

Regulations and Functions of Type Three and Type Six Secretion Systems in

Pseudomonas aeruginosa

by

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Abstract

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium associated with a wide range of acute and chronic infections, particularly in patients with cystic fibrosis. Its pathogenic success relies on complex regulatory networks that integrate environmental signals, cellular metabolism, and energy status to control virulence determinants. Central to these processes are the Type 3 secretion system (T3SS) and Type 6 secretion system (T6SS), which deliver effector proteins into host cells and competing microbes. The overall objective of this thesis was to elucidate the regulatory mechanisms that coordinate T3SS and T6SS functions in *P. aeruginosa*.

In this study, the nitric oxide reductase operon *norCBD* was identified as a critical regulator of the H2-T6SS. Disruption of *norCBD* led to excessive intracellular accumulation of nitric oxide, resulting in impaired Dnr-dependent transcription and significantly reduced H2-T6SS expression. This dysregulation was accompanied by defects in multiple virulence-associated phenotypes, including biofilm formation, pyocyanin production, motility, and proteolytic activity. These findings demonstrate that optimal nitric oxide homeostasis, rather than nitric oxide presence alone, is essential for proper regulation of H2-T6SS and virulence.

This work also reveals functional and regulatory crosstalk between the ATPases of the T3SS and T6SS, PscN and ClpV3, respectively. Deletion of either ATPase led to reduced transcription of T3SS and H3-T6SS genes, diminished interbacterial competitiveness, altered virulence phenotypes, and accumulation of intracellular ATP. Complementation studies demonstrated functional interchangeability between PscN and ClpV3, indicating that T3SS and T6SS are energetically and regulationally coupled and represent major ATP-consuming processes in the cell.

This study also characterizes an operon regulated by the Fem cell-surface signaling system.

Regulation of this operon links iron sensing to downstream control of virulence traits, highlighting convergence between nutrient sensing and T6SS regulation.

This work establishes the hybrid sensor kinase PA1611 as an environmental sensor that responds to host-derived mucin. PA1611 modulates virulence and T6SS and T3SS through the RetS/GacS/GacA-Rsm pathway, linking host signals to the acute to chronic infection transition.

Collectively, this thesis uncovers integrated regulatory layers that coordinate nitric oxide homeostasis, ATP-dependent energy balance, iron sensing, and host-derived signals to finely tune T3SS and T6SS activity in *P. aeruginosa*.

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Chapter 3

norCBD disruption affects the H2-type six secretion system and multiple virulence factors in *Pseudomonas aeruginosa*. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY).

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List of abbreviations

AAA⁺ = ATPases Associated with diverse cellular Activities

ABC = ATP-Binding Cassette

ADPRT = ADP-Ribosyltransferase

AMP = Adenosine Monophosphate

Amp^r = Ampicillin Resistance

ANOVA = Analysis of Variance

ANR = Anaerobic regulator of arginine metabolism and nitrate reduction

AprA = Alkaline Protease A

ATP = Adenosine Triphosphate

ATPase = Adenosine Triphosphatase

BC = Baseplate Component

BdlA = Biofilm Dispersion Locus A

cAMP = Cyclic Adenosine Monophosphate

Cb^r = Carbenicillin Resistance

C-di-AMP = Cyclic Diadenosine Monophosphate

C-di-GMP = Cyclic Diguanylate Monophosphate

CPS = Counts Per Second

CPTIO = 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide

CRK = CT10 Regulator of Kinase

CRP = cAMