, Hachani *et al.* 2007).

Utilized by many human pathogens, T3SS functions like a molecular syringe (Coburn, Sekirov *et al.* 2007). The syringe-like structure, called injectisome, allows T3SS to inject and translocate toxins directly into the eukaryotic host cells. Functionally, T3SS can be divided into five components: the needle complex, the translocation apparatus, the regulatory proteins, the effector proteins and the chaperones. These five components work together to inject effector proteins into host cells in a highly regulated manner (Figure 1.1).

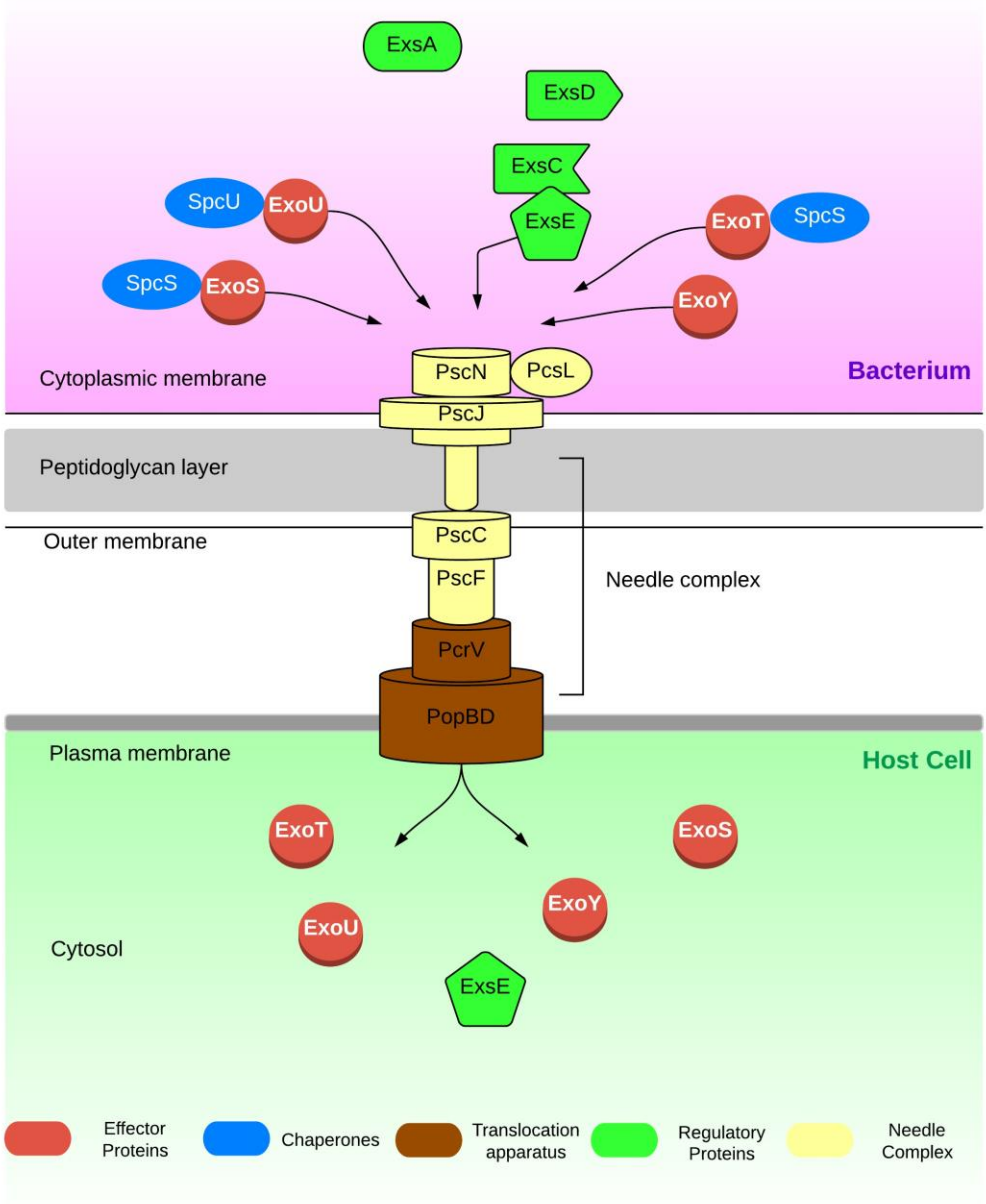
The multi-ring base and needle-like filament form the 60-120 nm long by 6-10 nm wide needle complex. The translocation apparatus is a proteinaceous membrane pore that takes charge of delivery in T3SS. More specifically, PopB, PopD and PcrV is responsible for translocation (Sundin, Wolfgang *et al.* 2002). Secreted by the type III needle complex, PopB and PopD interact with each other, but also with the host cell membrane to form the translocation pore in a poorly understood manner (Schoehn, Di Guilmi *et al.* 2003). On the other hand, PcrV is required for translocation, but is not involved in the formation of the pore component (Goure, Pastor *et al.* 2004). In addition of the transporting function, the translocation apparatus also causes cell death

by itself (Hauser 2009), either in a direct way through pore-mediated increases in membrane permeability or through the activation of defensive responses indirectly (Roy, Liston *et al.* 2004).

Some T3SS secreted proteins bind cognate chaperones to facilitate both storage and delivery (Parsot, Hamiaux *et al.* 2003). For some effectors, such as ExoS and ExoT, the chaperone SpcS is required for their maximal secretion (Shen, Quenee *et al.* 2008). SpcU is specific for ExoU, while no chaperone has been found for ExoY (Finck-Barbancon, Yahr *et al.* 1998). Many other T3SS secreted proteins also need chaperones, such as PcrH for PopB and PopD (Schoehn, Di Guilmi *et al.* 2003), PscE and PscG for PscF (Quinaud, Chabert *et al.* 2005), and ExsC for ExsE (Urbanowski, Lykken *et al.* 2005).

Contact with eukaryotic cells triggers the activation of T3SS, by which the effectors are released into the host. Once positioned into host cytoplasm, these molecules invade the tissue and spread systemically, resulting in cell rounding and cell death by necrosis or apoptosis (Kim, Ahn *et al.* 2005, Engel and Balachandran 2009). Compared to the highly conserved injectisome structure, different pathogens own exclusive sets of effectors in order to facilitate their unique lifestyles (Coburn, Sekirov *et al.* 2007) (Table 1.1).

**Figure 1.1 Structure of T3SS in *P. aeruginosa*.**




This figure was modified from the originally published work by Galle *et al*. (Galle, Carpentier *et al*. 2012).

**Table 1.1 Examples of effector proteins of known functions secreted via type III secretion systems.**

| Bacterial species | Secreted protein | Biochemical activity | Effect on host cell function |
|---|---|---|---|
| *E. coli* | Tir | Receptor for intimin | Effacement of the microvilli of the intestinal brush border |
| *P. aeruginosa* | ExoS | ADP-ribosyltransferase | Inhibition of phagocytosis |
| | ExoT | ADP-ribosyltransferase | Cytotoxicity |
| | ExoU | Phospholipase | Necrosis |
| | ExoY | Adenylate cyclase | Cytotoxicity |
| *Yersinia* spp. | YopE | Unknown | Disruption of the actin cytoskeleton; inhibition of phagocytosis |
| | YopH | Tyrosine phosphatase | Disruption of focal adhesions; inhibition of phagocytosis |
| | YpkA | Serine/threonine kinase | Unknown |
| | YpoJ (YopP) | Unknown | Apoptosis |

| | | | |
|---|---|---|---|
| *Shigella* spp. | IpaB | Activation of caspase-1; binds β1-integrins and CD44 | Apoptosis |
| | IpgA | Binds vinculin | Stimulation of bacterial entry |
| | IpgD | Putative inositol phosphatase | Unknown |

Note: the information in this table was collected and summarized from the literatures written by Mota *et al.* (Mota and Cornelis 2005), Lee (Lee 1997) and Hueck (Hueck 1998).

There are four effector proteins in *P. aeruginosa* T3SS. ExoS is a bifunctional toxin that has both GTPase-activating protein (GAP) activity and ADP ribosyltransferase (ADPRT) activity. The GAP domain targets Rho, Rac and cell division cycle 42 (CDC42), which are small GTPases that maintain host cell actin cytoskeleton (Goehring, Schmidt *et al.* 1999). These GTPases normally switch between an active GTP-bound form and an inactive GDP-bound form. The ADPRT activity of ExoS has several adverse effects on the host cell, including cell death, vesicular trafficking, endocytosis actin, cytoskeleton destruction and inhibition of DNA synthesis (Pederson and Barbieri 1998, Barbieri, Sha *et al.* 2001). A eukaryotic cofactor FAS (Factor Activating ExoS, was later shown to be a 14-3-3 protein) is necessary for the activation of ADPRT activity (Henriksson, Francis *et al.* 2002).

Sharing 76% amino acid identity with ExoS, ExoT is also a bifunctional toxin with N-terminal GAP activity and C-terminal ADPRT activity. Similar to ExoS, ExoT contains GAP activity towards Rac, Rho and CDC42 (Krall, Schmidt *et al.* 2000). ExoT possesses an ADPRT domain that requires binding of the host cell cofactor 14-3-3 for activation (Liu, Yahr *et al.* 1997). However, in contrast to ExoS, ExoT ADP ribosylates a distinct and limited number of host proteins, particularly the CT10 regulator of kinase (CRKI and CRKII) adaptor proteins (Sun and Barbieri 2003). Although it was initially thought that ExoT had limited ADPRT activity, evidences show that ExoT has a robust ADPRT activity that directs towards unique substrates, the CRK proteins. These properties of ExoT work together to alter the host's actin

cytoskeleton and to inhibit cell migration, adhesion and proliferation, therefore to block phagocytosis and disrupt epithelial barriers in the moment of bacterial dissemination (Shaver and Hauser 2004).

ExoU is a powerful phospholipase that leads to rapid cell death in eukaryotic cells (Phillips, Six *et al.* 2003). Like other *P. aeruginosa* type III effector proteins, ExoU requires a eukaryotic cofactor SOD1. SOD1 activates ExoU by inducing a conformational change or linking ExoU to other factors or to its phospholipid substrate (Sato, Feix *et al.* 2006). A fairly interesting aspect about ExoU is the ubiquitinylation process it undergoes once inside the host cell (Stirling, Cuzick *et al.* 2006). The net result of intoxication with ExoU is the death of the host cell characterized by a rapid loss of plasma membrane integrity and a consistent necrosis, which apparently differs from what happens with ExoS and ExoT (Finck-Barbancon, Goranson *et al.* 1997). ExoU killing may be directed against phagocytes, as well as epithelial barriers, thereby promoting bacterial persistence and spreading (Kurahashi, Kajikawa *et al.* 1999).

The last effector protein is a secreted adenylyl cyclase ExoY (Yahr, Vallis *et al.* 1998). Injection of ExoY into mammalian cells results in an elevation of intracellular cAMP concentration, which subsequently leads to interference to the actin cytoskeleton and bacterial uptake and augmentation of endothelial permeability (Sayner, Frank *et al.* 2004, Cowell, Evans *et al.* 2005).

T3SS can inhibit the host innate immune response efficiently and facilitate the infection and colonization of *P. aeruginosa* (Yamazaki, Li *et al.* 2012). A precondition for a successful infection is to avoid phagocytic clearance in the host. T3SS effectors possess the anti-phagocytic property (Frank 1997). Specifically, ExoT and ExoS inhibit phagocytosis by disrupting cytoskeleton arrangement, focal adhesions and the relevant signal transduction cascades, whereas ExoU and ExoY are cytotoxins with phospholipase and adenylate cyclase activities (Yahr, Vallis *et al.* 1998, Sato and Frank 2004, Barbieri and Sun 2005). Furthermore, effectors mediate host tissue dissemination, leading to systemic infection and septic shock (Kurahashi, Kajikawa *et al.* 1999).

A prevalence survey of type III effector genes in a large collection of *P. aeruginosa* clinical and environmental isolates indicates most strains do not carry the whole package of 4 effector genes (Feltman, Schulert *et al.* 2001). Even though all strains have the *exoS* or the *exoU* gene, they are never simultaneously activated in any individual strain. ExoS-secreting strains cause delayed cell death through apoptosis, on the other hand, ExoU-secreting strains are more likely linked to rapid cell lysis (Fleiszig, Wiener-Kronish *et al.* 1997, Schulert, Feltman *et al.* 2003). Generally speaking, ExoU and ExoS secretions are inverse actions and are related to opposite infections. By carrying the ExoU-encoding gene, the strains are likely involved in acute infection. Likewise, the ExoS-secreting strains are adaptable to the CF airway (Feltman, Schulert *et al.* 2001).

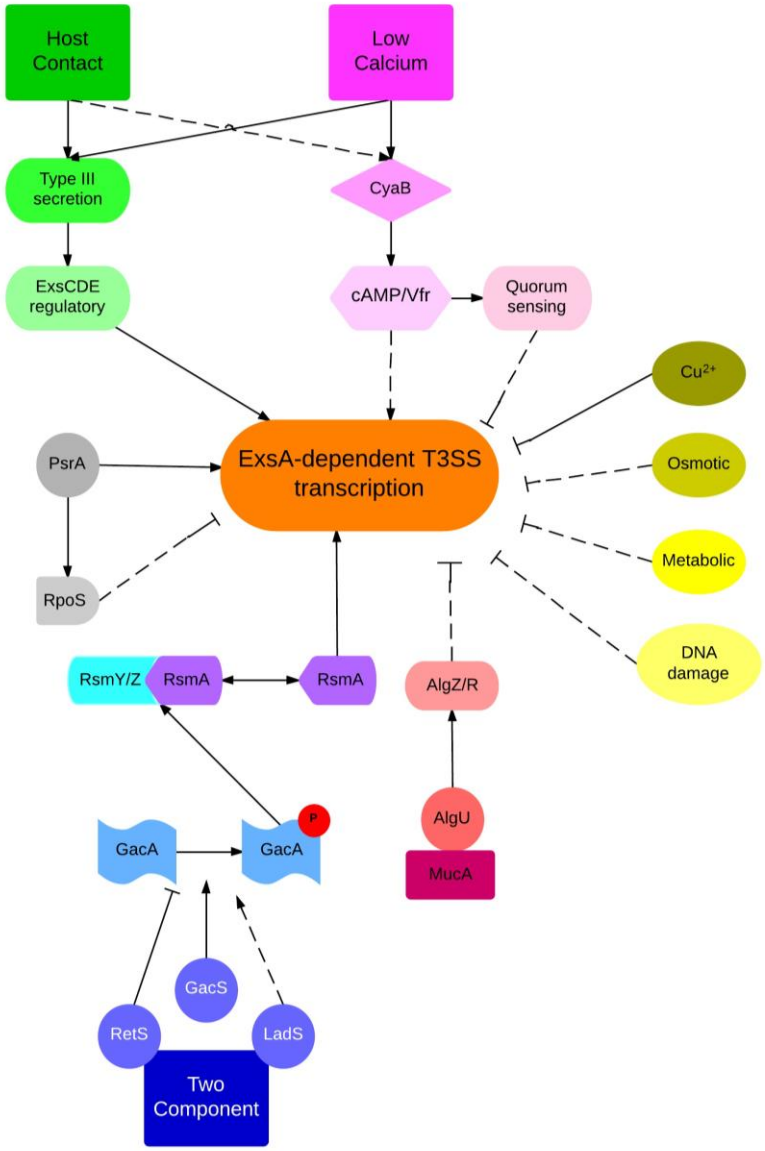
Defined as a contact-dependent secretion system, T3SS requires intimate mutual contact between host and pathogen *in vivo* (Hayes, Aoki *et al.* 2010). *In vitro*, the surrogates of the host-contact signal include growth under calcium depleting media and in the presence of serum (Frank 1997).

### 1.2.2 Regulation of T3SS

#### 1.2.2.1 ExsA and ExsADCE Cascade

T3SS is regulated by a DNA-binding protein ExsA, the central regulator of T3SS gene expression in *P. aeruginosa* (Figure 1.2). ExsA belongs to the AraC/XylS family of transcriptional activators, having an amino-terminal domain (NTD) and a carboxy-terminal domain (CTD). While NTD mediates ligand binding and oligomerization, CTD is the DNA-binding domain (Gallegos, Schleif *et al.* 1997, Brutinel, Vakulskas *et al.* 2009). ExsA recognizes a consensus sequence (TNAAAANA), usually located upstream of the transcriptional start site which promotes the expression of T3SS genes (Hovey and Frank 1995). Encoded last in the gene cluster *exsCEBA*, *exsA* can be auto-regulated (Yahr and Frank 1994). Inhibition of *exsA* expression represses the T3SS regulon, and T3SS is not expressed in the mutant which abolished *exsA* expression (Dacheux, Attree *et al.* 2001).

**Figure 1.2 Environmental signals and regulatory systems controlling expression of the T3SS.**




The regulation of T3SS occurs intrinsically or extrinsically through the presence of various signals. Solid lines indicate regulatory connections that have been demonstrated experimentally, whereas dashed lines indicate hypothetical regulatory connections. This figure was modified from the originally published work by Yahr and Wolfgang (Yahr and Wolfgang 2006).

Three additional players, namely ExsC, ExsD, and ExsE, work together with ExsA via a "partner-switching" mechanism to stimulate the type III secretion activity (Figure 1.3) (Brutinel and Yahr 2008). ExsD is an anti-activator which inhibits ExsA function by interrupting both the self-association and DNA-binding properties (Brutinel, Vakulskas *et al.* 2010). ExsC is known as an anti-anti-activator that sequester ExsD (Lykken, Chen *et al.* 2006). Meanwhile, ExsC can also function as a chaperone to form a complex with ExsE (Zheng, Chen *et al.* 2007). When ExsC interacts with ExsE, ExsD is allowed to sequester ExsA and T3SS regulon is repressed. The opposite scenario is when ExsE is secreted outside of the bacterium under inducing condition, favoring the formation of the ExsC-ExsD complex. In this way T3SS is activated by ExsA (Rietsch, Vallet-Gely *et al.* 2005, Urbanowski, Brutinel *et al.* 2007).

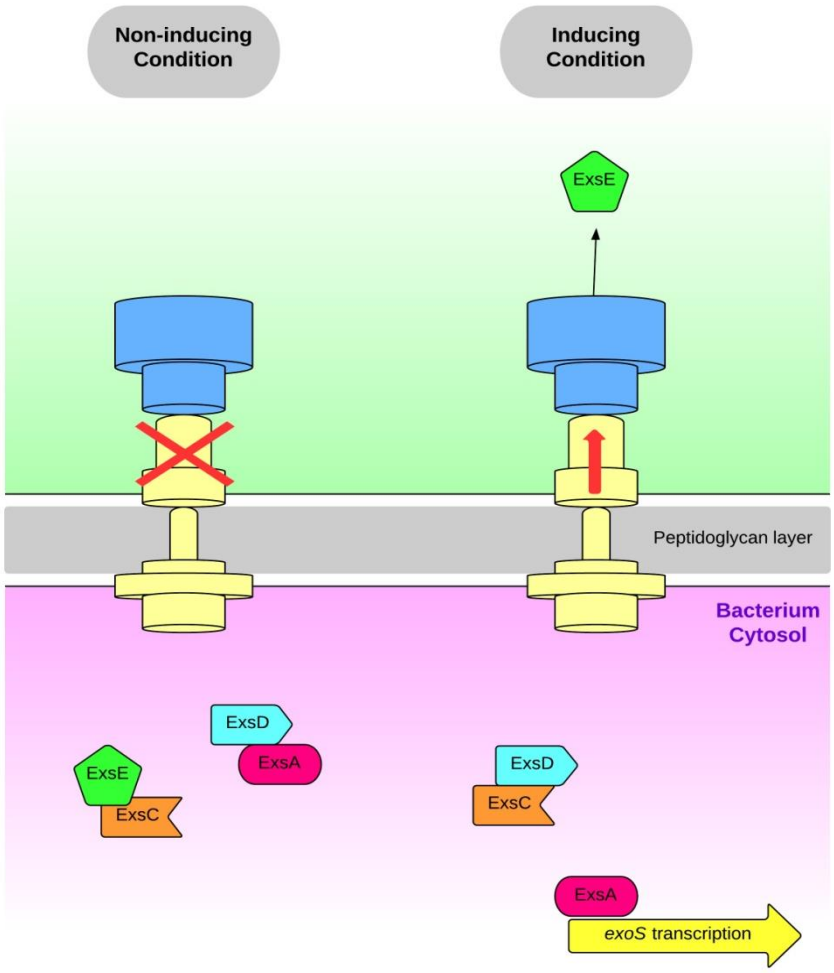
Coupling secretion with transcription is an extraordinary feature of T3SS (Figure 1.3). Prior to exposure to low calcium levels or contact with the host, ExsD usually sequesters ExsA and represses the T3SS machinery, since ExsC displays a stronger affinity for ExsE than ExsD (Vogelaar, Jing *et al.* 2010). Through this, the production of T3SS proteins, including needle complexes and effector proteins, is maintained at low basal levels (Rietsch and Mekalanos 2006). However, under inducing conditions, type III secretion channel is converted from a closed (inactive) state to an open (active) state (McCaw, Lykken *et al.* 2002). Once ExsE is secreted out, the T3SS gene transcription is magnified enormously. As a result, the profound effects on T3SS gene

expression are accomplished only by switching the partners among the regulators and a minor change of individual protein levels (Dasgupta, Lykken *et al.* 2004).

#### 1.2.2.2 cAMP-Dependent Pathway

The biosynthesis of intracellular cAMP may influence T3SS expression in *P. aeruginosa*. Two adenylate cyclases, CyaA and CyaB, take charge of the cAMP synthesis in this microorganism. CyaB is more potent, as its corresponding mutant is devoid of measurable intracellular cAMP and has a defective T3SS gene expression character (Smith, Wolfgang *et al.* 2004). Evidence indicates that low calcium and high salt conditions favors T3SS, as well as increases intracellular cAMP levels (Wolfgang, Lee *et al.* 2003, Rietsch and Mekalanos 2006). In addition, virulence factor regulator (Vfr), which is required for *P. aeruginosa* extracellular protease and exotoxin A production, joins in the cAMP regulation cascade to control the *exsA* expression along with quorum sensing (West, Sample *et al.* 1994, Nouwens, Beatson *et al.* 2003).

**Figure 1.3 Coupling of T3SS secretion with transcription.**




Coupling of transcription and secretion occurs by the "partner switching" mechanism of four regulatory proteins: ExsA, ExsD, ExsC and ExsE. Under non-inducing conditions, ExsA bounds ExsD, whereas ExsC sequesters ExsE. Through this, the transcriptional activation of T3SS is inhibited. Once secretion is activated under inducing conditions, ExsE is exported, allowing ExsA to bind to the promoter region of the T3SS gene such as *exoS*, thereby activating the process of transcription. This figure was modified from the originally published work by Galle *et al*. (Galle, Carpentier *et al.* 2012).

### 1.2.2.3 Two-Component System

Two-component system (TCS) has been found extensively in bacteria, eukaryotic microorganisms, and higher plants (Wolanin, Thomason *et al.* 2002). It is a predominant strategy for bacteria to monitor stimuli and couple their adaptive response to the environmental changes, such as oxygen availability, nutrient limitation, phosphate limitation and osmolarity (Sivaneson, Mikkelsen *et al.* 2011, Zhou, Keller *et al.* 2011). TCS has a highly conserved core architecture with a histidine kinase protein domain and a cognate receiver domain, termed as the sensor and the response regulator respectively (Stock, Robinson *et al.* 2000). The inner-membrane spanning sensor can detect environmental stimuli and phosphorylate the cytoplasmic response regulator, which in turn activates a group of physiological responses by altering gene expression (Rodrigue, Quentin *et al.* 2000, Stock, Robinson *et al.* 2000).

*P. aeruginosa* genome encodes a large number of putative genes for TCSs. 64 histidine kinases and 72 response regulators were identified, these large numbers may explain the ubiquity of this organism (Rodrigue, Quentin *et al.* 2000, Chen, Chang *et al.* 2004). By regulating virulence processes and antibiotic resistance, TCSs contribute to the versatility of *P. aeruginosa*, permitting the pathogen to survive under diverse circumstances. Major TCSs in *P. aeruginosa* include PhoQ/PhoP, CzcS/CzcR, CopS/CopR, PmrB/PmrA, AlgZ/AlgR, FleS/FleR, PilS/PilR, GacS/GacA, RetS and LadS (Gooderham and Hancock 2009).

A global regulatory network composed of two levels of TCSs regulates T3SS in *P. aeruginosa*. The first-level key regulatory components are LadS and RetS. Both these proteins contain internal domains encoding a sensor histidine kinase and response regulator-like receiver domains, but neither *ladS* nor *retS* are genetically linked to a typical regulator (Laskowski and Kazmierczak 2006). RetS activates T3SS gene expression, while repressing the *pel* and *psl* loci related to biofilm formation. LadS functions exactly opposite to RetS (Ventre, Goodman *et al.* 2006). These two hybrid sensor kinases are proposed to be responsible for the transition between acute and chronic infections reciprocally (Goodman, Merighi *et al.* 2009).

The second-level TCS is the GacS/GacA system that interacts with LadS and RetS. GacA is a response regulator, while GacS is the sensor kinase. By forming a RetS/GacS heterodimer, the self autophosphrylation of GacS and the phosphorylation of GacA would be blocked (Bordi, Lamy *et al.* 2010). Phosphorylated GacA promotes the expression of two non-coding regulatory small RNAs (sRNAs), RsmY and RsmZ (Brencic, McFarland *et al.* 2009). RsmY and RsmZ sequester RsmA, the central post-transcriptional regulator of *P. aeruginosa* pathogenesis (Lapouge, Schubert *et al.* 2008). Hypothetically, RsmA may directly promote the activators or inhibit the anti-activators in the ExsADCE cascade; otherwise, it indirectly modulates the extrinsic pathway of T3SS. RsmA influences a group of virulence determinants such as rhamnolipid production, lipase production, swarming motility, quorum sensing, lectin, pyocyanin, hydrogen cyanide, and the type VI secretion system (Pessi,

Williams *et al.* 2001, Heurlier, Williams *et al.* 2004, Mulcahy, O'Callaghan *et al.* 2006, Brencic and Lory 2009).

In addition, PA1611 encodes another hybrid sensor kinase that may alter the RetS and GacS interaction, which was recently discovered (Kong, Chen *et al.* 2013). In response to environmental cues, the interaction between RetS and PA1611 could shift the balance between RetS and GacS through the release of GacS from the RetS-GacS heterodimer.

#### 1.2.2.4 PsrA-RpoS Pathway

PsrA, a long-chain fatty acid sensory regulator, induces the expression of T3SS genes through its DNA-binding capacity. It can bind to the promoter region of *exsCEBA* operon directly, but high PsrA concentration may be required (Shen, Filopon *et al.* 2006). Alternatively, PsrA binds to the promoter region of *rpoS* to enhance the transcription of RpoS. RpoS is a stationary phase sigma factor which negatively affects T3SS (Hogardt, Roeder *et al.* 2004).

#### 1.2.2.5 Other Factors

The expression of T3SS genes is also influenced by osmolarity, DNA damage and metabolism.

The salt effect has been attributed to osmolarity, where a high salt concentration is capable of triggering T3SS toxic effector ExoS production (Hornef, Roggenkamp *et al.* 2000). Rietsch and colleagues indicated that maximal expression of T3SS genes occur when extracellular salt concentration is elevated to 200 mM (Rietsch and Mekalanos 2006). Similarly, microarray analysis shows T3SS related genes is induced in response to hyperosmotic shock (Aspedon, Palmer *et al.* 2006). Osmolarity and intracellular cAMP levels may be linked and may work together in T3SS regulation (Rietsch and Mekalanos 2006).

A newly discovered SOS-induced product, PtrB, has been identified to suppress T3SS gene expression (Wu and Jin 2005). SOS responses, which are the global responses based on DNA damage, cause cell cycle arrest, induction of DNA repair and synthesis of pyocins (McKenzie, Harris *et al.* 2000, Michel-Briand and Baysse 2002).

Signals that stimulate the overproduction or reduction of specific metabolites can affect T3SS expression. Depletion of pyruvate dehydrogenase in *aceA* or *aceB* mutant, as well as the lack of a glucose transport regulator gene *gltR* expression render the repression of T3SS genes under inducing conditions (Dacheux, Epaulard *et al.* 2002, Wolfgang, Lee *et al.* 2003). Similar results were observed with the over-expression of genes encoding multiple drug resistance efflux pumps or genes related to histidine transport and metabolism (Rietsch, Wolfgang *et al.* 2004, Linares, López *et al.* 2005). The regulation of T3SS gene expression by metabolic imbalance may be derived from

acetyl-CoA according to a recent report (Rietsch and Mekalanos 2006).

After entering the host environment, the pathogen experiences a temperature increase, with respect to the previous inhabited surrounding. T3SS virulence gene expression is induced by the increasing temperature in *Shigella* spp. and *Yersinia* spp. (Portnoy, Moseley *et al.* 1981, Maurelli, Blackmon *et al.* 1984). The high concentration of extracellular $Cu^{2+}$ is also a signal for T3SS regulon repression (Ha, Kim *et al.* 2004).

### 1.2.3 Reciprocal Regulation of T3SS

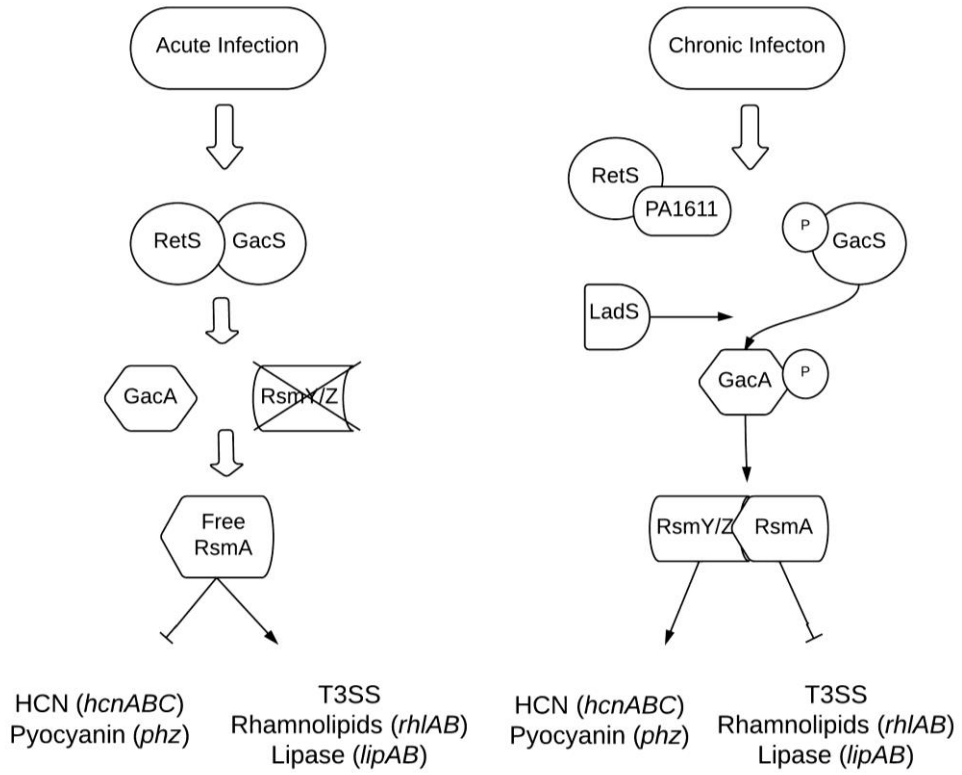
*P. aeruginosa* often undergoes a conversion from acute infection to chronic infection (Yahr and Greenberg 2004, Jain, Bar-Meir *et al.* 2008). In the well-established acute infection, *P. aeruginosa* causes tissue damage, sepsis and mortality (Engel and Balachandran 2009). On the other hand, for chronic infection in cystic fibrosis lungs, *P. aeruginosa* shows different traits such as biofilm formation, minimal tissue damage and restriction to the airways (Gómez and Prince 2007). Biofilm formation versus expression of the T3SS has been described as the hallmark characteristics of *P. aeruginosa* living styles (Kuchma, Connolly *et al.* 2005, Furukawa, Kuchma *et al.* 2006).

The genetic switch between acute and chronic infections is determined by the RetS/LadS/GacS/GacA pathway (Figure 1.4). Stimulated by unknown signals in favor

of acute infections, RetS is activated and a series of signals transductions are initiated. As a result, free RsmA level is relatively high. Modulated by two redundant small RNAs, RsmY and RsmZ, RsmA is a global post-transcriptional regulator that affects the expression of many genes in *P. aeruginosa* (Heeb, Kuehne *et al.* 2006, Brencic and Lory 2009). It promotes actin depolymerization, cytotoxicity, and anti-internalization of *P. aeruginosa* by positively regulating the virulence-associated T3SS (Mulcahy, O'Callaghan *et al.* 2006). It also negatively affects quorum sensing circuitry directly or indirectly (Pessi, Williams *et al.* 2001).

However, this kind of reciprocal regulation of T3SS and biofilm formation is not absolute. *P. aeruginosa* small colony variants (SCVs) isolated from chronically infected CF patients not only have increased antibiotic resistance, auto-aggregative growth behavior, and an enhanced ability to form biofilms, but also show up-regulated T3SS gene expression and cytotoxicity (von Götz, Häussler *et al.* 2004).

**Figure 1.4 RetS/LadS/GacS/GacA signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *P. aeruginosa.***




Environmental signals in acute infection favor activation of RetS and consequent elevation of free RsmA protein levels. T3SS, rhamnolipids and lipase production would be activated. A physiological equivalent of this shift occurs in chronic infection; PA1611 is expressed and interacts with RetS, leaving GacS to form homodimers and to phosphorylate GacA. LadS also promotes the phosphorylation of GacA. Phosphorylated GacA activates the small regulatory RNAs, RsmY and RsmZ, which cause the sequestration of RsmA. This figure was modified from the originally published works by Goodman *et al.* and by Kong *et al*. (Goodman, Kulasekara *et al.* 2004, Kong, Chen *et al.* 2013).

### 1.2.4 T3SS and CF Lung Infection

Mutation in the gene named cystic fibrosis transmembrane conductance regulator (CFTR) is the cause of cystic fibrosis (Kerem, Rommens *et al.* 1989). Abnormal CFTR function results in impaired mucociliary clearance, defective innate immunity, and infection of the respiratory tract by poly-microorganisms. Typically, infant patients are rapidly colonized by *Haemophilus influenzae* or *Staphylococcus aureus*, whereas *Pseudomonas aeruginosa* appears in teenage years, and becomes predominant afterwards (Rajan and Saiman 2002).

During the colonization of *P. aeruginosa* in CF patients' lungs, certain genomic modifications occur consistently to alter specific virulence traits. At the early stage, where the bacterial colonization has just occurred, isolated strains are entirely motile and express full-length lipopolysaccharide (LPS) side chains. In contrast, strains isolated from the chronic stage are deficient in LPS and are mucoid due to exopolysaccharide alginate overproduction (Goldberg and Pier 1996). Genetic adaptations, such as *mucA*, *exsA*, *vfr* and *cyaB* mutant accumulation, also contribute to the chronic colonization of *P. aeruginosa* in CF lungs (Smith, Buckley *et al.* 2006). Compared to a high percentage of type III secretion positive phenotypes ($T3SS^+$) isolated from patients with hospital-acquired pneumonia, bacteremia or the environment, CF patients have a strikingly low prevalence of $T3SS^+$ persistence (Roy-Burman, Savel *et al.* 2001, Hauser, Cobb *et al.* 2002, Berthelot, Attree *et al.* 2003). Accumulated evidences indicate that type III secretion gradually changes

during infection (Jain, Ramirez *et al.* 2004).

Since serum harbors antibodies against T3SS components, *P. aeruginosa* is thought to express the immuno-proteins during CF lung colonization (Moss, Ehrmantraut *et al.* 2001, Banwart, Splaingard *et al.* 2002). PcrV is assessable at the tip of the needle complex, as it is required for translocation (Goure, Pastor *et al.* 2004, Mueller, Broz *et al.* 2005). In animal models of *P. aeruginosa*-induced lungs, the anti-PcrV immunoglobulin G reduces the inflammatory reaction in the respiratory disease and greatly increases survival, seemingly by a direct barricade of T3SS injection behavior (Sawa, Yahr *et al.* 1999, Imamura, Yanagihara *et al.* 2007). The approach by engineering human anti-PcrV Fab fragments has been facilitated clinically (Baer, Sawa *et al.* 2009). Suggesting that T3SS can be a potential vaccine candidate, early prevention of *P. aeruginosa* with high T3SS activity may improve the clinical outcome for CF treatment (Engel and Balachandran 2009).

### 1.3 Interspecies Interaction

Infectious disease is the outcome of the interactions between pathogen and the host, which has been the focus of intensive studies. Not only do the invading bacteria make contact with host cells, but they also inevitably interact with the resident microflora in the host environment. However, knowledge of this interaction is limited to the protective roles of the resident microflora against pathogen invasion (Tancrède 1992).

CF airway disease is characterized by stable periods of stability and intermittent acute exacerbations that cause irreversible lung damage. Besides *P. aeruginosa*, which colonizes above 80% of patients by early adulthood, other pathogens such as *Staphylococcus aureus*, *Burkholderia cepacia*, *Haemophilus influenzae* and other respiratory viruses also cause periodic infections (Hart and Winstanley 2002, Rajan and Saiman 2002, Soni, Marks *et al.* 2002). A group of non-pathogenic, indigenous oropharyngeal flora (OF) is also present along with *P. aeruginosa* in the lung of adult CF patients (Coenye, Goris *et al.* 2002).

Animal experiments have been done, which demonstrated that increased lung damage was due to the presence of OF, not the *P. aeruginosa* load. Therefore, OF seem to contribute to disease progression, while enhancing the pathogenicity of *P. aeruginosa*. The up-regulation of a considerable number of virulence genes in *P. aeruginosa* by OF strains is a probable explanation of these observations. It has been reported that the T3SS in *P. aeruginosa* is affected by *Staphylococcus* spp. (Duan, Dammel *et al.* 2003). Interestingly, a recent study points out that the probiotic *Saccharomyces boulardii* affects the T3SS in *Citrobacter rodentium* (Wu, Vallance *et al.* 2008), which suggests T3SS may be involved in interspecies interaction.

As the communication between pathogen and host may determine the evolution of the virulence determinants, it is conceivable that interactions among microbes have the similar contribution (Cotter and DiRita 2000). Bacteria are believed to rely on

interspecies interaction to maintain their virulence, when adapting into diverse niches without hosting. Given that *P. aeruginosa* usually lives in a non-host environment, the process of interspecies communication may have a function in preventing the loss of T3SS.

The traditional investigation approach involves bacterial isolation under defined laboratory conditions on the subjects of pathogenesis. Nevertheless, for polymicrobial infectious diseases, the interactions among various causative agents in a microbial community often result in new physiological functions. Apparently, the molecular mechanisms of synergistic virulence can hardly be verified through pure culture. New approach, in light of this, is necessary to be designed to reach the underlying mechanism of interspecies interaction.

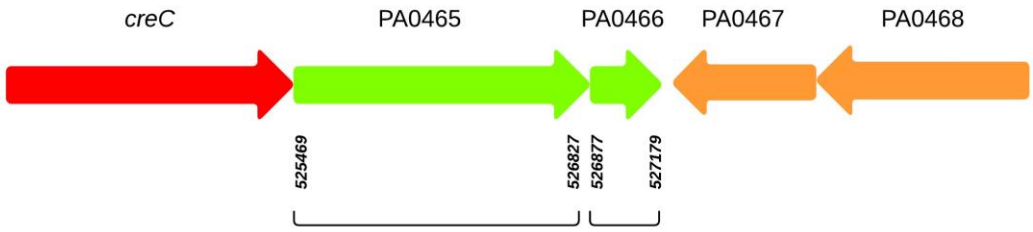
### 1.4 Putative T3SS Regulator PA0465

T3SS plays a key role in *P. aeruginosa* acute infection. The alteration of T3SS genes expression may significantly influence disease progression. In order to obtain a complete picture of the T3SS regulatory pathway, a *P. aeruginosa* transposon insertion library of about 20,000 clones was constructed in our laboratory and screened for genes involved in T3SS regulation using the T3SS effector gene *exoS* as a reporter (Kong, Chen *et al.* 2013). A strain carrying the *exoS-luxCDABE* reporter on the chromosome was constructed and used in the screening, and transposon mutants with altered transcription of *exoS* were selected by observing changes in light

production. The sites of transposon insertions in the selected mutants were determined by semi-arbitrary primed PCR and subsequent sequencing of the PCR products. Among the mutants obtained, a decreased expression of *exoS* was observed in transposon mutant F9, suggesting that the genes affected may play a role in the regulation of T3SS. The disrupted gene has been identified as PA0465 (Kong 2013).

Located in the PA0456-0466 operon, PA0465 is 1358 bp in length (Figure 1.5). It is also called *creD*, shares homology with a well-studied gene in *E. coli*. Also known as *cet*, *creD* was previously predicated to encode an inner membrane protein in *E. coli* responsible for the tolerance of colicin E2, however, its actual function remains to be revealed (Cariss, Constantinidou *et al.* 2010). PA0465 encodes protein CreD in *P. aeruginosa*, and the function of this protein is to be defined.

**Figure 1.5 The location of PA0465-0466 on *P. aeruginosa* genome.**




The gene PA0465 is a positive strand located at 525469 – 526827 and the gene PA0466 is a positive strand located at 526877-527179. This figure was drawn according to the information in the Pseudomonas Genome Database (Winsor, Lam *et al.* 2011).

## 1.5 Hypothesis and Objectives

According to the prior observations that T3SS gene expression was decreased in F9 strain, a PA0465 knock-out mutation has been constructed from a PAO1 strain, carrying an *exoS-lux* reporter fused on the chromosome, in order to prove its putative function on T3SS regulation (Zhao, Kong *et al*. 2013). However, gene expression measurements indicate that the PA0465 mutant did not affect *exoS* expression (data not shown). Considering the fact that the transposon harbors an outward directed promoter, we presumed that the decreased *exoS* expression in the F9 transposon mutant is due to the enhanced expression of the downstream gene PA0466, rather than the inactivation of PA0465 or PA0466. In summary, our hypothesis is that PA0466 plays a role in the regulation of T3SS gene expression.

On the other hand, living in a biofilm community and a non-host environment, *P. aeruginosa* still possess T3SS. Our hypothesis is that T3SS plays a role in bacterial interspecies interaction, which can be an ecological function that prevents the system from being lost in a non-host environment, where *P. aeruginosa* is mostly found.

Based on the hypotheses above, two objectives are proposed:

Objective I. Characterization of the Regulatory Function of PA0466

Objective II. Identification of the Potential Role of T3SS in Interspecies Interaction

# CHAPTER II. MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Bacteria, Plasmids and Primers

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

**Table 2.1 Bacterial strains and plasmids used in this study.**

| Strain or plasmid | Relevant characteristics | Source |
|---|---|---|
| *E. coli* | | |
| DH5α | *F⁻φ80lacZ ΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 ($rk^-$, $mk^+$) phoA supE44 thi-1 gyrA96 relA1 tonA* | Invitrogen |
| SM10-λ*pir* | Mobilizing strain, RP4 integrated in the chromosome; $Kn^r$ | (Simon, Priefer *et al.* 1983) |
| *Staphylococcus* spp. | | (Duan, Dammel *et al.* 2003) |
| *P. aeruginosa* | | |

| | | |
|---|---|---|
| PAO1 | Wild type | This lab |
| Δ*exsA* | *exsA* knockout mutant of PAO1; $Gm^r$ | This lab |
| Δ*exsE* | *exsE* knockout mutant of PAO1 | This study |
| Δ*exoS* | PA4594 knockout mutant of PAO1; $Gm^r$ | This lab |
| Δ*exoT* | PA3019 knockout mutant of PAO1; $Gm^r$ | This lab |
| Δ*gacA* | *gacA* knockout mutant of PAO1; $Gm^r$ | This lab |
| Plasmids | | |
| pBT20 | Mini-Tn*M* delivery vector; $Gm^r$ | (Kulasekara, Ventre *et al.* 2005) |
| pEX18Tc | *oriT*$^+$ *sacB*$^+$ gene replacement vector with MCS from pUC18; $Tc^r$ | (Hoang, Kutchma *et al.* 2000) |
| pRK2013 | Broad-host-range helper vector; $Tra^+$, $Kn^r$ | (Ditta, Sharon *et al.* 1980) |
| pMS402 | Expression reporter plasmid carrying the promoterless *luxCDABE* gene; $Kn^r$, $Tmp^r$ | (Duan, Dammel *et al.* 2003) |
| pKD-*exoS* | pMS402 containing *exoS* promoter region; $Kn^r$, $Tmp^r$ | (Duan, Dammel *et al.* 2003) |
| pKD-*rhlA* | pMS402 containing *rhlA* promoter region; $Kn^r$, $Tmp^r$ | (Duan, Dammel *et al.* 2003) |
| CTX6.1 | Integration plasmid origins of plasmid Mini-CTX-*lux*; $Tc^r$ | This lab |

| | | |
|---|---|---|
| Mini-CTX-*luxCDABE* | Integration plasmid containing *attP* site for integration at chromosomal *attB* site; $Tc^r$ | (Hoang, Kutchma *et al.* 2000) |
| pAK1900 | *E. coli-P. aeruginosa* shuttle cloning vector carrying p*lac* upstream of MCS; $Ap^r$, $Cb^r$ | (Williams, Zlosnik *et al.* 2006) |
| pAK-0466 | pAK1900 with a 293 bp fragment of PA0466 between *Sal*I and *Hin*dIII; $Ap^r$, $Cb^r$ | This study |
| CTX-*exoS* | Integration plasmid, CTX6.1 with a fragment of pKD-*exoS* containing *exoS* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX-*exoT* | Integration plasmid, CTX6.1 with a fragment of pKD-*exoT* containing *exoT* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX-*exoY* | Integration plasmid, CTX6.1 with a fragment of pKD-*exoY* containing *exoY* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX-*exsC* | Integration plasmid, CTX6.1 with a fragment of pKD-*exsC* containing *exsCEBA* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX-*exsD* | Integration plasmid, CTX6.1 with a fragment of pKD-*exsD* containing *exsD-pscB-L* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |

| | | |
|---|---|---|
| CTX-*rsmA* | Integration plasmid, CTX6.1 with a fragment of pKD-*rsmA* containing *rsmA* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX-*rsmY* | Integration plasmid, CTX6.1 with a fragment of pKD-*rsmY* containing *rsmY* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX-*rsmZ* | Integration plasmid, CTX6.1 with a fragment of pKD-*rsmZ* containing *rsmZ* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX-*rhlA* | Integration plasmid, CTX6.1 with a fragment of pKD-*rhlA* containing *rhlA* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This study |
| CTX-*rhlI* | Integration plasmid, CTX6.1 with a fragment of pKD-*rhlI* containing *rhlI* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX-*rhlR* | Integration plasmid, CTX6.1 with a fragment of pKD-*rhlR* containing *rhlR* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX-*lasI* | Integration plasmid, CTX6.1 with a fragment of pKD-*lasI* containing *lasI* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |

| | | |
|---|---|---|
| CTX-*lasR* | Integration plasmid, CTX6.1 with a fragment of pKD-*lasR* containing *lasR* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX-*phzA1* | Integration plasmid, CTX6.1 with a fragment of pKD- *phzA1* containing *phzA1* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | This lab |
| CTX- *phzA2* | Integration plasmid, CTX6.1 with a fragment of pKD- *phzA2* containing *phzA2* promoter region and *luxCDABE* gene; $Kn^r$, $Tmp^r$, $Tc^r$ | 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 |

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.

**Table 2.2 Primers used in this study.**

| **Primer** | **Sequence (5'→3')** | **Restriction site** |
|---|---|---|
| PA0466-S | GAGaagcttGTGATGGTGCTGACCCGC | *Hin*dIII |
| PA0466-A | TATgtcgacGGAACAGCAGGCCGATCC | *Sal*I |
| PA1711-upstream-S | ACCgaattcCAGTCCTTCGTCCAGATG | *Eco*RI |
| PA1711-upstream-A | ATAggatccAGCGTCTTGGGACGGC | *Bam*HI |
| PA1711-downstream-S | TTggatccTGAGGTGCTGGATGCTGTTG | *Bam*HI |
| PA1711-downstream-A | GTTCGCCctcgagCAGAACATATACG | *Xho*I/*Sal*I |
| PAO1-S | GCCTGCCTGAAGATCGAGGAG | |
| PAO1-A | GCAACTGATGGGCATAGAGGATTC | |
| Δ*exsA*-S | GCCAGTGAATCCGTAATCATGGTC | |
| Δ*exsA*-A | GCAACTGATGGGCATAGAGGATTC | |

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

### 2.1.2 Culture Media, Chemicals and Bacterial Growth Conditions

1. Luria-Bertani (LB) Medium

Per 1 L: To 950 ml of deionized $H_2O$, add:

| | |
|---|---|
| Tryptone | 10 g |
| Yeast extract | 5 g |
| NaCl | 10 g |

The solutes were shaken 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. Pseudomonas Isolation Agar (PIA) Medium

Per 1 L: To 950 ml of deionized $H_2O$, add:

| | |
|---|---|
| PIA agar powder | 45 g |
| Glycerol | 20 ml |

The solutes were shaken 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.

3. Swarming Medium

Per 1 L: To 950 ml of deionized $H_2O$, add:

| | |
|---|---|
| Ager 0.5% | 5 g |
| Nutrient Broth | 8 g |

| | |
|---|---|
| Glucose | 5 g |

The solutes were shaken 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.

4. SOC medium

Per 1 L: To 950 ml of deionized $H_2O$, add:

| | |
|---|---|
| Tryptone 2% | 20 g |
| Yeast extract 0.5% | 5 g |
| NaCl 0.05% | 0.5 g |

The solutes were shaken 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. Just before use, 20 ml of a sterile solution of 1 M glucose and 5 ml of a sterile solution of 2 M $MgCl_2$ were added.

5. Phosphate-Buffered Saline (PBS) buffer

Per 1 L:

| | |
|---|---|
| NaCl 137 mM | 8 g |
| KCl 2.7 mM | 0.2 g |

| | |
|---|---|
| $Na_2HPO_4$ 10 mM | 1.44 g |
| $KH_2PO_4$ 2 mM | 0.24 g |

In 800 ml of distilled $H_2O$, the pH was adjust to 7.4 with HCl. $H_2O$ was added to reach to a final volume of 1 L. The solution was dispensed into aliquots and sterilized by autoclaving for 20 minutes on liquid cycle. The buffer was stored at room temperature.

6. TAE 50×

| | |
|---|---|
| Tris base | 242 g |
| Glacial acetic acid | 57.1 ml |
| EDTA 0.5 M (pH 8.0) | 100 ml |

### 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

*Staphylococcus* spp., *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 $MgCl_2$ was used as a T3SS inducing medium (calcium-deplete).

Antibiotics were used at the following conditions: 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. For *E. coli*, kanamycin (Kn) at 50 μg $ml^{-1}$, ampicillin (Ap) at 100 μg $ml^{-1}$, chloramphenicol (Cm) at 25 μg $ml^{-1}$, Tc at 15 μg $ml^{-1}$ and Gm at 15 μg $ml^{-1}$ in LB.

### 2.2.2 *P. aeruginosa* Genomic DNA Isolation

E.Z.N.A. Bacterial DNA Kit (OMEGA bio-tek) was used in this study for *P. aeruginosa* 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 were 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 centrifuge tube, thereby disturbing the pellet. Incubating 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 inserted to a new 2 ml collection tube and 500 µl HBC buffer was added. After subjecting to centrifugation similar to the previous step, 700 µl DNA wash buffer was added and then centrifuged again. The wash step was repeated twice. The empty column was centrifuged at a maximum speed for 2 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

AxyPrep Plasmid Miniprep Kit (Axygen Biosciences) was used in this study for plasmid isolation. Cultures were grown overnight in media supplemented with the appropriate antibiotic. 1 ml of the overnight culture was pelleted in a centrifuge tube at room temperature, and then re-suspended in 250 µl ice-cold re-suspension buffer. 250 µl of lysis buffer was added, and the tube was gently mixed by inverting 4-6 times. 350 µl neutralization buffer was added within 5 minutes, and the solution was mixed by gently inverting 6-8 times, followed by a 10 minutes centrifugation at 12,000×*g* to clarify the lysate. An AxyPrep column was placed into an uncapped 2 ml centrifuge tube, and the clarified supernatant was transferred to the column followed by centrifugation at 12,000×*g* for 1 minute. 700 µl Desalting 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 again for drying. Pre-warmed elution buffer was used to purify the plasmid DNA by centrifugation. 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 8,000 rpm for 3 minutes at room temperature. The supernatant was discarded and the wash step was repeated once. After that, the cells were washed again with 1 ml 10% glycerol. The amount of 10% glycerol added was based on the concentration of competent cells, and 100 µl aliquots of the resulting solution were stored at -80°C.

### 2.2.5 Preparation of *E. coli* Competent Cells

In the process of chemical transformation, treatment of *E. coli* cells with the calcium cation induces competence and allows the cells to take up DNA from the environment. A tip with a few cells from a single DH5α colony was transferred to a tube with 3 ml of LB medium. The culture was then incubated for 18 hours at 37°C with agitation at 220 rpm. Afterwards, the culture was transferred into 50 ml of LB broth. The cells were further grown until the optical density reached 0.4. After cooling on ice for 10 minutes, cells were centrifuged at 5,000 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 left on ice for 10 minutes and centrifuged again. Once again, the pellet was gently re-suspended in 4 ml 100 mM $CaCl_2$ and left on ice for 30 minutes. After addition of 1 ml 80% glycerol, aliquots of 50 µl were transferred into 1.5 ml centrifuge tubes with a cut pipette tip. The aliquots were frozen immediately in liquid $N_2$ and stored at -80°C.

For electroporation, *E. coli* cells were grown overnight in LB broth at 37°C with agitation at 225 rpm. 100 ml of fresh LB broth was inoculated with 1 ml of inoculum from the overnight culture, and then incubated for 3 hours while shaking to the logarithmic phase. 50 ml of 3 hours culture was transferred to falcon tubes, and then centrifuged at 4,500 rpm for 7 minutes at 4°C.The cells were re-suspended in 25 ml 1 mM HEPES and centrifuged as the previous step. The supernatant was discarded and the process is repeated once more. After that, the cells were washed again with 1ml 10% glycerol. The amount of 10% glycerol added was based on the concentration of competent cells. At the end, 100 µl aliquots were stored at -80°C.

### 2.2.6 Transformation and Electroporation

#### 2.2.6.1 Transformation

DH5α competent cells were thawed on ice, to which 5 µl of ligation mixture was added. This mixture was mixed gently. After incubation on ice for 30 minutes, the mixture was heat-shocked for 20 seconds in a 42°C water bath without shaking, and the tubes were placed on ice for 2 minutes for cold shocking afterwards. 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. 20-200 µl of cell culture from each transformation was spread on pre-warmed selective media and the plates were

incubated overnight at 37°C. The remaining transformation reaction was stored at 4°C.

### 2.2.6.2 Electroporation

The transformation of *P. aeruginosa* by electroporation was described previously (Smith and Iglewski 1989). Electroporation cuvettes and centrifuge tubes were pre-chilled on ice. Electro-competent cells were thawed on ice and suspended by carefully flicking the tubes. 1 µl plasmid was added into the competent cells, and the entire 20 µl of solution was transferred to the cuvette gently to avoid bubbles. Once DNA was added to the cells, electroporation was carried out immediately by setting the electroporator to 2,500 V. 500 µl of 37°C pre-warmed SOC media were added into the cuvette, followed by a transfer to centrifuge tube. After culturing for about 1 hour at 37°C, the cells were spread on the pre-warmed stand-by plates and cultured overnight. The unused cells were stored at -80°C.

### 2.2.7 PCR

Polymerase chain reaction (PCR) is a method that uses a thermo stable DNA polymerase enzyme to amplify a 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 was denatured at 95°C,

while annealing occurred at a range from 55-65°C. Lastly, elongation was carried out at 72°C for 30 seconds per every 1 Kb of product expected. Ready PCR mix kit from AMRESCO was used to perform standard PCR and colony screening. Supplied as a 2× mixture of reaction buffer, AMRESCO's Extender *Taq* polymerase blend, dNTPs, electrophoresis tracking dye and a non-mutagenic EZ-Vision visualization dye was used.

The size of the PCR products was validated by running agarose gel electrophoresis, which was also used to separate the fragments from the template DNA, unincorporated nucleotides, polymerase and buffer salts. After PCR amplification, 1 µl of 10× DNA loading buffer was added to the reaction mixtures containing the amplified fragments, and then loaded on to the pre-made gel with 2-10 µl molecular weight marker (GeneRular$^{TM}$ 1 Kb DNA ladder, Fermentas). The gel was run at 80 V for 60 minutes. The results were visualized by UV light.

#### 2.2.7.1 Standard PCR Reactions

(1). Primers, template DNA, and Ready Master Mix were thawed on ice;

(2). The components were assembled on ice according to the Table 2.3;

(3). Standard PCR amplification was performed according to the Table 2.4.

**Table 2.3 Standard regular PCR reaction mix.**

| 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 |

**Table 2.4 Standard regular PCR program.**

| 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 |

### 2.2.7.2 Colony Screening

Bacteria originating from a single colony can be used directly as template for PCR because the initial de-naturating heat disrupts the cell walls and makes DNA accessible.

(1). Primers, template DNA, and Ready Master Mix were thawed on ice;

(2). The components were assembled on ice according to Table 2.5;

(3). Bacteria were added directly into the PCR mixture and the standard PCR amplification was performed according to 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 program 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 |

### 2.2.8 Digestion and Ligation

#### 2.2.8.1 Digestion

Restriction endonucleases are bacterial enzymes that cleave dsDNA at specific sites by recognizing specific short sequences and producing restriction fragments. A pair of enzymes (Thermo Scientific) can be used for the excision of the target DNA from plasmids or chromosomes, and also to create sticky overhangs of the PCR products for future ligation steps. The double digestion for plasmids or chromosomes was carried out at 37°C for 4 hours, while it was carried out at 37°C overnight for PCR

products. In this study, the restriction enzymes and corresponding buffers used are shown in the Table 2.7.

**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 | Tango Buffer 2× |
| PA1711 upstream fragment | *Bam*HI, *Eco*RI | Tango Buffer 2× |
| PA1711 downstream fragment | *Bam*HI, *Sal*I | *Bam*HI Buffer 1× |
| pAK1900 | *Hin*dIII,*Sal*I | Tango Buffer 2× |
| pAK-0466 | *Hin*dIII, *Sal*I | Tango Buffer 2× |
| pKD-*rhlA* | *Pac*I | *Pac*I Buffer 1× |
| CTX6.1 | *Pac*I | *Pac*I Buffer 1× |

### 2.2.8.2 Ligation

DNA ligases transfer an adenyl residue to the phosphate located at the 5’ terminus of one DNA molecule. It then becomes vulnerable to nucleophilic attack, which is to be conducted by hydroxyl groups on the 3’ terminus of neighboring DNA molecules in close proximity. T4 DNA ligase (Thermo Scientific) originated from bacteriophage T4 can be used for a wide range of ligation reactions of both cohesive and blunt DNA

termini. After assembly, the reaction mixture was incubated at room temperature for 1 hour and then overnight at 4°C. The amount of inserted DNA and vector DNA were calculated according to the recommended 4:1 molecular ratio, based on the DNA concentration measured with NanoDrop. The reaction mixture was assembled according to Table 2.8.

**Table 2.8 Standard ligation reaction.**

| Components | Volume (20 µl Reaction) |
|---|---|
| Linear Vector DNA | 20-100 ng |
| Insert DNA | 4:1 molar ratio over vector |
| 10× T4 DNA Ligase Buffer | 2 µl |
| T4 DNA Ligase | 1 u |
| Nuclease-Free Water | As needed |

### 2.2.9 DNA Purification

Fragments amplified by PCR can be purified directly from the PCR reaction or from the gel, after agarose gel electrophoresis. Gel/PCR Extraction Kit (Geneaid) was used for purification of the DNA fragments. For gel extraction, the correct sized amplified fragments were cut out of the gel with a scalpel, while being illuminated with UV-light. The agarose gel was dissolved and the polymerase enzyme was denatured

with guanidine thiocyanante, a chaotropic salt that interferes with hydrogen-bonds and other stabilizing non-covalent forces. Only the DNA bonded to the glass fiber matrix of the spin column, while other substances passed through. After the washing step, the DNA fragments were detached from the matrix and eluted with elution buffer.

### 2.2.10 Swarming Motility Test

The media used for the swarming motility assay consisted of 0.5% agar, 8 g/l nutrient broth mix, and 5 g/l glucose. The bacteria PAO1 (pAK1900), PAO1 (pAK-0466), Δ*gacA* (pAK1900) and Δ*gacA* (pAK-0466) were inoculated onto plates from the 2 µl aliquots taken directly from overnight LB (Cb250) cultures. After inoculation, plates were incubated at 37°C for 12-14 hours.

### 2.2.11 Protein Isolation

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. Following the removal of bacteria 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 finally visualized with Coomassie blue stain.

### 2.2.12 SDS-PAGE

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed as described by Laemmli (Laemmli 1970). It is a method used for separating proteins according to their sizes. The SDS-PAGE resolving gel (12%) and stacking gel (5%) were prepared freshly. The solution for the resolving gel was initially applied up to 1 cm beneath the combs and then sealed from surrounding air with $ddH_2O$ until the gel solidified. The $ddH_2O$ was removed and the stacking gel was cast on the top of the resolving gel with combs mounted on top.

Protein samples were boiled in the presence of loading buffer for 10 minutes prior to loading on 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 utilized, and electrophoresis was carried out at 100 V. Gels were 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 initially de-stained for 45 minutes. Afterwards, it was submerged in fresh de-stain solution overnight (7.5% acetic acid, 20% methanol). Typically, 50-100 µg protein samples were analyzed on mini-gels. The gels were made according to Table 2.9.

**Table 2.9 SDS-PAGE gels.**

| Components | SDS-PAGE Resolving Gel (12%) Per 10 ml | SDS-PAGE Stacking Gel (5%) Per 5 ml |
|---|---|---|
| Sterile Deionized $H_2O$ | 4.0 ml | 3.4 ml |
| Acrylamide Mix 30% | 3.3 ml | 0.83 ml |
| Tris 1.5 M (pH 8.8) | 2.5 ml | |
| Tris 1.0 M (pH 6.8) | | 0.63 ml |
| SDS 10% | 0.1 ml | 0.05 ml |
| Ammonium Persulfate 10% | 0.1 ml | 0.05 ml |
| TEMED | 0.004 ml | 0.005 ml |

### 2.2.13 Construction of PA1711 Deletion Mutant

For engineering the *exsE* deletion mutant, a *sacB*-based strategy was employed (Schweizer and Hoang 1995). To construct the *exsE*-null mutant (ΔPA1711), the upstream sequence (863 Kb) and downstream sequence (848 Kb) were amplified from PAO1 genomic DNA using the primers indicated in Table 2.2. The PCR products of the upstream sequence were digested with restriction enzymes *Bam*HI and *Eco*RI and cloned in tandem into vector No.1 pEX18Tc. The resultant plasmid with the linked DNA fragments was transformed into *E. coli* for large quality harvest. The

recombinant plasmid was isolated and digested with *Bam*HI and *Sal*I for use as vector No.2. The digested downstream fragment using the same restriction enzymes was sub-cloned into the vector No.2 and put through transformation, yielding plasmid pEX18Tc-ΔPA1711 into *E. coli*. Triparental mating was performed by using the helper strain *E. coli* with conjugative plasmid pRK2013, the donor strain *E. coli* (pEX18Tc-ΔPA1711) and the recipient strain PAO1, in order to replace the original gene piece with the mutant PA1711 on the PAO1 chromosome. The suicide plasmid pEX18Tc has a counter-selectable marker *sacB*, which allow for positive selection of true mutants from the more frequently occurring mero-diploids on medium containing 5% sucrose (Hoang, Karkhoff-Schweizer *et al.* 1998). The forward primer from the upstream sequence and the reverse primer from the downstream sequence were used to perform standard PCR for final confirmation, and the length of the correct PCR product was less than PA1711 control.

### 2.2.14 Triparental Mating

*P. aeruginosa* PAO1 recipient cells were grown overnight in LB broth, while the 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. The inoculum was collected in a falcon tube and centrifuged at 8,000 rpm for 3 minutes. The supernatant was discarded and the bacterial pellets were re-suspended completely using 1 ml PBS buffer. 1,000 μl of

each the recipient culture, the donor culture and helper culture were added to different centrifuge tubes, which were centrifuged at 8,000 rpm for 3 minutes. The supernatants from this process were discarded and the wash step was repeated twice. At the end, the weights of the cell pellets were measured.

SOC media was added with appropriate volumes according to the obtained weight for each strain. For *P. aeruginosa*, the final concentration was about 250 μg/μl, while for *E. coli* it was about 500 μg/μl. The cell suspensions were mixed with equal volumes. 25-30 μl of the mixtures, as well as PAO1 negative control were plated on LB plates, followed by overnight culturing. The following day, the developed pellets were collected in 500 μl SOC media, and 100-200 μl was spread on PIA (Gm150) plus 10% sucrose for selection. The final confirmation was carried out by PCR.

### 2.2.15 Biparental Mating

A broad host mobilizing strain SM10 containing plasmid CTX-*exoS* was grown in LB (Tc15) (Simon, Priefer *et al.* 1983). Δ*gacA* was grown in LB overnight at 37°C with agitation at 225 rpm. The inoculum was collected into falcon tubes and centrifuged at 8,000 rpm for 3 minutes. The supernatant was discarded and the bacterial pellets were re-suspended by 1 ml PBS buffer completely. 1,000 μl each of the recipient culture, the donor culture and the helper culture were added to centrifuge tubes for centrifugation at 8,000 rpm for 3 minutes, their respective supernatants were

discarded. The wash step was repeated twice. After that, the weights of cell pellets were measured.

SOC media was added with appropriate volumes according to the obtained weight for each strain. For *P. aeruginosa*, the final concentration was about 250 μg/μl, while it was about 500 μg/μl for *E. coli*. The cell suspensions were mixed with equal volumes, 25-30 μl of the mixtures, as well as PAO1 negative control were plated on LB plates, followed by overnight culturing. The next day, the developed pellets were collected in 500 μl SOC media, and 100-200 μl was spread on PIA (Gm150) plus 10% sucrose for selection. The final confirmation was carried out by PCR.

### 2.2.16 Over-expression of PA0466

The PA0466 gene was amplified by PCR from PAO1 genomic DNA using the primers GAGaagcttGTGATGGTGCTGACCCGC and TATgtcgacGGAACAGCAGGCCGATCC. Digested by *Hin*dIII and *Sal*I, PA0466 was engineered on the multi-copy-number *E. coli-P. aeruginosa* shuttle vector pAK1900 (Poole, Neshat *et al.* 1993), which has an origin replicon and multiple cloning sites, yielding the PA0466 over-expression plasmid pAK-0466.

### 2.2.17 Construction of CTX-*rhlA* Reporter

The promoter region of *rhlA* was cloned into the upstream *Bam*HI-*Xho*I site of the *lux* genes on pMS402, carrying a promoterless *luxCDABE* reporter gene cluster, in order to construct the promoter-*luxCDABE* reporter fusion (Duan, Dammel *et al.* 2003). The resultant plasmid pKD-*rhlA* was transformed into *P. aeruginosa* by electroporation.

An integration plasmid CTX6.1 originating from the plasmid mini-CTX-*luxCDABE* was used to construct a chromosomal fusion reporter (Becher and Schweizer 2000). This plasmid has all the elements required for integration, the origin of replication, and a tetracycline-resistance marker. Two steps lead to the Mini-CTX-mediated integration at the *attB* site: the integrase-mediated recombination and the Flp recombinase-mediated excision of unwanted plasmid sequences (Hoang, Kutchma *et al.* 2000).

The pKD-*rhlA* was isolated and digested by *Pac*I, as well as CTX6.1. After ligation and transformation, CTX-*rhlA* in *E. coli* was gotten. PAO1 (CTX-*rhlA*) was obtained using the triparental mating procedure in which the strain carrying the helper plasmid, pRK2013 was used in conjunction with the donor and recipient (Ditta, Sharon *et al.* 1980). The resultant plasmid was verified by restriction enzyme digestion and electrophoresis.

Since the target strain should have both the tetracycline and kanamycin resistances,

the PIA medium containing both antibiotics was used for selection. The single colony which carries the CTX-*rhlA* on its chromosome has chemi-luminescence emission.

### 2.2.18 Gene Expression Detection

The promoter fragments of selected genes were cloned into Mini-CTX-*luxCDABE* (Hoang, Kutchma *et al.* 2000), yielding different reporters, namely CTX-*exoS*, CTX-*exoT*, CTX-*exoY*, CTX-*exsC*, CTX-*exsD*, CTX-*phzA1*, CTX-*phzA2*, CTX-*pqsA*, CTX-*rhlA*, CTX-*rhlI*, CTX-*rhlR*, CTX-*lasI*, CTX-*lasR*, CTX-*rsmA*, CTX-*rsmY*, and CTX-*rsmZ*. These reporters were then integrated into the PAO1 or *gacA* mutant *att* site to obtain the reporter strains. pAK-0466 and the control pAK1900 have been transformed into those bacterial cells by electroporation.

Using the *lux*-based reporters, gene expression in liquid cultures was examined as counts per second (cps) of light production using a Synergy 2 Multimode Microplate Reader (Bio Tek). Expression was measured every 30 minutes for 24 hours. Bacterial growth was monitored at the same time by measuring the $OD_{600}$ in the Microplate Reader. Overnight cultures of the reporter strains were diluted to an optical density at 600 nm of 0.2 and cultivated for three additional hours before being used as inoculants. The cultures were inoculated into parallel wells on 96-well white plate with a transparent bottom. 5 µl of fresh culture was inoculated into the wells containing a total of 95 µl medium plus other components. 50 µl of filter-sterilized

mineral oil was added to prevent evaporation during the assay.

### 2.2.19 Competitive Index Assay

The overnight cultures of the three strains were collected and their respective $OD_{600}$ values were adjusted to the same value (around 0.20). The three groups were divided and the bacteria were mixed in 50 ml LB broth based on ratio. They were: control group: the ratio of wild type and mutant was 1:1; 1:1:1 group: the ratio of wild type, mutant and *Staphylococcus* was 1:1:1; and 1:1:10 group: the ratio of wild type, mutant and *Staphylococcus* was 1:1:10.

Twelve time points were tested through collected cultured samples, performing serial dilutions, and dropping 10 μl mixtures on PIA or PIA (Gm150). The zero hour point stands for the initial inoculum. Plated immediately after mixing, the bacteria dose and the relative population were confirmed as the input number. For the other time points, CFU was accounted, followed by a CI calculation based on the present data. The functions are listed below:

Initial inoculation: $R_I=CFU_{mutant}/CFU_{wt}=1:1$

Mixed inoculation: $R_M=CFU_{mutant'}/CFU_{wt'}$

$CI= R_I/ R_M$

### 2.2.20 Real-time PCR

All real-time PCR reactions were carried out in Real-time PCR System (MBI Lab Equipment). MBI EVOlution EvaGreen (R) qPCR master mixes kit from MBI Lab Equipment was used to perform real-time PCR. MBI hotstart DNA polymerase was activated by a 15 minutes incubation step at 95°C. This prevents extension of non-specific annealed primers and primer-dimmers formed at low temperatures during qPCR setup. EvaGreen is a DNA-binding dye with many features that make it a superior alternative to SYBR Green I for qPCR. Apart from having similar spectra, EvaGreen has three important features that set it apart from SYBR Green I: less PCR inhibition, extremely stability and is shown to be non-mutagenic and non-cytotoxic.

(1). Drawing the standard curve by performing real-time PCR

Wild type PAO1 and mutant Δ*exsA* were grown overnight in LB broth at 37°C with agitation at 225 rpm. 5 ml of fresh LB broth was inoculated with 100 µl inoculum from the overnight culture, and incubated for 3 hours with shaking. After $OD_{600}$ adjustment, two kinds of bacteria with the same $OD_{600}$ values were then collected in centrifuge tubes and washed with nuclease-free water several times. By using the PCR standard water to re-suspend the pellet, they were mixed together based on the certain ratios: 1:1, 1:2, 1:3, 1:4, 4:1, 3:1, and 2:1. The components were assembled on ice according to Table 2.10. Real-time PCR amplification was performed according to Table 2.11.

(2). Obtaining the bacterial ratio by performing real-time PCR

Wild type PAO1, mutant Δ*exsA* and *Staphylococcus* spp. were grown overnight in LB broth at 37°C with agitation at 225 rpm. The $OD_{600}$ of overnight cultures, PAO1, Δ*exsA* and *Staphylococcus* spp., were adjusted to the same value (around 0.20), and then mixed together in 50 ml LB broth media using different groups. Two groups were tested in this experiment: 1:1 group stands for the addition of 100 µl PAO1 and 100 µl Δ*exsA*; while the 1:1:10 group means 1000 µl of *Staphylococcus* were co-cultured with 100 µl PAO1 and 100 µl Δ*exsA*. Bacteria mixtures were cultured for a long time and 1 ml samples were collected to centrifuge tubes at four various times: 0 hour (starting point), 5 hours, 30 hours and 48 hours. Sample collection was followed, by cell washing 3 times by nuclease-free water. The components were assembled on ice according to Table 2.10. Real-time PCR amplification was performed according to Table 2.11.

**Table 2.10 Real-time PCR reaction mix.**

| Components | Volume (10 µl Reaction) |
|---|---|
| MBI EVOlution 5× EvaGreen qPCR Mix | 2 µl |
| Forward Primer | 0.25 µl |
| Reverse Primer | 0.25 µl |
| Bacteria Mix | 1 µl |
| Nuclease-Free Water | 6.5 µl |

**Table 2.11 Real-time PCR reaction program.**

| **Steps** | **Time (minutes)** | **Temperature (°C)** |
|---|---|---|
| Initial Denaturation | 15:00 | 95 |
| Denaturation | 0:10 | 95 |
| Annealing | 0:15 | 60 |
| Elongation | 0:15 | 72 |
| Repeat Steps Denaturation to Elongation 40× cycles | | |
| Melt Curve | 0:15 | 95 |
| | 0:15 | 55 |
| | 0:15 | 95 |

Note: For quantification experiments, a standard melt was performed that collected fluorescence data every 0.3°C during melting.

# CHAPTER III. RESULTS

## 3.1 Characterization of the Regulatory Function of PA0466

Previously in our lab, a transposon-insertion-mutant library of *P. aeruginosa* PAO1 was constructed and mutants with altered promoter activity of the T3SS effector gene *exoS* were collected (Kulasekara, Ventre *et al.* 2005, Kong, Chen *et al.* 2013). *exoS* is part of the type III secretion regulons, and its expression requires ExsA (Yahr, Hovey *et al.* 1995). The sites of transposon insertion in the selected mutants were determined by semi-arbitrary PCR and subsequent sequencing of the PCR products (Friedman and Kolter 2004, Liang, Li *et al.* 2008). One of the mutants, F9, exhibited decreased *exoS* promoter activity about five-fold, with PA0465 being identified as the affected gene (Kong, Chen *et al.* 2013).

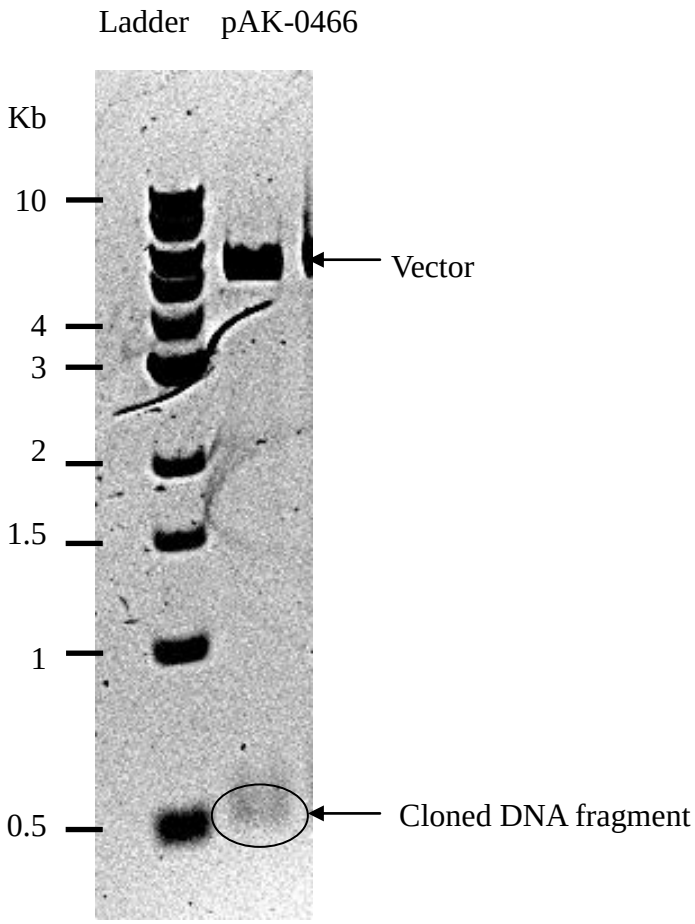
The observed effect may have resulted from the disruption of PA0465 or promoter affecting the downstream gene PA0466. However, because an outward *tac* promoter is present in the transposon pBT-20, the genes located downstream of the insertion site may have been over-expressed instead, which in turn causes the down-regulation of *exoS*. Previous study has ruled out the involvement of PA0465 in the down-regulation of T3SS, therefore, we decided to investigate the involvement of PA0466 in affecting *exoS* expression.

### 3.1.1 PA0466 over-expression represses T3SS

To verify that the over-expression of PA0466 was responsible for the decreased *exoS* expression, a PA0466 over-expression plasmid was generated. The amplified DNA fragment containing PA0466 was cloned into the *E. coli-P. aeruginosa* shuttle vector, pAK1900, under the control of a *lac* promoter (Poole, Neshat *et al.* 1993), yielding pAK-0466. As shown in Figure 3.1, restriction enzyme DNA digest confirmed that PA0466 had been cloned into the vector successfully.

The PA0466 over-expression strain, PAO1 (pAK-0466), was obtained by introducing pAK-0466 into PAO1 through electroporation. The effect of PA0466 over-expression on T3SS was checked using five T3SS genes. The five T3SS genes selected include *exoS*, *exoT*, *exoY*, *exsC* and *exsD*. Their promoter regions were cloned into Mini-CTX-*luxCDABE*. These genes were integrated into the PAO1 *att* site on the PAO1 genome, yielding different reporter strains (Table 3.1). The $Ca^{2+}$-specific chelator, EGTA, was added in the media to create a T3SS inducing condition of low calcium level (Chuang, Lin *et al.* 2013). The promoter activity was measured by the intensity of luminescence emission detected by a Microplate reader. Compared to those in the PAO1 (pAK1900) control strains, the expressions of all these genes were reduced in the PA0466 over-expression strains (Figure 3.2).

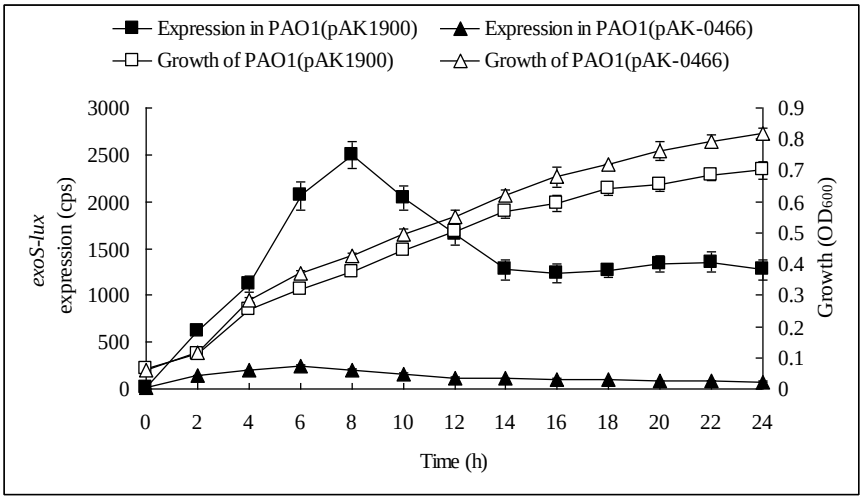
**Figure 3.1 Restriction enzyme digestion of pAK1900 vector containing the PA0466 DNA.**



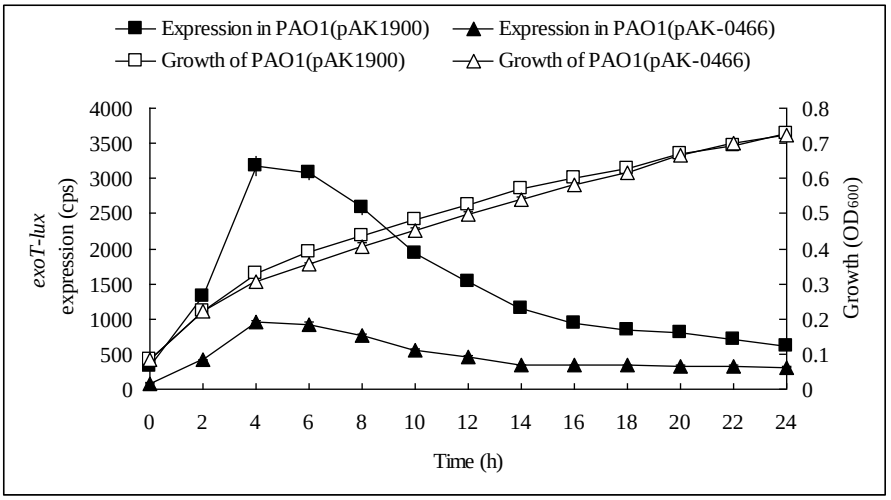

The plasmid pAK1900 harboring the PA0466 and renamed as pAK-0466 was digested by *Hin*dIII and *Sal*I. The correct subcloned DNA fragments are marked in the circle.

**Figure 3.2 The T3SS genes promoter activities in PA0466 expression strain.**

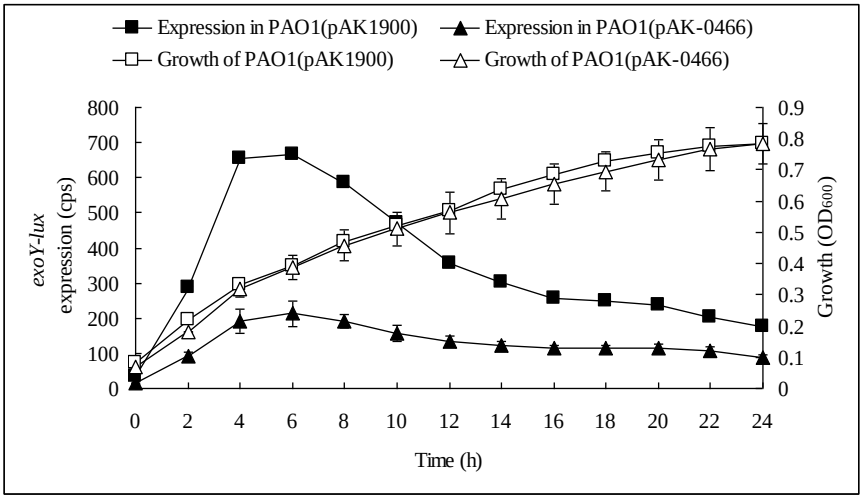
**A**



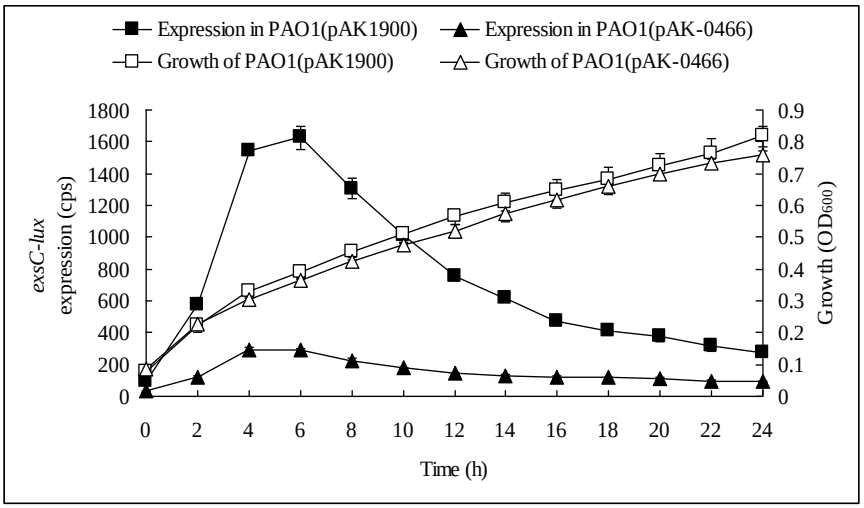

**B**



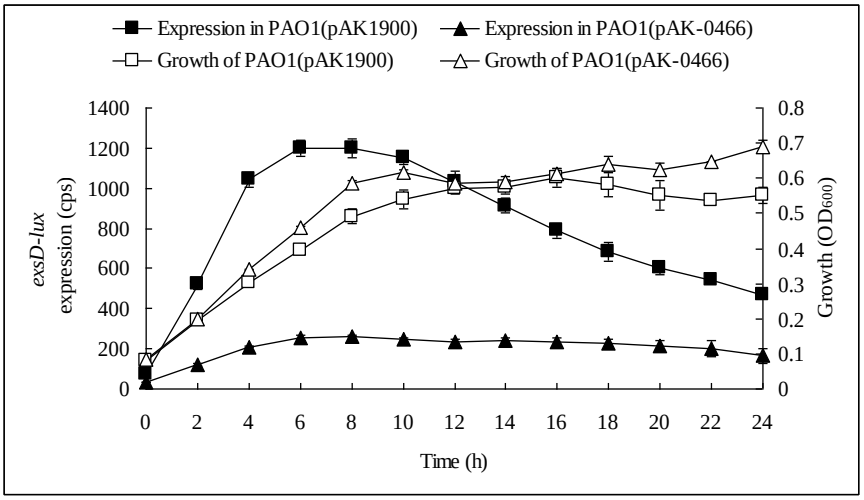

**C**



**D**




**E**



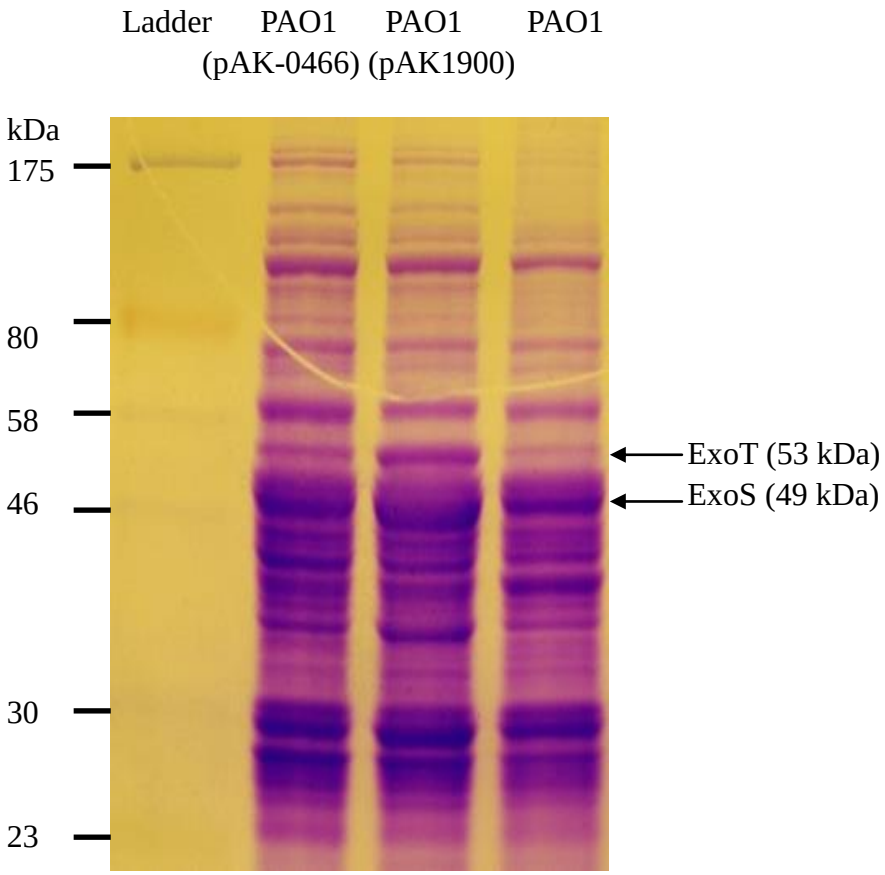

A-E: CTX-*exoS*, CTX-*exoT*, CTX-*exoY*, CTX-*exsC*, and CTX-*exsD* reporter fusions integrated on the chromosome were used to measure the promoter activity of *exoS*, *exoT*, *exoY*, *exsC* and *exsD*. The result represents the mean of triplicate experiments and the error bars indicate standard deviations.

The effect of PA0466 over-expression on T3SS was confirmed by examining the secretion of T3SS effectors under inducing conditions (Figure 3.3). Among the proteins secreted by the wild-type PAO1, two proteins correspond to the T3SS effectors, ExoT and ExoS, which are approximately 53 kDa and 49 kDa in size, respectively (Dacheux, Attree *et al.* 2001, Hauser 2009). The secretion of these proteins was reduced in the PA0466 over-expression strain PAO1 (pAK-0466), confirming that the expression of T3SS is down-regulated when PA0466 is over-expressed.

### 3.1.2 PA0466 over-expression increases *rsmY* and *rsmZ* expression

Along with T3SS genes, the promoter activities of the ten other virulence related genes of *P. aeruginosa* were also tested by using the *lux*-based reporter (Table 3.1). Eight genes proved to be unaffected, including *rsmA* (RNA-binding protein), *rhlI/R* (quorum sensing *rhl* system), *lasI/R* (quorum sensing *las* system), *phzA1/A2* (phenazine synthesis) and *pqsA* (PQS synthesis). However, the expression of two small regulatory RNA genes, *rsmY* and *rsmZ*, were increased in PA0466 over-expression strains (Figure 3.4). Both of them are GacA-dependent and control swarming ability (Kay, Dubuis *et al.* 2005, Kay, Humair *et al.* 2006).

**Figure 3.3 Effects of PA0466 over-expression on T3SS effector secretion.**



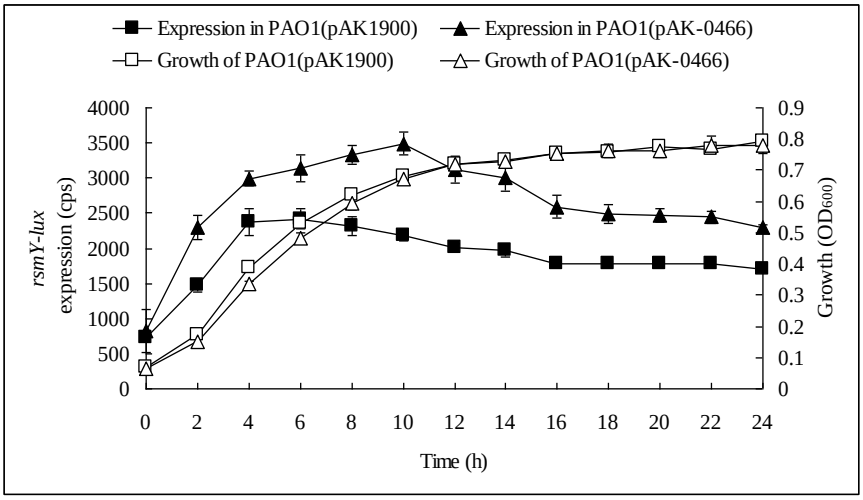

Culture supernatants of different strains after 6 hours of growth in T3SS inducing media were analyzed by 12% SDS-PAGE, followed by staining with Coomassie blue. Bands corresponding to T3SS effectors, ExoT and ExoS, are indicated by the arrows.

**Table 3.1 The reporter systems used in this project.**

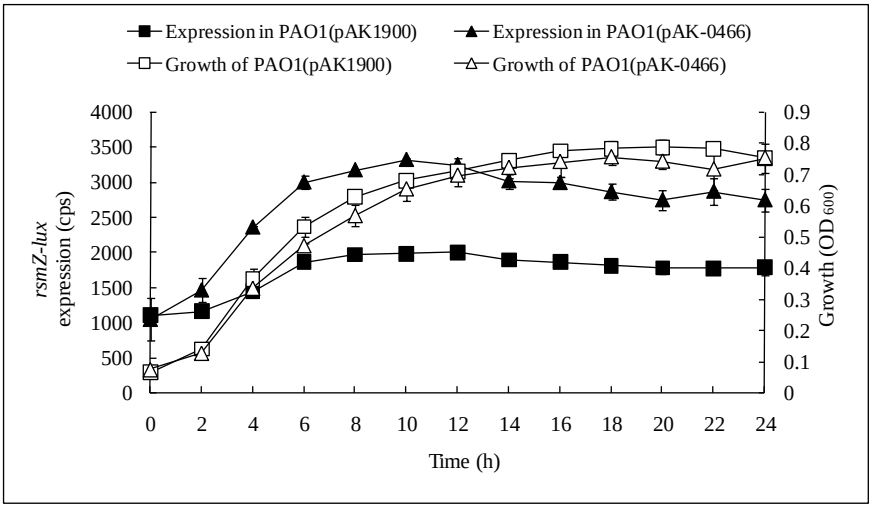
| **Target Gene** | **Reporters in PAO1** | **Protein/Gene Function** |
|---|---|---|
| *exoS* | CTX-*exoS-luxCDABE* | T3SS toxin |
| *exoT* | CTX-*exoT-luxCDABE* | T3SS toxin |
| *exoY* | CTX-*exoY-luxCDABE* | T3SS toxin |
| *exsC* | CTX-*exsC-luxCDABE* | T3SS regulator |
| *exsD* | CTX-*exsD-luxCDABE* | T3SS regulator |
| *phzA1* | CTX-*phzA1-luxCDABE* | Phenazine synthesis |
| *phzA2* | CTX-*phzA2-luxCDABE* | Phenazine synthesis |
| *rsmA* | CTX-*rsmA-luxCDABE* | Small RNA-binding regulatory protein |
| *rsmY* | CTX-*rsmY-luxCDABE* | Small RNA |
| *rsmZ* | CTX-*rsmZ-luxCDABE* | Small RNA |
| *rhlA* | CTX-*rhlA-luxCDABE* | Rhamnosyltransferase chain A |
| *rhlI* | CTX-*rhlI-luxCDABE* | Homoserine lactone synthase |
| *rhlR* | CTX-*rhlR-luxCDABE* | Quorum sensing regulator protein |
| *lasI* | CTX-*lasI-luxCDABE* | Homoserine lactone synthase |
| *lasR* | CTX-*lasR-luxCDABE* | Quorum sensing regulator protein |
| *pqsA* | CTX-*pqsA-luxCDABE* | PQS synthesis |

**Figure 3.4 The *rsmY* and *rsmZ* gene promoter activities in PA0466 expression strain.**

**A**




**B**




A and B: CTX-*rsmY* and CTX-*rsmZ* reporter fusions integrated on the chromosome were used to measure the promoter activity of *rsmY* and *rsmZ*. The result represents the mean of triplicate experiments and the error bars indicate standard deviations.

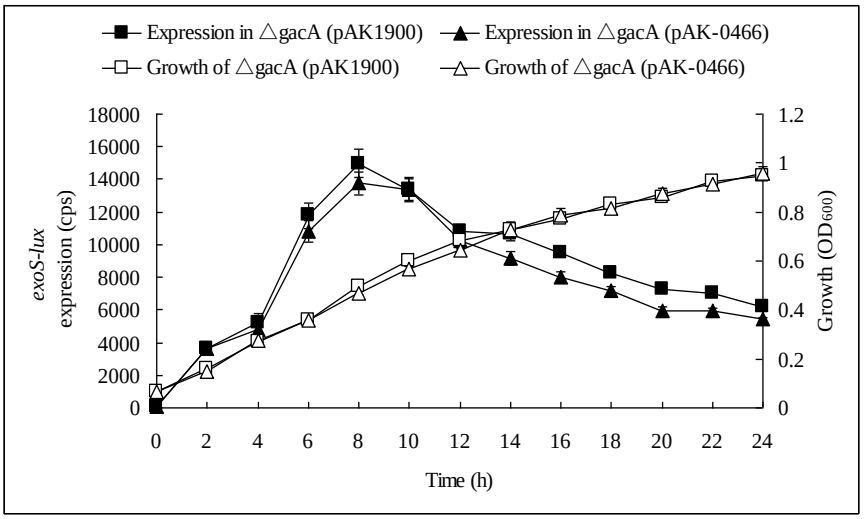
### 3.1.3 PA0466 does not affect T3SS in g*acA* mutant

As the expression of *rsmY* and *rsmZ* increased in the PA0466 over-expression strain, it is probable that the PA0466 functions through the GacA-RsmA system.

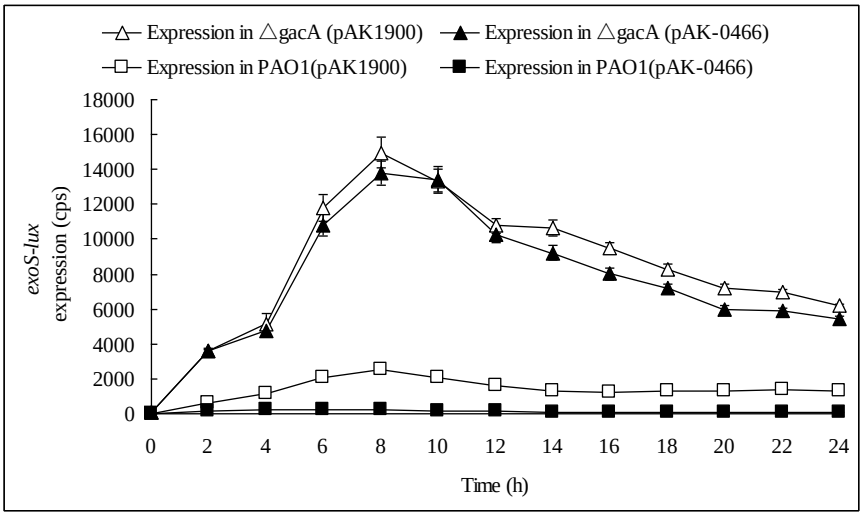
To address this possibility, the effect of PA0466 was tested in a *gacA* mutant background. CTX-*exoS* reporter was integrated on the chromosome of Δ*gacA*, and the PA0466 over-expression plasmid pAK-0466 and control pAK1900 plasmid were electroporated into the strain respectively, yielding Δ*gacA* (pAK-0466) and Δ*gacA* (pAK1900). As *gacA* negatively regulates *rsmA*, the *exoS* expression level, as expected, was much higher in the *gacA* mutant compared to the wild type (Figure 3.5). However, over-expression of PA0466 had no effect on *exoS* expression in the *gacA* mutant background, in contrast to the observation that it dramatically down-regulated *exoS* expression in the wild-type strain. Similarly, PA0466 expression had no effect on swarming motility in Δ*gacA* (Figure 3.6). Analysis of the secreted T3SS protein confirmed the results (Figure 3.7). These observations suggest that PA0466 might exert its regulation on the target genes through the GacA-RsmA pathway.

**Figure 3.5 Effect of *gacA* mutation on *exoS* promoter activity in PA0466 expression strain.**

**A**

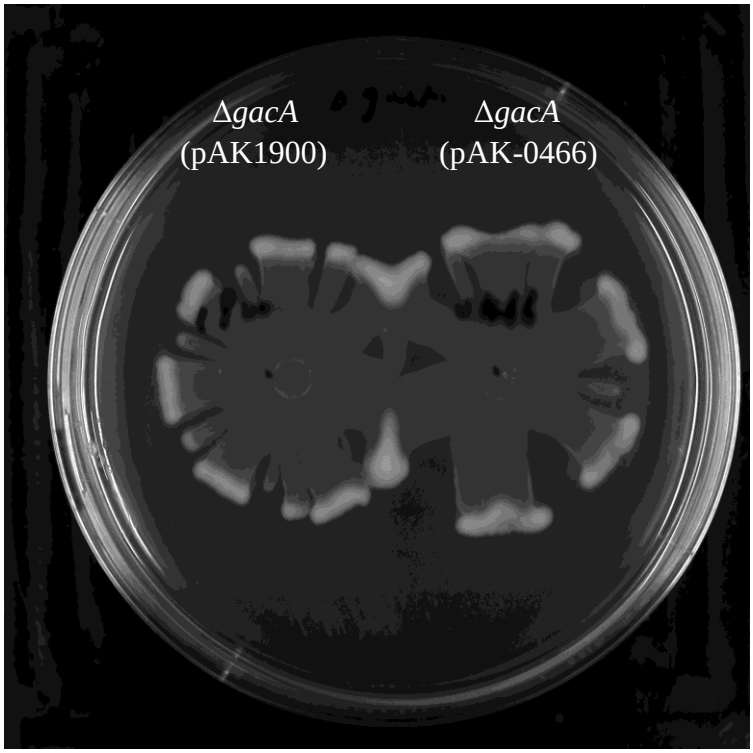



**B**



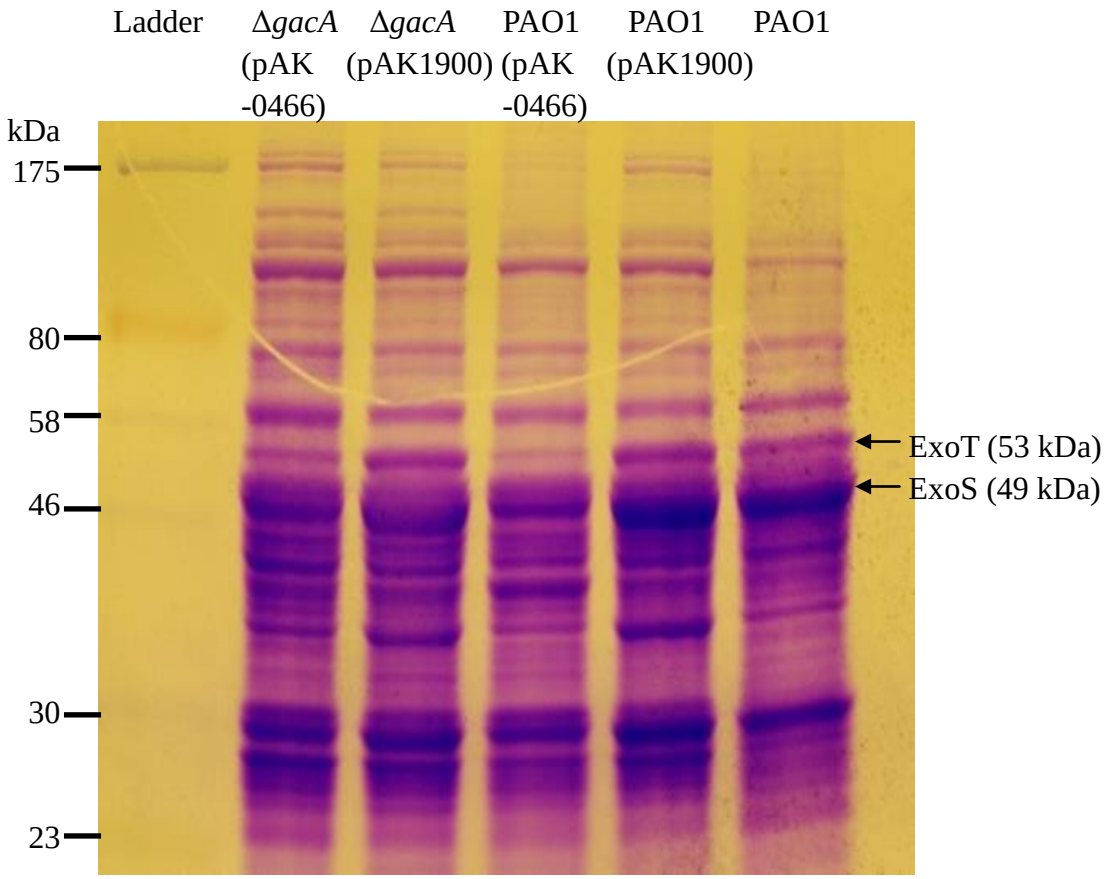

A CTX-*exoS* reporter fusion integrated on the chromosome was used to measure expression levels under inducing conditions. The result represents the mean of triplicate experiments and the error bars indicate standard deviations.

**Figure 3.6 Effect of *gacA* mutation on swarming motility in PA0466 expression strain.**




2 µl of overnight culture were spotted onto swarm plates. After inoculation, the plates were incubated at 37°C and images captured after 12-14 hours of growth. The experiments were repeated at least three times and similar results were observed.

**Figure 3.7 Effect of PA0466 over-expression in Δ*gacA* and PAO1 on T3SS effectors secretion.**



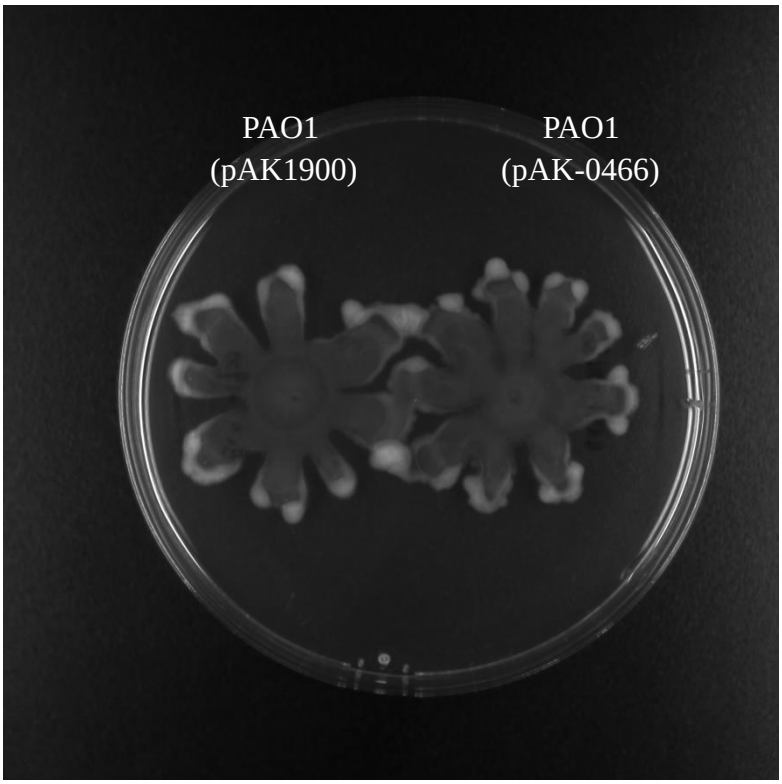

Culture supernatants of different strains after 6 hours of growth in T3SS inducing media were analyzed by 12% SDS-PAGE, followed by staining with Coomassie blue. Bands corresponding to T3SS effectors, ExoT and ExoS, are indicated by the arrows.

### 3.1.4 PA0466 affects T3SS but not swarming motility

It is known that swarming motility is under the control of RsmY and RsmZ. Based on this, it is expected that the increase of *rsmY/Z* gene expression by PA0466 over-expression should result in a change in swarming motility. However, as shown in Figure 3.8, no significant difference was observed between the PA0466 over-expression strain PAO1 (pAK-0466) and the control strain PAO1 (pAK1900).

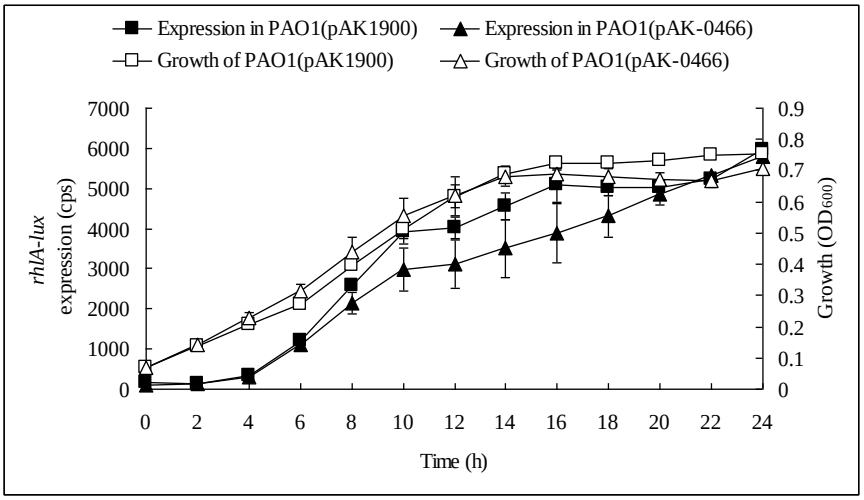
Rhamnolipid is required for swarming. To further test the effect of PA0466 on swarming, the expression of *rhlA* was tested with the PA0466 over-expression strain. *rhlA* encodes rhamnosyltransferase involved in rhamnolipid synthesis (Heurlier, Williams *et al.* 2004). The *rhlA-luxCDABE* reporter was integrated to the chromosome using the CTX system, which resulted to the CTX-*rhlA* reporter strain. The *rhlA* promoter activity was measured under the PA0466 over-expression background, and compared with the pAK1900 control (Figure 3.9). The results show that *rhlA* expression was not affected by the over-expression of PA0466, which is consistent with the unchanged swarming motility observed.

**Figure 3.8 Influence of PA0466 over-expression on swarming motility.**




2 µl of overnight culture were spotted onto swarm plates. After inoculation, the plates were incubated at 37°C and images captured after 12-14 hours of growth. The experiments were repeated at least three times and similar results were observed.

**Figure 3.9 The promoter activity of *rhlA* in PA0466 expression strain.**




CTX-*rhlA* reporter fusions integrated on the chromosome were used to measure the promoter activity of *rhlA*. The result represents the mean of triplicate experiments and the error bars indicate standard deviations.

## 3.2 Potential Ecological Role of T3SS

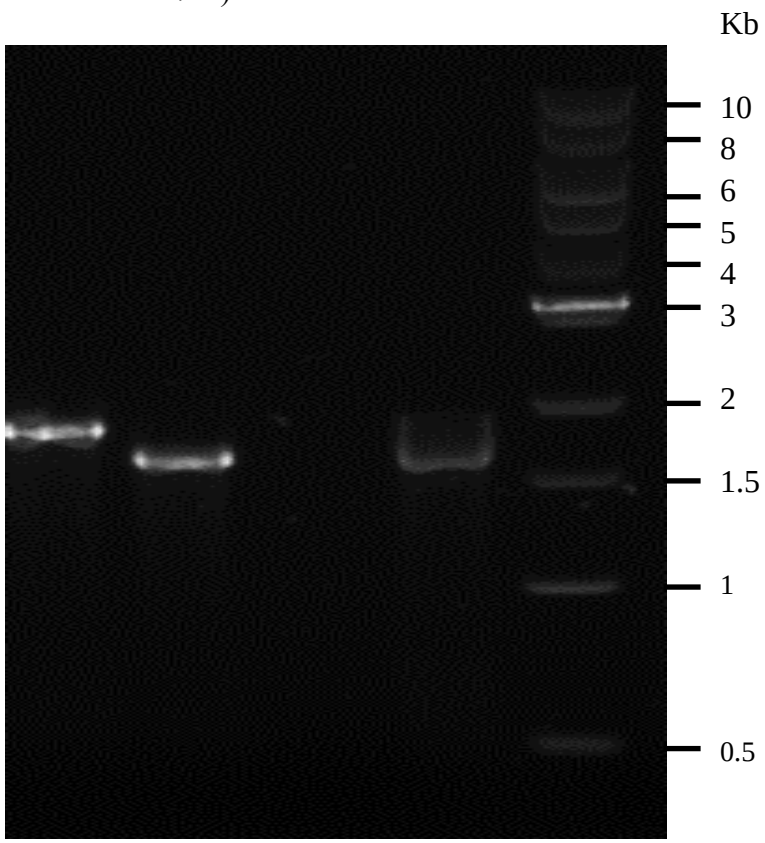
In the natural environment, diverse microbial species compete for nutritional resources. In addition, many studies have shown that the interspecies interaction between bacteria have profound impacts on the outcome of competition in nature (Hibbing, Fuqua *et al.* 2010).

It has been reported that the T3SS in *P. aeruginosa* is affected by the presence of other bacteria species, such as *Staphylococcus* spp. (Duan, Dammel *et al.* 2003). Since *P. aeruginosa* usually live in non-host environments, interspecies interaction may be important in preventing the loss of T3SS. In this study, T3SS mutants are used to probe the potential involvement of T3SS in the interaction between *P. aeruginosa* and *Staphylococcus* spp..

### 3.2.1 T3SS expressed at similar levels in *exsE* mutant and the wild type

To investigate the role of T3SS in interspecies interaction, Δ*exsA* and Δ*exsE* mutants together with Δ*exoS* and Δ*exoT* mutants were used. Δ*exsA* abolishes T3SS function (Dacheux, Attree *et al.* 2001), whereas Δ*exsE* has the opposite effect. The mutants Δ*exsA*, Δ*exoS* and Δ*exoT* are available in our lab, and *exsE* mutant was constructed in this study. Figure 3.10 shows the confirmation of the *exsE* deletion mutant by PCR.

**Figure 3.10 PCR analysis of the construction of PA1711 (*exsE*) deletion mutant.**

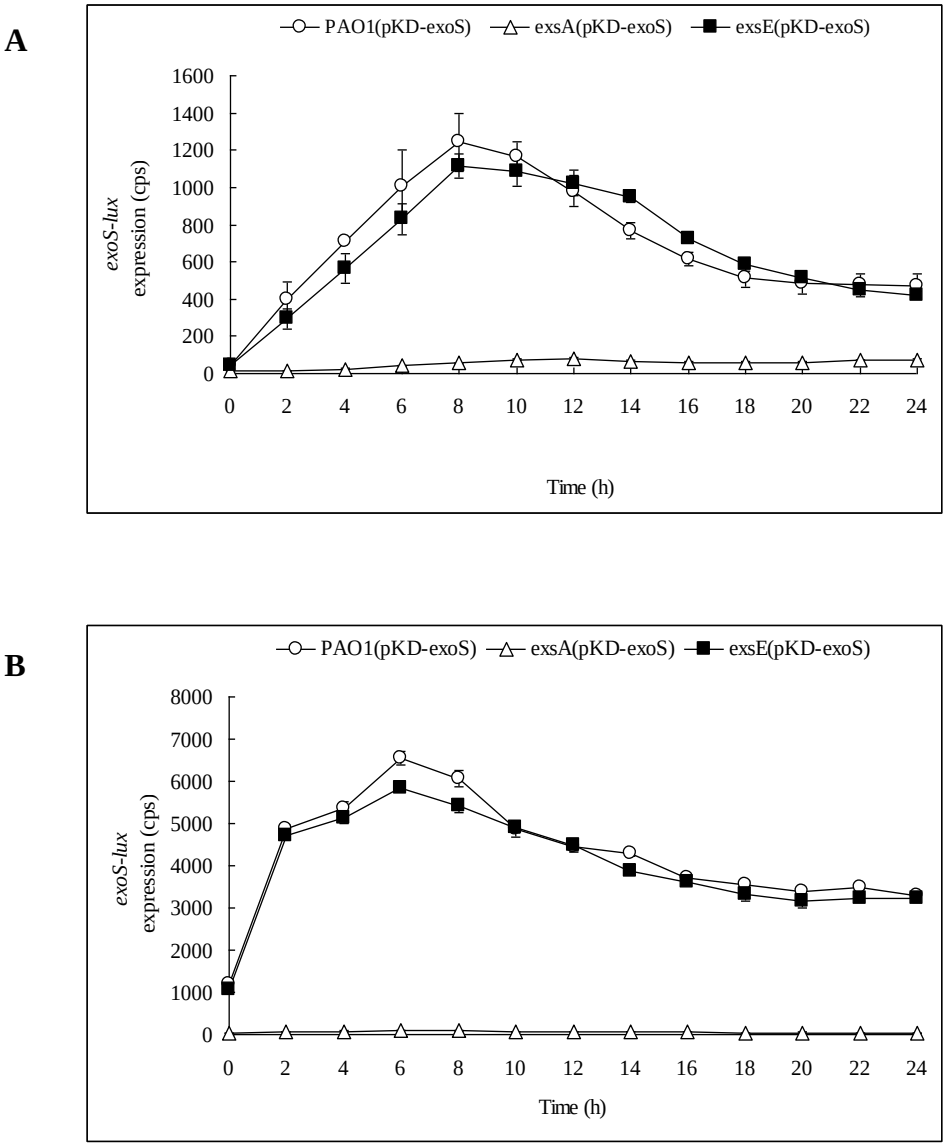



To confirm the successful construction of PA1711 deletion mutant, the length of the corresponded PCR product was compared with different controls.

One of the remarkable features of T3SS is the coupling of secretion with transcription. Stimulated by certain environmental signals, type III secretion channels are switched from an inactive state to an active state, permitting the release of ExsE (McCaw, Lykken *et al.* 2002). Through the construction of the PA1711 (*exsE*) deletion mutant, the resultant strain should have an activated T3SS phenotype, irrespective of the calcium level.

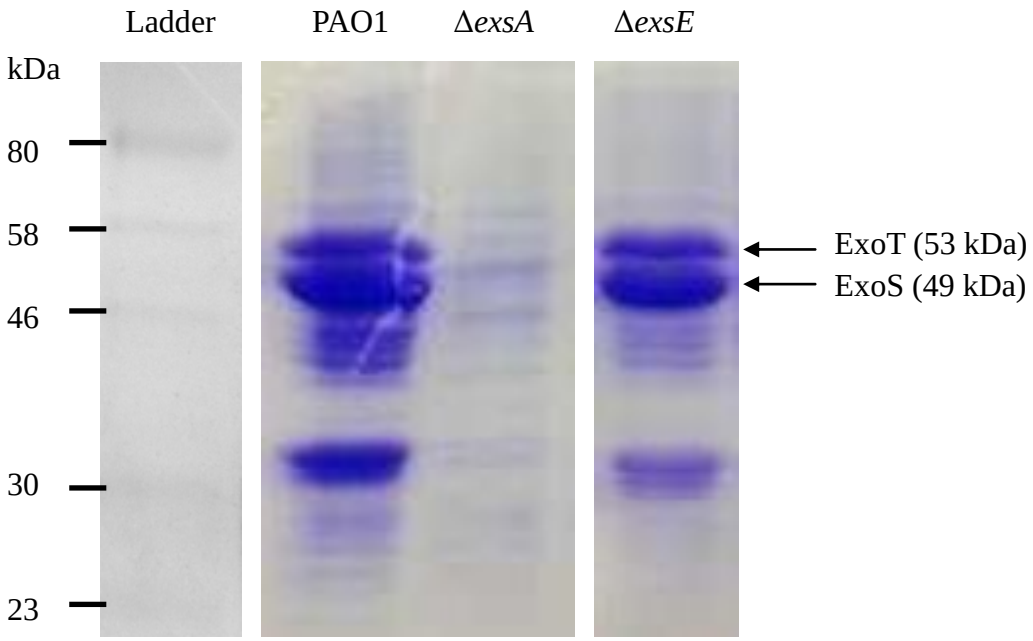
To verify the effect of the *exsA* and *exsE* mutants, the expression of *exoS* was compared in PAO1 (pKD-*exoS*), Δ*exsA* (pKD-*exoS*) and Δ*exsE* (pKD-*exoS*) under both non-inducing and inducing conditions. In agreement with findings in the literature, *exoS* expression in Δ*exsA* (pKD-*exoS*) was completely abolished. However, *exoS* expression unexpectedly reached similar levels in Δ*exsE* (pKD-*exoS*) and PAO1 (pKD-*exoS*) (Figure 3.11). The results were confirmed by protein secretion analysis. While Δ*exsA* produced no T3SS proteins, no difference in T3SS protein secretion was observed between PAO1 and Δ*exsE* (Figure 3.12). The result is somewhat different from what would be expected, as the absence of *exsE* would activate T3SS expression (Urbanowski, Lykken *et al.* 2005).

**Figure 3.11 The promoter activity of *exoS* in mutant and wild type strains.**




pKD-*exoS* reporters transferred into the bacteria cells by electroporation were used to measure the promoter activity of *exoS*. A: the *exoS* expression for each strain was detected under non-inducing condition; B: the *exoS* expression for each strain was detected under inducing condition. The result represents the mean of triplicate experiments and the error bars indicate standard deviations.

**Figure 3.12 Effect of e*xsE* mutation on T3SS effectors secretion.**




Culture supernatants of various strains after 6 hours of growth in T3SS inducing media were concentrated and analyzed by SDS-PAGE, followed by staining with Coomassie blue. Bands corresponding to T3SS effectors, ExoT and ExoS, are indicated by the arrows.

### 3.2.2 Comparison of competitiveness between PAO1 and T3SS mutants by mixed cultures and CFU counts

To compare the competitiveness between the wild type and the T3SS-related mutants in mixed cultures, co-culturing and subsequent cell counting were carried out with or without the addition of *Staphylococcus* spp.. Colony Forming Units (CFU) counting, a classical approach in the determination of bacterial cell numbers, was done by simply performing serial dilution, and viable bacterial number enumeration (Hazan, Que *et al.* 2012).

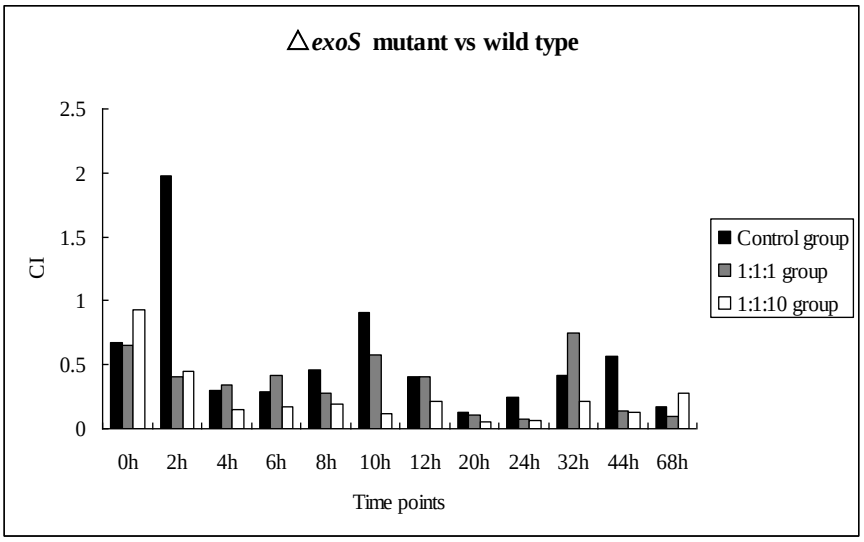
Mixed inoculums containing equal CFU of wild type PAO1 and mutant Δ*exoS* or Δ*exoT* were co-cultured with or without the addition of *Staphylococcus* spp.. During a 68 hours period of cultivation, bacterial samples were collected and the input and output mutant-to-wild type ratios were determined at different time points. Strains were distinguished using different antibiotic markers and selective media and CFU counted after serial dilution and plating. The competitive index (CI) was calculated (Table 3.2). Due to difficulties of accurately counting the different colonies after co-culturing, major variations were observed in this result (Figure 3.13), and no conclusion could be drawn from this experiment. It seems that such conventional method is not suitable for this experiment.

**Table 3.2 CI values of each group at different time points.**

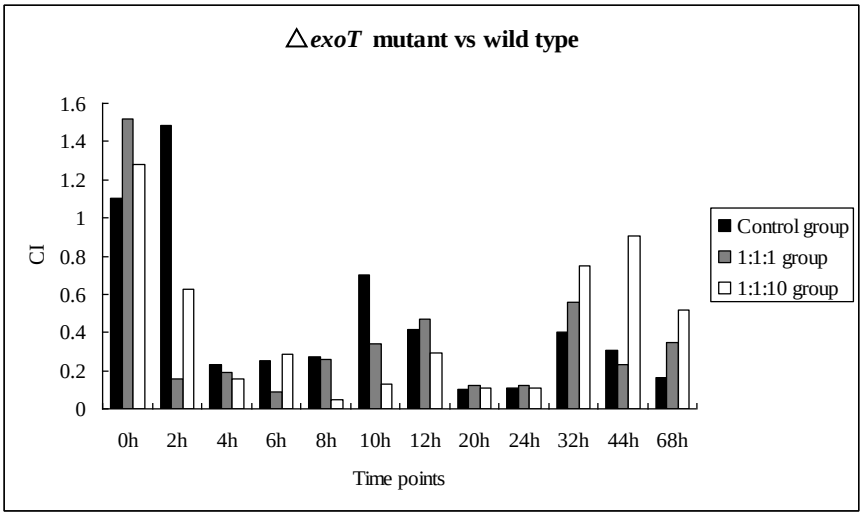
| CI / Time Points | Control group | | 1:1:1 group | | 1:1:10 group | |
|---|---|---|---|---|---|---|
| | **Δ*exoS*** | **Δ*exoT*** | **Δ*exoS*** | **Δ*exoT*** | **Δ*exoS*** | **Δ*exoT*** |
| **0 h** | 0.676 | 1.103 | 0.656 | 1.520 | 0.933 | 1.280 |
| **2 h** | 1.972 | 1.483 | 0.401 | 0.155 | 0.447 | 0.625 |
| **4 h** | 0.299 | 0.231 | 0.345 | 0.189 | 0.146 | 0.154 |
| **6 h** | 0.293 | 0.254 | 0.416 | 0.086 | 0.169 | 0.286 |
| **8 h** | 0.460 | 0.272 | 0.280 | 0.258 | 0.191 | 0.046 |
| **10 h** | 0.907 | 0.704 | 0.581 | 0.339 | 0.118 | 0.127 |
| **12 h** | 0.411 | 0.418 | 0.407 | 0.470 | 0.214 | 0.293 |
| **20 h** | 0.125 | 0.104 | 0.108 | 0.125 | 0.058 | 0.111 |
| **24 h** | 0.244 | 0.111 | 0.079 | 0.124 | 0.068 | 0.111 |
| **32 h** | 0.417 | 0.400 | 0.750 | 0.556 | 0.214 | 0.750 |
| **44 h** | 0.571 | 0.304 | 0.136 | 0.234 | 0.124 | 0.905 |
| **68 h** | 0.176 | 0.163 | 0.101 | 0.348 | 0.275 | 0.516 |

**Figure 3.13 The CIs of each group over the time course.**

**A**




**B**




A: CIs of co-culturing Δ*exoS* with PAO1 in different amounts of *Staphylococcus*; B: CIs of co-culturing Δ*exoT* with PAO1 in different amounts of *Staphylococcus*. Control group: PAO1:mutant=1:1; 1:1:1 group: PAO1:mutant:*Staphylococcus*=1:1:1; 1:1:10 group: PAO1:mutant:*Staphylococcus*=1:1:10.

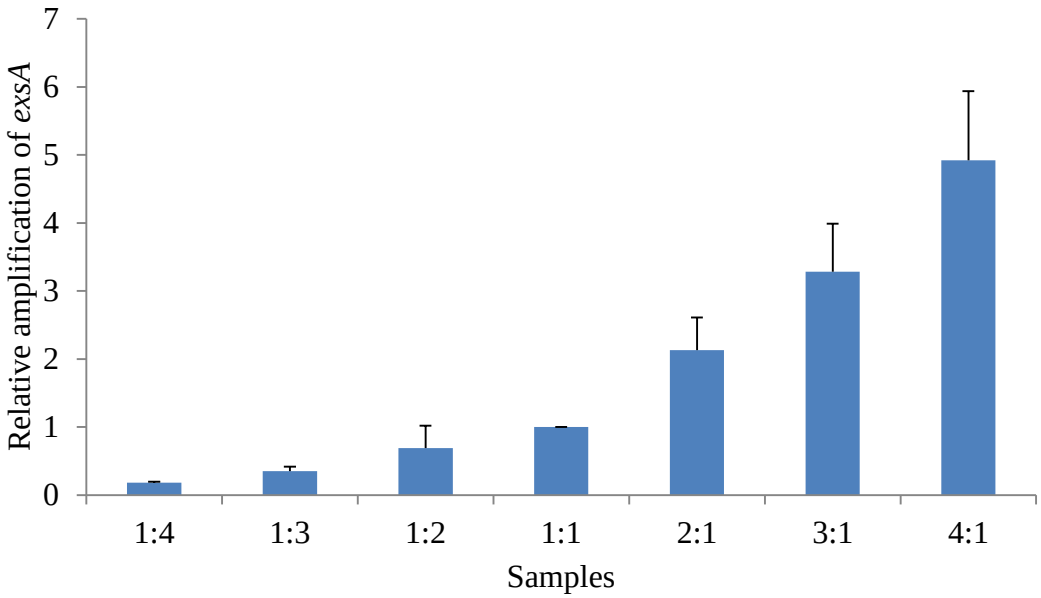
### 3.2.3 Bacterial ratio determined by real-time PCR

A novel method for the competitive index assay is required to replace the classical but inaccurate method previously used. The relative quantification by real-time PCR, which can determine the initial amount of templates or cell numbers by the $C_t$ value was used as an alternative method. The ratio of different bacteria in the co-cultures was determined by differentially amplifying the unique DNA fragments in the mutants. Using the PAO1 and Δ*exsA* genomes as DNA templates, the relative copy number of a specific gene on the chromosome of the wild type was assessed (Figure 3.14).

Two pairs of primers have been designed specifically to amplify the similar lengths of DNA sequences when using the chromosomes of PAO1 and Δ*exsA* as templates (Table 2.2). In this study, the PCR product for PAO1 is part of *exsA*, and the one for Δ*exsA* is part of the *lacZ* gene located in the middle of *exsA* on the mutant chromosome.

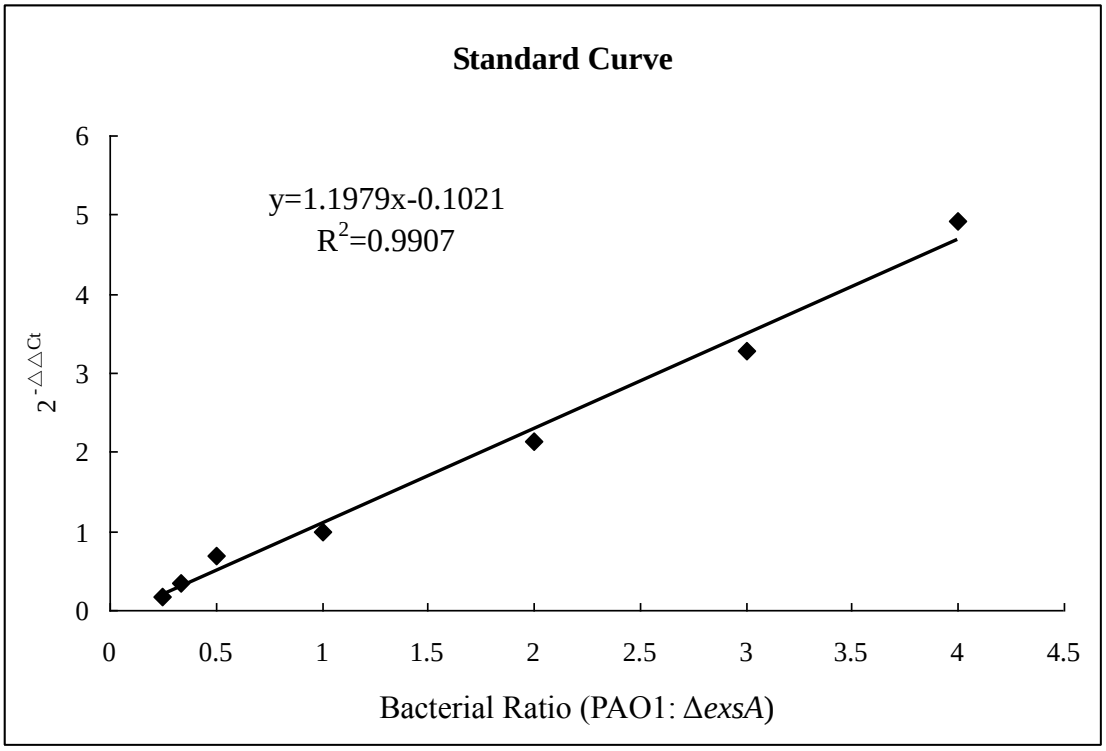
In a high throughput setting, a standard curve was made by plotting the DNA amplification levels against the corresponding starting inoculum ratios, which were also assessed by the CFU counts. Based on the results, the $2^{-\Delta\Delta Ct}$ is proportional to the corresponding input bacterial ratio (Figure 3.15). The standard curve can be represented by a function, y=1.1979x-0.1021.

**Figure 3.14 Relative ratio of *exsA* mutant to the wild type.**




X axis: the bacterial ratios of wild type to mutant; Y axis: the $2^{-\Delta\Delta Ct}$ of *exsA* on the chromosome of PAO1, when using the $2^{-\Delta\Delta Ct}$ of *lacZ* on the chromosome of Δ*exsA* as reference. Error bars indicate standard deviations.

**Figure 3.15 Standard curve for the competitive investigation between T3SS related mutants and wild type strains.**

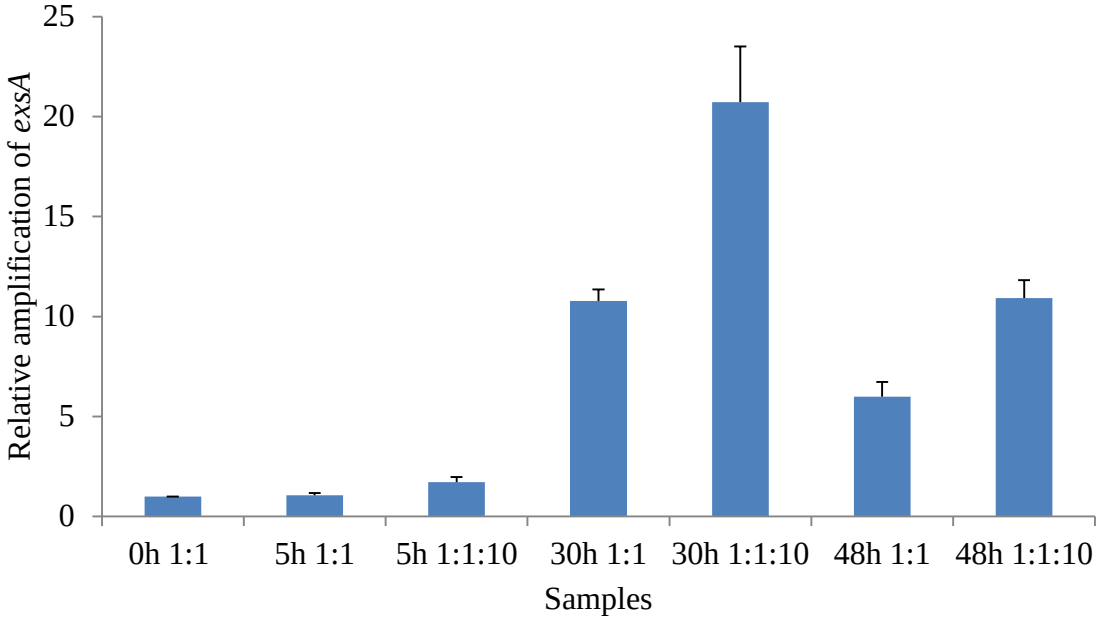



Bacterial ratio has a linear relationship with the relative DNA amplification. The function indicated can be utilized for relative amplification calculation. The result represents the mean of triplicate experiments.

As the standard curve which reflects the relationship between gene copy number and initial amount of DNA template has been obtained previously, it was utilized to assess the bacterial ratio in the co-cultured sample. Equal initial CFU of PAO1 and Δ*exsA* strains were co-cultured in 50 ml LB broth for long term, with the addition of 10-fold amount of *Staphylococcus* spp. (1:1:10 group) or without it (1:1 group). At the time point of 5 hours, 30 hours and 48 hours, the mixtures were collected and the relative quantification of *exsA* copy numbers by real-time PCR was performed (Figure 3.16).

Substituting the obtained $2^{-\triangle\triangle Ct}$ into the function y=1.1979x-0.1021, bacterial ratio in those samples were determined and shown in Table 3.3. Apparently, PAO1 was more competitive than Δ*exsA*. More interestingly, with the addition of *Staphylococcus* spp., the competitiveness of PAO1 was increased when compared to the 1:1 group.

**Figure 3.16 Relative ratio of *exsA* mutant to wild type in co-cultured samples.**




1:1 group: PAO1-Δ*exsA* co-culturing group; 1:1:10 group: PAO1-Δ*exsA*-*Staphylococcus* spp. co-culturing group. X axis: the samples collected at different time points in 1:1 group and 1:1:10 group; Y axis: the the $2^{-\Delta\Delta Ct}$ of *exsA* on the chromosome of PAO1, when using the $2^{-\Delta\Delta Ct}$ of *lacZ* on the chromosome of Δ*exsA* as reference. Error bars indicate standard deviations.

**Table 3.3 PAO1 to Δ*exsA* ratios at different time points in different groups.**

| Groups / Time Points | 1:1 group | 1:1:10 group |
|---|---|---|
| **0 h** | 0.920027 | 0.920027 |
| **5 h** | 0.968842 | 1.522895 |
| **30 h** | 9.078475 | 17.37571 |
| **48 h** | 5.082731 | 12.04461 |

PAO1 to Δ*exsA* ratios were determined by substituting the $2^{-\triangle\triangle Ct}$ of *exsA* into the function y=1.1979x-0.1021. 1:1 group: PAO1-Δ*exsA* co-culturing group; 1:1:10 group: PAO1-Δ*exsA*-*Staphylococcus* spp. co-culturing group.

# CHAPTER IV. DISCUSSION

## Part I

*Pseudomonas aeruginosa* is a human pathogen that causes serious and often life-threatening infections. Type III secretion system (T3SS) is an important virulence factor for *P. aeruginosa*'s successful establishment of infection. T3SS is a novel target for the development of new therapeutic strategies against *P. aeruginosa* infections. Although dozens of genes have been identified in the past 10 years that are implicated in regulation of the T3SS in *P. aeruginosa*, the complete picture of the molecular basis of the central regulatory pathways has not been fully revealed.

As part of a broader attempt to investigate the regulatory mechanisms that govern *P. aeruginosa* disease progression, the *P. aeruginosa* genome for genes that affect T3SS expression were screened through the construction of a transposon-insertion-mutant library, which carries the reporter *exoS-lux* on its chromosome. It was elucidated that transposon insertion in PA0465-0466 gene cluster has a dramatic negative effect on the promoter activity of the T3SS effector gene, *exoS*. But because PA0465 disruption did not affect *exoS*, the hypothesis of this study was that the downstream gene PA0466 may be involved in the regulation of T3SS.

The PA0466 over-expression plasmid pAK-0466 was constructed and T3SS effector and regulator genes were tested under the PA0466 over-expression background. The

results showed that the expression of *exoS*, *exoT*, *exoY*, *exsC* and *exsD* decreased remarkably, indicating PA0466, rather than PA0465, affects T3SS in *P. aeruginosa*.

To investigate the regulatory pathways that PA0466 asserts its regulation on T3SS, the promoter activities of several genes known to regulate T3SS were tested while PA0466 was over-expressed. Interestingly, while the *rsmY* and *rsmZ* gene expressions are apparently up-regulated, the other genes including *rsmA, rhlI/R, lasI/R, phzA1/A2*, and *pqsA* did not show significant differences.

The RsmY/Z/A post-transcriptional regulatory system controls over 500 gene expressions in *P. aeruginosa* (Burrowes, Baysse *et al.* 2006). Theoretically, RsmY and RsmZ act by antagonizing RsmA, and their transcription depends on the GacA-RsmA pathway (Babitzke and Romeo 2007, Goodman, Merighi *et al.* 2009). As PA0466 affects *rsmY/Z* and T3SS, experiments were done to dissect the relationship between PA0466 and the GacSA-RsmYZ-RsmA pathway. GacA is believed to directly control the transcription of *rsmY* and *rsmZ* exclusively (Brencic, McFarland *et al.* 2009), and Δ*gacA* has a sRNAs-deleted phenotype in *Erwinia caroovora*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* (Liu, Cui *et al.* 1998, Kay, Dubuis *et al.* 2005, Kay, Humair *et al.* 2006). T3SS expression was tested in PA0466 over-expression strain. It is expected that if the expression of T3SS reporter was decreased in the Δ*gacA* (pAK-0466), it would mean that PA0466 regulates T3SS through the other pathways. If no significant difference was seen between Δ*gacA*

(pAK-0466) and Δ*gacA* (pAK1900), the possible explanation of the T3SS down-regulation might be due to the involvement of PA0466 in the GacSA-RsmYZ-RsmA pathway.

In our study, the *exoS* promoter activity in the *gacA* mutant showed no difference in the PA0466 over-expression background, suggesting PA0466 works through the GacA-RsmA pathway. Swarming motility assay and T3SS protein secretion measurement further confirmed the inability of PA0466 over-expression to affect T3SS in Δ*gacA*. In conjunction with the results from above, it is very possible that PA0466 functions through the GacSA-RsmYZ-RsmA signaling pathway.

The increased sRNA production may give rise to low RsmA level in the cytosol and may affect other genes besides those involved in T3SS, such as *rhlA* (Heurlier, Williams *et al.* 2004). *P. aeruginosa* shows a swarming motility on semisolid media that require rhamnolipids production (Reimmann, Ginet *et al.* 2002). Our result shows that swarming motility is irrespective to the over-expression of PA0466. Additionally, the promoter activities monitored in the *rhlA-lux* reporter were also observed to keep a similar level in both the control strain and the over-expression strain.

The unaffected *rhlA* expression and swarming motility are in conflict with the enhanced *rsmY* and *rsmZ* activities, which indicate that the regulatory effect of PA0466 on T3SS is complex. It is very possible that PA0466 primarily acts through an

unknown pathway to affect T3SS directly without the participation of RsmA. At the same time, it also exerts a minor impact on the GacSA-RsmYZ-RsmA signaling pathway by up-regulating the expression of *rsmY* and *rsmZ*. Moreover, it is likely that the deletion of *gacA* has a dramatic influence on T3SS expression; therefore, such an effect would mask the weaker impact by PA0466. This might be the reason that no effect of PA0466 on *exoS* expression in *gacA* mutant. Apparently, further investigations are required to investigate the regulatory pathway through which PA0466 influences T3SS.

**Part II**

It has been shown that the expressions of T3SS effector genes *exoS*, *exoT* and *exoY* were up-regulated by the presence of CF lung residential flora OF strains (Duan, Dammel *et al.* 2003). A recent study also points out that the probiotic *Saccharomyces boulardii* affects the T3SS in *Citrobacter rodentium* (Wu, Vallance *et al.* 2008), which suggests that T3SS may be involved in the interspecies interaction. Our hypothesis is that through interacting with the environmental microflora, for example *Staphylococcus* spp., *P. aeruginosa* can prevent T3SS to be lost and retain its invasive function, especially in the biofilm living mode. In the light of this, the role T3SS played in the interspecies interaction needs to be identified.

Two mutants, Δ*exsE* and Δ*exsA*, with opposite effects on T3SS were used to probe the potential involvement of T3SS in the interaction between *P. aeruginosa* and

*Staphylococcus* spp.. Besides the mutants of regulatory genes (Δ*exsA* and Δ*exsE*), the effector gene mutants (Δ*exoS* and Δ*exoT*) were also used along with the wild type PAO1. As Δ*exsE* was not available in the lab previously, the construction of the PA1711 deletion mutant was carried out.

The direct consequence of the disruption of *exsE* would be the activation of T3SS genes, regardless of the culturing conditions (inducing or non-inducing). More T3SS proteins should also be secreted out by the mutant strain under inducing conditions. Intriguingly, our results showed that no significant difference of T3SS gene expression had been observed between the Δ*exsE* and PAO1 with or without calcium depletion. The reason might be because both Δ*exsE* and wild type released sufficient ExsA to promote the gene expression by reaching an equivalent level.

The definition of competitive index (CI) is the mutant-to-wild type output ratio divided by the mutant-to-wild type input ratio (Macho, Zumaquero *et al.* 2007). Competitive index reveals different competitiveness between the co-cultured T3SS mutant and wild type strain, therefore reflecting the importance of the roles of the gene mutated in such culture conditions. It provides a clue about how T3SS functions in the conditions with or without a neighborhood flora subsist. According to the CI equation, CI<1 means mutant is more competitive, CI>1 means wild type grows better, and CI=1 means to reach a balance. Using the CFU counting method, however, it seems hard to observe an apparent trend the CI follows within the time course of an

individual group, or to conclude any straightforward linkage the CI conveyed among three groups. This method is easy and classic, but also time-consuming. Miscounting is hard to avoid. For example, cell clumps are frequently regarded as single units. Inevitably, the limitations of CFU method restrict accuracy and efficiency (Hazan, Que *et al.* 2012).

Assessing gene expression quantity through real-time PCR for each type of strain will reflect indirect cell numbers. Due to the difference between mutant and wild type, distinct DNA fragments were amplified, followed by quantity determination and comparison. Two pairs of primers have been designed to amplify similar lengths of DNA sequences when using the chromosomes of PAO1 and Δ*exsA* as templates. For Δ*exsA*, the forward primer locates on the Gm cassette exclusively, whereas the reverse primer is same to the one for wild type. Meanwhile, although both of the primers can recognize the mutant genome in PAO1, the whole gene sequence between them is too long to be amplified. In this case, a couple of different PCR products were generated with similar lengths for maintaining PCR efficiency closely. In this experiment, the PCR product for PAO1 is part of *exsA*, and the one for Δ*exsA* is part of *lacZ*.

Relative quantification of real-time PCR can monitor minor gene expression changes based on a reference transcript. The basis for DNA copy number calculation is that $C_t$ values are directly proportionate to the amount of template at the starting point (Ginzinger 2002). In this assay, the DNA copy number of *lacZ* was regarded as the

internal control to facilitate the relative quantification of *exsA*. The resultant difference in cycle number $\Delta\Delta C_t$ is exponent of the base 2 due to the doubling function of PCR, representing the fold difference of the template of two genes. Our data shows a function, y=1.1979x-0.1021, which indicates the relationship between cell ratios and $2^{-\Delta\Delta Ct}$ numbers. In the context of this, simply acquiring the relevant $C_t$ values and substituting into the equation can reach the unknowing bacteria ratio in the co-cultured sample.

Using the standard curve, samples were analyzed at three time points from the PAO1-Δ*exsA* co-culturing group (1:1 group) and PAO1-Δ*exsA*-*Staphylococcus* spp. co-culturing group (1:1:10 group). The obtained bacterial ratios in co-cultured samples indicate that PAO1 is more competitive than Δ*exsA*, which suggests that the bacteria with T3SS grow better. With the addition of *Staphylococcus* spp., the competitiveness of PAO1 was enhanced even more when compared to the condition without *Staphylococcus* spp.. Having T3SS seems to be advantageous in the presence of other species. The future tasks may focus on how T3SS is involved in the interspecies interaction.

# CHAPTER V. CONCLUSION

In this study, PA0466 was shown to regulate T3SS negatively, as the expressions of *exoS, exoT, exoY, exsC* and *exsD* were decreased when PA0466 was over-expressed. Since PA0466 over-expression also affected *rsmY* and *rsmZ* gene expression, it provided evidence that this gene might function through the GacSA-RsmYZ-RsmA signaling pathway. Observations including unaffected swarming motility, T3SS protein secretion and *exoS* expression in *gacA* mutant suggested that the elimination of RsmY and RsmZ blocked the PA0466 regulatory function. Theoretically, the decreased RNA transcription renders a high free RsmA level in cytosol, which in turn down-regulates T3SS and *rhlA* expression. According to our results, however, the over-expression of PA0466 did not affect swarming motility and *rhlA* expression. The unchanged RsmA-controlled factors in the mutant indicate that PA0466 might pass through a non GacA-RsmA pathway to regulate T3SS directly.

In order to study how T3SS is involved in the interspecies interaction, T3SS mutant and wild type were co-cultured and the competitiveness was compared, with *Staphylococcus* spp. or not. Two methods had been selected. While the classical CFU counting in the competitive index assay is tedious and inaccurate, bacterial ratios in a co-cultured sample can be determined by performing the relative quantification of real-time PCR. Our results indicated that wild type possessing functional T3SS is more competitive than *exsA* mutant. The addition of *Staphylococcus* spp. was also

shown to enhance the competitiveness of wild type. The results suggest that T3SS may play a significant role in bacterium-bacterium interaction.

**REFERENCES**


Allen, L., Dockrell, D. H., *et al.* "Pyocyanin Production by Pseudomonas aeruginosa Induces Neutrophil Apoptosis and Impairs Neutrophil-Mediated Host Defenses In Vivo." The Journal of Immunology (2005).**174**(6): 3643-3649.

Ash, C. "Antibiotic resistance: The new apocalypse?" Trends in Microbiology (1996).**4**(10): 371-372.

Aspedon, A., Palmer, K., *et al.* "Microarray Analysis of the Osmotic Stress Response in Pseudomonas aeruginosa." Journal of Bacteriology (2006).**188**(7): 2721-2725.

Babitzke, P. and Romeo, T. "CsrB sRNA family: sequestration of RNA-binding regulatory proteins." Current Opinion in Microbiology (2007).**10**(2): 156-163.

Baer, M., Sawa, T., *et al.* "An Engineered Human Antibody Fab Fragment Specific for Pseudomonas aeruginosa PcrV Antigen Has Potent Antibacterial Activity." Infection and Immunity (2009).**77**(3): 1083-1090.

Banwart, B., Splaingard, M. L., *et al.*"Children with Cystic Fibrosis Produce an Immune Response against Exoenzyme S, a Type III Cytotoxin of Pseudomonas aeruginosa." Journal of Infectious Diseases (2002).**185**(2): 269-270.

Barbieri, A. M., Sha, Q., *et al.* "ADP-ribosylation of Rab5 by ExoS of Pseudomonas aeruginosa affects endocytosis." Infect Immun (2001).**69**(9): 5329-5334.

Barbieri, J. T. and Sun, J. (2005). Pseudomonas aeruginosa ExoS and ExoT. Reviews of Physiology, Biochemistry and Pharmacology, Springer Berlin Heidelberg. **152:** 79-92.

Becher, A. and Schweizer, H. P. "Integration-proficient Pseudomonas aeruginosa vectors for isolation of single-copy chromosomal lacZ and lux gene fusions." Biotechniques (2000).**29**(5): 948-952.

Berthelot, P., Attree, I., *et al.* "Genotypic and Phenotypic Analysis of Type III Secretion System in a Cohort of Pseudomonas aeruginosa Bacteremia Isolates: Evidence for a Possible Association between O Serotypes and exo Genes." Journal of Infectious Diseases (2003).**188**(4): 512-518.

Beuzón, C. R. and Holden, D. W. "Use of mixed infections with Salmonella strains to study virulence genes and their interactions in vivo." Microbes and Infection (2001).**3**(14): 1345-1352.

Bleves, S., Viarre, V., *et al.*"Protein secretion systems in Pseudomonas aeruginosa: A wealth of pathogenic weapons." International Journal of Medical Microbiology (2010).**300**(8): 534-543.

Bordi, C., Lamy, M.-C., *et al.* "Regulatory RNAs and the HptB/RetS signalling pathways fine-tune Pseudomonas aeruginosa pathogenesis." Molecular Microbiology (2010).**76**(6): 1427-1443.

Branda, S. S., Vik, Å., *et al.*"Biofilms: the matrix revisited." Trends in Microbiology (2005).**13**(1): 20-26.

Breidenstein, E., de la Fuente-Núñez, C., *et al.*"Pseudomonas aeruginosa: all roads lead to resistance." Trends in Microbiology (2011).**19**(8): 419-426.

Brencic, A. and Lory, S. "Determination of the regulon and identification of novel mRNA targets of Pseudomonas aeruginosa RsmA." Molecular Microbiology (2009).**72**(3): 612-632.

Brencic, A., McFarland, K. A., *et al.* "The GacS/GacA signal transduction system of Pseudomonas aeruginosa acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs." Molecular Microbiology (2009).**73**(3): 434-445.

Brutinel, E. D., Vakulskas, C. A., *et al.*"Functional Domains of ExsA, the Transcriptional Activator of the Pseudomonas aeruginosa Type III Secretion System." Journal of Bacteriology (2009).**191**(12): 3811-3821.

Brutinel, E. D., Vakulskas, C. A., *et al.* "ExsD Inhibits Expression of the Pseudomonas aeruginosa Type III Secretion System by Disrupting ExsA Self-Association and DNA Binding Activity." Journal of Bacteriology (2010).**192**(6): 1479-1486.

Brutinel, E. D. and Yahr, T. L. "Control of gene expression by type III secretory activity." Curr Opin Microbiol (2008).**11**(2): 128-133.

Burrowes, E., Baysse, C., *et al.* "Influence of the regulatory protein RsmA on cellular functions in Pseudomonas aeruginosa PAO1, as revealed by transcriptome analysis." Microbiology (2006).**152**(2): 405-418.

Cámara, M., Hardman, A., *et al.*"Quorum sensing in Vibrio cholerae." nature genetics (2002).**32**(2).

Cao, H., Krishnan, G., *et al.*"A quorum sensing-associated virulence gene of

Pseudomonas aeruginosa encodes a LysR-like transcription regulator with a unique self-regulatory mechanism." Proceedings of the National Academy of Sciences (2001).**98**(25): 14613-14618.

Cariss, S. J. L., Constantinidou, C., *et al.* "YieJ (CbrC) Mediates CreBC-Dependent Colicin E2 Tolerance in Escherichia coli." Journal of Bacteriology (2010).**192**(13): 3329-3336.

Chen, Y.-T., Chang, H., *et al.* "Evolutionary Analysis of the Two-Component Systems in Pseudomonas aeruginosa PAO1." Journal of Molecular Evolution (2004).**59**(6): 725-737.

Chuang, E.-Y., Lin, K.-J., *et al.* "Calcium depletion-mediated protease inhibition and apical-junctional-complex disassembly via an EGTA-conjugated carrier for oral insulin delivery." Journal of Controlled Release (2013).**169**(3): 296-305.

Coburn, B., Sekirov, I., *et al.* "Type III Secretion Systems and Disease." Clinical Microbiology Reviews (2007).**20**(4): 535-549.

Coenye, T., Goris, J., *et al.* "Characterization of unusual bacteria isolated from respiratory secretions of cystic fibrosis patients and description of Inquilinus limosus gen. nov., sp. nov." J Clin Microbiol (2002).**40**(6): 2062-2069.

Cohen, T. S. and Prince, A. "Cystic fibrosis: a mucosal immunodeficiency syndrome." Nat Med (2012).**18**(4): 509-519.

Cotter, P. A. and DiRita, V. J. "Bacterial virulence gene regulation: an evolutionary perspective." Annu Rev Microbiol (2000).**54**: 519-565.

Cowell, B. A., Evans, D. J., *et al.* "Actin cytoskeleton disruption by ExoY and its effects on Pseudomonas aeruginosa invasion." FEMS Microbiol Lett (2005).**250**(1): 71-76.

Déziel, E., Lépine, F., *et al.*"Analysis of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication." Proceedings of the National Academy of Sciences of the United States of America (2004).**101**(5): 1339-1344.

Dacheux, D., Attree, I., *et al.*"Expression of ExsA in trans Confers Type III Secretion System-Dependent Cytotoxicity on Noncytotoxic Pseudomonas aeruginosa Cystic Fibrosis Isolates." Infection and Immunity (2001).**69**(1): 538-542.

Dacheux, D., Epaulard, O., *et al.* "Activation of the Pseudomonas aeruginosa Type III Secretion System Requires an Intact Pyruvate Dehydrogenase aceAB Operon."

Infection and Immunity (2002).**70**(7): 3973-3977.

Dasgupta, N., Lykken, G. L., *et al.* "A novel anti-anti-activator mechanism regulates expression of the Pseudomonas aeruginosa type III secretion system." Molecular Microbiology (2004).**53**(1): 297-308.

Davey, M. E. and O'Toole, G. A. "Microbial biofilms: From ecology to molecular genetics." Microbiology and Molecular Biology Reviews (2000).**64**(4): 847-867.

Davies, D. G., Parsek, M. R., *et al.* "The Involvement of Cell-to-Cell Signals in the Development of a Bacterial Biofilm." Science (1998).**280**(5361): 295-298.

De Kievit, T. R., Gillis, R., *et al.* "Quorum-Sensing Genes in Pseudomonas aeruginosa Biofilms: Their Role and Expression Patterns." Applied and Environmental Microbiology (2001).**67**(4): 1865-1873.

Dietrich, L. E. P., Price-Whelan, A., *et al.* "The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of Pseudomonas aeruginosa." Molecular Microbiology (2006).**61**(5): 1308-1321.

Diggle, S. P., Winzer, K., *et al.* "The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR." Molecular Microbiology (2003).**50**(1): 29-43.

Ditta, G., Sharon, S., *et al.* "Broad Host Range DNA Cloning System for Gram-Negative Bacteria: Construction of a Gene Bank of Rhizobium meliloti." Proceedings of the National Academy of Sciences of the United States of America (1980).**77**(12): 7347-7351.

Duan, K., Dammel, C., *et al.*"Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication." Molecular Microbiology (2003).**50**(5): 1477-1491.

Duan, K. and Surette, M. G. "Environmental Regulation of Pseudomonas aeruginosa PAO1 Las and Rhl Quorum-Sensing Systems." Journal of Bacteriology (2007).**189**(13): 4827-4836.

Engel, J. and Balachandran, P. "Role of Pseudomonas aeruginosa type III effectors in disease." Current Opinion in Microbiology (2009).**12**(1): 61-66.

Feltman, H., Schulert, G., *et al.* "Prevalence of type III secretion genes in clinical and environmental isolates of Pseudomonas aeruginosa." Microbiology (2001).**147**(10): 2659-2669.

Figurski, D. H. and Helinski, D. R. "Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans." Proceedings of the National Academy of Sciences (1979).**76**(4): 1648-1652.

Filloux, A. "Protein secretion systems in Pseudomonas aeruginosa: an essay on diversity, evolution and function." Frontiers in Microbiology (2011).**2**.

Finck-Barbancon, V., Goranson, J., *et al.* "ExoU expression by Pseudomonas aeruginosa correlates with acute cytotoxicity and epithelial injury." Mol Microbiol (1997).**25**(3): 547-557.

Finck-Barbancon, V., Yahr, T. L., *et al.*"Identification and characterization of SpcU, a chaperone required for efficient secretion of the ExoU cytotoxin." J Bacteriol (1998).**180**(23): 6224-6231.

Fleiszig, S. M., Wiener-Kronish, J. P., *et al.* "Pseudomonas aeruginosa-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S." Infection and Immunity (1997).**65**(2): 579-586.

Frank, D. W. "The exoenzyme S regulon of Pseudomonas aeruginosa." Molecular Microbiology (1997).**26**(4): 621-629.

Friedman, L. and Kolter, R. "Genes involved in matrix formation in Pseudomonas aeruginosa PA14 biofilms." Molecular Microbiology (2004).**51**(3): 675-690.

Furukawa, S., Kuchma, S. L., *et al.* "Keeping Their Options Open: Acute versus Persistent Infections." Journal of Bacteriology (2006).**188**(4): 1211-1217.

Gómez, M. I. and Prince, A. "Opportunistic infections in lung disease: Pseudomonas infections in cystic fibrosis." Current Opinion in Pharmacology (2007).**7**(3): 244-251.

Galan, J. E. and Wolf-Watz, H. "Protein delivery into eukaryotic cells by type III secretion machines." Nature (2006).**444**(7119): 567-573.

Gallagher, L. A., McKnight, S. L., *et al.* "Functions Required for Extracellular Quinolone Signaling by Pseudomonas aeruginosa." Journal of Bacteriology (2002).**184**(23): 6472-6480.

Galle, M., Carpentier, I., *et al.* "Structure and Function of the Type III Secretion System of Pseudomonas aeruginosa." Current Protein and Peptide Science (2012).**13**(8): 831-842.

Gallegos, M. T., Schleif, R., *et al.* "Arac/XylS family of transcriptional regulators."

Microbiology and Molecular Biology Reviews (1997).**61**(4): 393-410.

Gibson, R. L., Burns, J. L., *et al.* "Pathophysiology and Management of Pulmonary Infections in Cystic Fibrosis." American Journal of Respiratory and Critical Care Medicine (2003).**168**(8): 918-951.

Ginzinger, D. G. "Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream." Experimental hematology (2002).**30**(6): 503-512.

Goehring, U. M., Schmidt, G., *et al.* "The N-terminal domain of Pseudomonas aeruginosa exoenzyme S is a GTPase-activating protein for Rho GTPases." J Biol Chem (1999).**274**(51): 36369-36372.

Goldberg, J. B. and Pier, G. B. "Pseudomonas aeruginosa lipopolysaccharides and pathogenesis." Trends in Microbiology (1996).**4**(12): 490-494.

Gooderham, W. J. and Hancock, R. E. W. "Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa." FEMS Microbiology Reviews (2009).**33**(2): 279-294.

Goodman, A. L., Kulasekara, B., *et al.* "A Signaling Network Reciprocally Regulates Genes Associated with Acute Infection and Chronic Persistence in Pseudomonas aeruginosa." Developmental Cell (2004).**7**(5): 745-754.

Goodman, A. L., Merighi, M., *et al.* "Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen." Genes & Development (2009).**23**(2): 249-259.

Goure, J., Pastor, A., *et al.* "The V antigen of Pseudomonas aeruginosa is required for assembly of the functional PopB/PopD translocation pore in host cell membranes." Infect Immun (2004).**72**(8): 4741-4750.

Ha, U.-H., Kim, J., *et al.* "An in vivo inducible gene of Pseudomonas aeruginosa encodes an anti-ExsA to suppress the type III secretion system." Molecular Microbiology (2004).**54**(2): 307-320.

Hauser, A. R. "The type III secretion system of Pseudomonas aeruginosa: infection by injection." Nat Rev Micro (2009).**7**(9): 654-665.

Hauser, A. R., Cobb, E., *et al.*"Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by Pseudomonas aeruginosa." Critical care medicine (2002).**30**(3): 521-528.

Hayes, C. S., Aoki, S. K., *et al.* "Bacterial contact-dependent delivery systems."

Annual review of genetics (2010).**44**: 71-90.

Hazan, R., Que, Y.-A., *et al.* "A method for high throughput determination of viable bacteria cell counts in 96-well plates." BMC microbiology (2012).**12**(1): 259.

Heeb, S., Kuehne, S. A., *et al.* "Functional Analysis of the Post-transcriptional Regulator RsmA Reveals a Novel RNA-binding Site." Journal of Molecular Biology (2006).**355**(5): 1026-1036.

Henriksson, M. L., Francis, M. S., *et al.* "A nonphosphorylated 14-3-3 binding motif on exoenzyme S that is functional in vivo." Eur J Biochem (2002).**269**(20): 4921-4929.

Heurlier, K., Williams, F., *et al.* "Positive Control of Swarming, Rhamnolipid Synthesis, and Lipase Production by the Posttranscriptional RsmA/RsmZ System in Pseudomonas aeruginosa PAO1." Journal of Bacteriology (2004).**186**(10): 2936-2945.

Hibbing, M. E., Fuqua, C., *et al.* "Bacterial competition: surviving and thriving in the microbial jungle." Nat Rev Micro (2010).**8**(1): 15-25.

Hoang, T. T., Karkhoff-Schweizer, R. R., *et al.* "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 (1998).**212**(1): 77-86.

Hoang, T. T., Kutchma, A. J., *et al.*"Integration-Proficient Plasmids for Pseudomonas aeruginosa: Site-Specific Integration and Use for Engineering of Reporter and Expression Strains." Plasmid (2000).**43**(1): 59-72.

Hogardt, M., Roeder, M., *et al.* "Expression of Pseudomonas aeruginosa exoS is controlled by quorum sensing and RpoS." Microbiology (2004).**150**(4): 843-851.

Hornef, M. W., Roggenkamp, A., *et al.* "Triggering the ExoS regulon of Pseudomonas aeruginosa: A GFP-reporter analysis of exoenzyme (Exo) S, ExoT and ExoU synthesis." Microbial Pathogenesis (2000).**29**(6): 329-343.

Hovey, A. K. and Frank, D. W. "Analyses of the DNA-binding and transcriptional activation properties of ExsA, the transcriptional activator of the Pseudomonas aeruginosa exoenzyme S regulon." Journal of Bacteriology (1995).**177**(15): 4427-4436.

Hueck, C. J. "Type III Protein Secretion Systems in Bacterial Pathogens of Animals and Plants." Microbiology and Molecular Biology Reviews (1998).**62**(2): 379-433.

Imamura, Y., Yanagihara, K., *et al.* "Effect of anti-PcrV antibody in a murine chronic airway Pseudomonas aeruginosa infection model." European Respiratory Journal (2007).**29**(5): 965-968.

Jain, M., Bar-Meir, M., *et al.* "Evolution of Pseudomonas aeruginosa type III secretion in cystic fibrosis: a paradigm of chronic infection." Translational Research (2008).**152**(6): 257-264.

Jain, M., Ramirez, D., *et al.* "Type III Secretion Phenotypes of Pseudomonas aeruginosa Strains Change during Infection of Individuals with Cystic Fibrosis." Journal of Clinical Microbiology (2004).**42**(11): 5229-5237.

Kay, E., Dubuis, C., *et al.*"Three small RNAs jointly ensure secondary metabolism and biocontrol in Pseudomonas fluorescens CHA0." Proceedings of the National Academy of Sciences of the United States of America (2005).**102**(47): 17136-17141.

Kay, E., Humair, B., *et al.*"Two GacA-Dependent Small RNAs Modulate the Quorum-Sensing Response in Pseudomonas aeruginosa." Journal of Bacteriology (2006).**188**(16): 6026-6033.

Kerem, B., Rommens, J. M., *et al.* "Identification of the cystic fibrosis gene: genetic analysis." Science (1989).**245**(4922): 1073-1080.

Kerr, K. and Snelling, A. "Pseudomonas aeruginosa: a formidable and ever-present adversary." Journal of Hospital Infection (2009).**73**(4): 338-344.

Keyser, P., Elofsson, M., *et al.* "Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against Gram-negative bacteria." Journal of Internal Medicine (2008).**264**(1): 17-29.

Kim, J., Ahn, K., *et al.*"Factors triggering type III secretion in Pseudomonas aeruginosa." Microbiology (2005).**151**(11): 3575-3587.

Kipnis, E., Sawa, T., *et al.* "Targeting mechanisms of Pseudomonas aeruginosa pathogenesis." Medecine et maladies infectieuses (2006).**36**(2): 78-91.

Kong, W., Chen, L., *et al.*"Hybrid sensor kinase PA1611 in Pseudomonas aeruginosa regulates transitions between acute and chronic infection through direct interaction with RetS." Molecular Microbiology (2013).**88**(4): 784-797.

Kong, W, "Transcriptional regulation mechanism of the type III secretion system in *Pseudomonas aeruginosa*." PhD thesis, Northwest University (2013).

Krall, R., Schmidt, G., *et al.* "Pseudomonas aeruginosa ExoT is a Rho

GTPase-activating protein." Infect Immun (2000).**68**(10): 6066-6068.

Kuchma, S. L., Connolly, J. P., *et al.* "A Three-Component Regulatory System Regulates Biofilm Maturation and Type III Secretion in Pseudomonas aeruginosa." Journal of Bacteriology (2005).**187**(4): 1441-1454.

Kulasekara, H. D., Ventre, I., *et al.* "A novel two‐component system controls the expression of Pseudomonas aeruginosa fimbrial cup genes." Molecular Microbiology (2005).**55**(2): 368-380.

Kurahashi, K., Kajikawa, O., *et al.*"Pathogenesis of septic shock in Pseudomonas aeruginosa pneumonia." The Journal of Clinical Investigation (1999).**104**(6): 743-750.

Lépine, F., Déziel, E., *et al.*"A stable isotope dilution assay for the quantification of the Pseudomonas quinolone signal in Pseudomonas aeruginosa cultures." Biochimica et Biophysica Acta (BBA) - General Subjects (2003).**1622**(1): 36-41.

Laemmli, U. K. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature (1970).**227**(5259): 680-685.

Lapouge, K., Schubert, M., *et al.* "Gac/Rsm signal transduction pathway of γ-proteobacteria: from RNA recognition to regulation of social behaviour." Molecular Microbiology (2008).**67**(2): 241-253.

Laskowski, M. A. and Kazmierczak, B. I. "Mutational Analysis of RetS, an Unusual Sensor Kinase-Response Regulator Hybrid Required for Pseudomonas aeruginosa Virulence." Infection and Immunity (2006).**74**(8): 4462-4473.

Laursen, J. B. and Nielsen, J. "Phenazine Natural Products: Biosynthesis, Synthetic Analogues, and Biological Activity." Chemical Reviews (2004).**104**(3): 1663-1686.

Lee, C. A. "Type III secretion systems: machines to deliver bacterial proteins into eukaryotic cells?" Trends in Microbiology (1997).**5**(4): 148-156.

Liang, H., Duan, J., *et al.* "Identification of mutants with altered phenazine production in Pseudomonas aeruginosa." Journal of Medical Microbiology (2011).**60**(1): 22-34.

Liang, H., Li, L., *et al.*"The YebC Family Protein PA0964 Negatively Regulates the Pseudomonas aeruginosa Quinolone Signal System and Pyocyanin Production." Journal of Bacteriology (2008).**190**(18): 6217-6227.

Linares, J. F., López, J. A., *et al.* "Over-expression of the Multidrug Efflux Pumps MexCD-OprJ and MexEF-OprN Is Associated with a Reduction of Type III Secretion in Pseudomonas aeruginosa." Journal of Bacteriology (2005).**187**(4): 1384-1391.

Liu, S., Yahr, T. L., *et al.* "Biochemical relationships between the 53-kilodalton (Exo53) and 49-kilodalton (ExoS) forms of exoenzyme S of Pseudomonas aeruginosa." J Bacteriol (1997).**179**(5): 1609-1613.

Liu, Y., Cui, Y., *et al.* "Characterization of a novel RNA regulator of Erwinia carotovora ssp. carotovora that controls production of extracellular enzymes and secondary metabolites." Molecular Microbiology (1998).**29**(1): 219-234.

Lyczak, J. B., Cannon, C. L., *et al.*"Establishment of Pseudomonas aeruginosa infection: Lessons from a versatile opportunist." Microbes and Infection (2000).**2**(9): 1051-1060.

Lykken, G. L., Chen, G., *et al.*"Characterization of ExsC and ExsD Self-Association and Heterocomplex Formation." Journal of Bacteriology (2006).**188**(19): 6832-6840.

Macho, A. P., Zumaquero, A., *et al.* "Competitive index in mixed infections: a sensitive and accurate assay for the genetic analysis of Pseudomonas syringae–plant interactions." Molecular Plant Pathology (2007).**8**(4): 437-450.

Maurelli, A. T., Blackmon, B., *et al.* "Temperature-dependent expression of virulence genes in Shigella species." Infection and Immunity (1984).**43**(1): 195-201.

Mavrodi, D. V., Bonsall, R. F., *et al.*"Functional Analysis of Genes for Biosynthesis of Pyocyanin and Phenazine-1-Carboxamide from Pseudomonas aeruginosa PAO1." Journal of Bacteriology (2001).**183**(21): 6454-6465.

Mayansky, A. N., Chebotar, I. V., *et al.* "Pseudomonas aeruginosa: Characteristics of the biofilm process." Molecular Genetics, Microbiology and Virology (2012).**27**(1): 1-6.

McCaw, M. L., Lykken, G. L., *et al.* "ExsD is a negative regulator of the Pseudomonas aeruginosa type III secretion regulon." Molecular Microbiology (2002).**46**(4): 1123-1133.

McKenzie, G. J., Harris, R. S., *et al.*"The SOS response regulates adaptive mutation." Proceedings of the National Academy of Sciences (2000).**97**(12): 6646-6651.

Michel-Briand, Y. and Baysse, C. "The pyocins of Pseudomonas aeruginosa." Biochimie (2002).**84**(5–6): 499-510.

Moss, J., Ehrmantraut, M. E., *et al.* "Sera from Adult Patients with Cystic Fibrosis Contain Antibodies to Pseudomonas aeruginosa Type III Apparatus." Infection and Immunity (2001).**69**(2): 1185-1188.

Mota, L. J. and Cornelis, G. R. "The bacterial injection kit: Type III secretion systems." Annals of Medicine (2005).**37**(4): 234-249.

Mueller, C. A., Broz, P., *et al.* "The V-Antigen of Yersinia Forms a Distinct Structure at the Tip of Injectisome Needles." Science (2005).**310**(5748): 674-676.

Mulcahy, H., O'Callaghan, J., *et al.* "The Posttranscriptional Regulator RsmA Plays a Role in the Interaction between Pseudomonas aeruginosa and Human Airway Epithelial Cells by Positively Regulating the Type III Secretion System." Infection and Immunity (2006).**74**(5): 3012-3015.

Nouwens, A. S., Beatson, S. A., *et al.* "Proteome analysis of extracellular proteins regulated by the las and rhl quorum sensing systems in Pseudomonas aeruginosa PAO1." Microbiology (2003).**149**(5): 1311-1322.

O'Toole, G., Kaplan, H. B., *et al.*"Biofilm formation as microbial development." Annual Reviews in Microbiology (2000).**54**(1): 49-79.

Palmer, K. L., Mashburn, L. M., *et al.*"Cystic Fibrosis Sputum Supports Growth and Cues Key Aspects of Pseudomonas aeruginosa Physiology." Journal of Bacteriology (2005).**187**(15): 5267-5277.

Parsot, C., Hamiaux, C., *et al.* "The various and varying roles of specific chaperones in type III secretion systems." Curr Opin Microbiol (2003).**6**(1): 7-14.

Pearson, J. P., Pesci, E. C., *et al.*"Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes." Journal of Bacteriology (1997).**179**(18): 5756-5767.

Pederson, K. J. and Barbieri, J. T. "Intracellular expression of the ADP-ribosyltransferase domain of Pseudomonas exoenzyme S is cytotoxic to eukaryotic cells." Mol Microbiol (1998).**30**(4): 751-759.

Pesci, E. C., Milbank, J. B. J., *et al.*"Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa." Proceedings of the National Academy of Sciences (1999).**96**(20): 11229-11234.

Pessi, G., Williams, F., *et al.* "The Global Posttranscriptional Regulator RsmA Modulates Production of Virulence Determinants andN-Acylhomoserine Lactones in Pseudomonas aeruginosa." Journal of Bacteriology (2001).**183**(22): 6676-6683.

Phillips, R. M., Six, D. A., *et al.* "In vivo phospholipase activity of the Pseudomonas aeruginosa cytotoxin ExoU and protection of mammalian cells with phospholipase A2

inhibitors." J Biol Chem (2003).**278**(42): 41326-41332.

Poole, K., Neshat, S., *et al.* "Cloning and nucleotide sequence analysis of the ferripyoverdine receptor gene fpvA of Pseudomonas aeruginosa." Journal of Bacteriology (1993).**175**(15): 4597-4604.

Portnoy, D. A., Moseley, S. L., *et al.* "Characterization of plasmids and plasmid-associated determinants of Yersinia enterocolitica pathogenesis." Infection and Immunity (1981).**31**(2): 775-782.

Quinaud, M., Chabert, J., *et al.* "The PscE-PscF-PscG complex controls type III secretion needle biogenesis in Pseudomonas aeruginosa." J Biol Chem (2005).**280**(43): 36293-36300.

Rajan, S. and Saiman, L. "Pulmonary infections in patients with cystic fibrosis." Semin Respir Infect (2002).**17**(1): 47-56.

Reimmann, C., Ginet, N., *et al.* "Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in Pseudomonas aeruginosa PAO1." Microbiology (2002).**148**(4): 923-932.

Richards, M. J. M. F., Edwards, J. R. M. S., *et al.* "Nosocomial Infections in Combined Medical - Surgical Intensive Care Units in the United States •." Infection Control and Hospital Epidemiology (2000).**21**(8): 510-515.

Rietsch, A. and Mekalanos, J. J. "Metabolic regulation of type III secretion gene expression in Pseudomonas aeruginosa." Molecular Microbiology (2006).**59**(3): 807-820.

Rietsch, A., Vallet-Gely, I., *et al.* "ExsE, a secreted regulator of type III secretion genes in Pseudomonas aeruginosa." Proceedings of the National Academy of Sciences (2005).**102**(22): 8006-8011.

Rietsch, A., Wolfgang, M. C., *et al.* "Effect of Metabolic Imbalance on Expression of Type III Secretion Genes in Pseudomonas aeruginosa." Infection and Immunity (2004).**72**(3): 1383-1390.

Rocchetta, H. L., Burrows, L. L., *et al.* "Genetics of O-Antigen Biosynthesis inPseudomonas aeruginosa." Microbiology and Molecular Biology Reviews (1999).**63**(3): 523-553.

Rodrigue, A., Quentin, Y., *et al.* "Cell signalling by oligosaccharides. Two-component systems in Pseudomonas aeruginosa: why so many?" Trends in Microbiology (2000).**8**(11): 498-504.

Roy-Burman, A., Savel, R. H., *et al.*"Type III Protein Secretion Is Associated with Death in Lower Respiratory and Systemic Pseudomonas aeruginosa Infections." The Journal of Infectious Diseases (2001).**183**(12): 1767-1774.

Roy, D., Liston, D. R., *et al.* "A process for controlling intracellular bacterial infections induced by membrane injury." Science (2004).**304**(5676): 1515-1518.

Salmond, G. P. C. and Reeves, P. J. "Membrance traffic wardens and protein secretion in Gram-negative bacteria." Trends in Biochemical Sciences (1993).**18**(1): 7-12.

Sato, H., Feix, J. B., *et al.* "Identification of superoxide dismutase as a cofactor for the pseudomonas type III toxin, ExoU." Biochemistry (2006).**45**(34): 10368-10375.

Sato, H. and Frank, D. W. "ExoU is a potent intracellular phospholipase." Molecular Microbiology (2004).**53**(5): 1279-1290.

Sauer, K., Camper, A. K., *et al.* "Pseudomonas aeruginosa Displays Multiple Phenotypes during Development as a Biofilm." Journal of Bacteriology (2002).**184**(4): 1140-1154.

Sawa, T., Yahr, T. L., *et al.*"Active and passive immunization with the Pseudomonas V antigen protects against type III intoxication and lung injury." Nature Medicine (1999).**5**(4): 392.

Sayner, S. L., Frank, D. W., *et al.* "Paradoxical cAMP-induced lung endothelial hyperpermeability revealed by Pseudomonas aeruginosa ExoY." Circ Res (2004).**95**(2): 196-203.

Schoehn, G., Di Guilmi, A. M., *et al.* "Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in Pseudomonas." Embo j (2003).**22**(19): 4957-4967.

Schulert, G. S., Feltman, H., *et al.* "Secretion of the toxin ExoU is a marker for highly virulent Pseudomonas aeruginosa isolates obtained from patients with hospital-acquired pneumonia." J Infect Dis (2003).**188**(11): 1695-1706.

Schweizer, H. P. and Hoang, T. T. "An improved system for gene replacement and xylE fusion analysis in Pseudomonas aeruginosa." Gene (1995).**158**(1): 15-22.

Shaver, C. M. and Hauser, A. R. "Relative contributions of Pseudomonas aeruginosa ExoU, ExoS, and ExoT to virulence in the lung." Infect Immun (2004).**72**(12): 6969-6977.

Shen, D. K., Filopon, D., *et al.* "PsrA Is a Positive Transcriptional Regulator of the Type III Secretion System in Pseudomonas aeruginosa." Infection and Immunity (2006).**74**(2): 1121-1129.

Shen, D. K., Quenee, L., *et al.* "Orf1/SpcS chaperones ExoS for type three secretion by Pseudomonas aeruginosa." Biomed Environ Sci (2008).**21**(2): 103-109.

Sibley, C. D., Rabin, H., *et al.*"Cystic fibrosis: a polymicrobial infectious disease." Future Microbiology (2006).**1**(1): 53-61.

Simon, R., Priefer, U., *et al.* "A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria." Nat Biotech (1983).**1**(9): 784-791.

Singh, P. K., Schaefer, A. L., *et al.* "Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms." Nature (2000).**407**(6805): 762-764.

Sivaneson, M., Mikkelsen, H., *et al.* "Two-component regulatory systems in Pseudomonas aeruginosa: an intricate network mediating fimbrial and efflux pump gene expression." Molecular Microbiology (2011).**79**(5): 1353-1366.

Smith, A. W. and Iglewski, B. H. "Transformation of Pseudomonas aeruginosa by electroporation." Nucleic Acids Research (1989).**17**(24): 10509.

Smith, E. E., Buckley, D. G., *et al.* "Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients." Proceedings of the National Academy of Sciences (2006).**103**(22): 8487-8492.

Smith, R. S., Wolfgang, M. C., *et al.* "An Adenylate Cyclase-Controlled Signaling Network Regulates Pseudomonas aeruginosa Virulence in a Mouse Model of Acute Pneumonia." Infection and Immunity (2004).**72**(3): 1677-1684.

Soni, R., Marks, G., *et al.* "Effect of Burkholderia cepacia infection in the clinical course of patients with cystic fibrosis: A pilot study in a Sydney clinic." Respirology (2002).**7**(3): 241-245.

Soscia, C., Hachani, A., *et al.*"Cross talk between type III secretion and flagellar assembly systems in Pseudomonas aeruginosa." Journal of Bacteriology (2007).**189**(8): 3124-3132.

Stirling, F. R., Cuzick, A., *et al.* "Eukaryotic localization, activation and ubiquitinylation of a bacterial type III secreted toxin." Cell Microbiol (2006).**8**(8): 1294-1309.

Stock, A. M., Robinson, V. L., *et al.* "Two-component signal transduction." Annual review of biochemistry (2000).**69**(1): 183-215.

Stover, C. K., Pham, X. Q., *et al.*"Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen." Nature (2000).**406**(6799): 959-964.

Sun, J. and Barbieri, J. T. "Pseudomonas aeruginosa ExoT ADP-ribosylates CT10 regulator of kinase (Crk) proteins." J Biol Chem (2003).**278**(35): 32794-32800.

Sundin, C., Wolfgang, M. C., *et al.* "Type IV pili are not specifically required for contact dependent translocation of exoenzymes by Pseudomonas aeruginosa." Microb Pathog (2002).**33**(6): 265-277.

Sutherland, I. W. "The biofilm matrix - An immobilized but dynamic microbial environment." Trends in Microbiology (2001).**9**(5): 222-227.

Tancrède, C. "Role of human microflora in health and disease." European Journal of Clinical Microbiology and Infectious Diseases (1992).**11**(11): 1012-1015.

Urbanowski, M. L., Brutinel, E. D., *et al.* "Translocation of ExsE into Chinese Hamster Ovary Cells Is Required for Transcriptional Induction of the Pseudomonas aeruginosa Type III Secretion System." Infection and Immunity (2007).**75**(9): 4432-4439.

Urbanowski, M. L., Lykken, G. L., *et al.* "A secreted regulatory protein couples transcription to the secretory activity of the Pseudomonas aeruginosa type III secretion system." Proceedings of the National Academy of Sciences of the United States of America (2005).**102**(28): 9930-9935.

Veesenmeyer, J. L., Hauser, A. R., *et al.* "Pseudomonas aeruginosa virulence and therapy: evolving translational strategies." Critical care medicine (2009).**37**(5): 1777.

Ventre, I., Goodman, A. L., *et al.* "Multiple sensors control reciprocal expression of Pseudomonas aeruginosa regulatory RNA and virulence genes." Proceedings of the National Academy of Sciences of the United States of America (2006).**103**(1): 171-176.

Vogelaar, N. J., Jing, X., *et al.*"Analysis of the Crystal Structure of the ExsC-ExsE Complex Reveals Distinctive Binding Interactions of the Pseudomonas aeruginosa Type III Secretion Chaperone ExsC with ExsE and ExsD." Biochemistry (2010).**49**(28): 5870-5879.

von Götz, F., Häussler, S., *et al.* "Expression Analysis of a Highly Adherent and

Cytotoxic Small Colony Variant of Pseudomonas aeruginosa Isolated from a Lung of a Patient with Cystic Fibrosis." Journal of Bacteriology (2004).**186**(12): 3837-3847.

West, S. E., Sample, A. K., *et al.* "The vfr gene product, required for Pseudomonas aeruginosa exotoxin A and protease production, belongs to the cyclic AMP receptor protein family." Journal of Bacteriology (1994).**176**(24): 7532-7542.

Whiteley, M., Bangera, M. G., *et al.* "Gene expression in Pseudomonas aeruginosa biofilms." Nature (2001).**413**(6858): 860-864.

Whiteley, M., Lee, K. M., *et al.* "Identification of genes controlled by quorum sensing in Pseudomonas aeruginosa." Proceedings of the National Academy of Sciences (1999).**96**(24): 13904-13909.

Williams, H. D., Zlosnik, J. E. A., *et al.* (2006). Oxygen, Cyanide and Energy Generation in the Cystic Fibrosis Pathogen Pseudomonas aeruginosa. Advances in Microbial Physiology, Academic Press. **Volume 52:** 1-71.

Wilson, R., Pitt, T., *et al.* "Pyocyanin and 1-hydroxyphenazine produced by Pseudomonas aeruginosa inhibit the beating of human respiratory cilia in vitro." J Clin Invest (1987).**79**(1): 221-229.

Winsor, G. L., Lam, D. K., *et al.* "Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes." Nucleic Acids Res (2011).**39**(Database issue): D596-600.

Winzer, K. and Williams, P. "Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria." International Journal of Medical Microbiology (2001).**291**(2): 131-143.

Wolanin, P. M., Thomason, P. A., *et al.* "Histidine protein kinases: key signal transducers outside the animal kingdom." Genome Biol (2002).**3**(10): 3013.

Wolfgang, M. C., Lee, V. T., *et al.* "Coordinate Regulation of Bacterial Virulence Genes by a Novel Adenylate Cyclase-Dependent Signaling Pathway." Developmental Cell (2003).**4**(2): 253-263.

Wu, W. and Jin, S. "PtrB of Pseudomonas aeruginosa Suppresses the Type III Secretion System under the Stress of DNA Damage." Journal of Bacteriology (2005).**187**(17): 6058-6068.

Wu, X., Vallance, B. A., *et al.* "Saccharomyces boulardii ameliorates Citrobacter rodentium-induced colitis through actions on bacterial virulence factors." American Journal of Physiology - Gastrointestinal and Liver Physiology (2008).**294**(1):

G295-G306.

Yahr, T. L. and Frank, D. W. "Transcriptional organization of the trans-regulatory locus which controls exoenzyme S synthesis in Pseudomonas aeruginosa." Journal of Bacteriology (1994).**176**(13): 3832-3838.

Yahr, T. L. and Greenberg, E. P. "The Genetic Basis for the Commitment to Chronic versus Acute Infection in Pseudomonas aeruginosa." Molecular Cell (2004).**16**(4): 497-498.

Yahr, T. L., Hovey, A. K., *et al.* "Transcriptional analysis of the Pseudomonas aeruginosa exoenzyme S structural gene." Journal of Bacteriology (1995).**177**(5): 1169-1178.

Yahr, T. L., Vallis, A. J., *et al.* "ExoY, an adenylate cyclase secreted by the Pseudomonas aeruginosa type III system." Proceedings of the National Academy of Sciences (1998).**95**(23): 13899-13904.

Yahr, T. L. and Wolfgang, M. C. "Transcriptional regulation of the Pseudomonas aeruginosa type III secretion system." Molecular Microbiology (2006).**62**(3): 631-640.

Yamazaki, A., Li, J., *et al.* "Derivatives of Plant Phenolic Compound Affect the Type III Secretion System of Pseudomonas aeruginosa via a GacS-GacA Two-Component Signal Transduction System." Antimicrobial Agents and Chemotherapy (2012).**56**(1): 36-43.

Yoon, S. S., Hennigan, R. F., *et al.* "Pseudomonas aeruginosa Anaerobic Respiration in Biofilms: Relationships to Cystic Fibrosis Pathogenesis." Developmental Cell (2002).**3**(4): 593-603.

Zhao, J., Kong, W, *et al*. "Functional studies of the regulation of the gene of type III secretion system in *Pseudomonas aeruginosa*." Unpublished manuscript.

Zheng, Z., Chen, G., *et al.* "Biochemical Characterization of a Regulatory Cascade Controlling Transcription of the Pseudomonas aeruginosa Type III Secretion System." Journal of Biological Chemistry (2007).**282**(9): 6136-6142.

Zhou, X., Keller, R., *et al.* "Structural Basis for Two-component System Inhibition and Pilus Sensing by the Auxiliary CpxP Protein." Journal of Biological Chemistry (2011).**286**(11): 9805-9814.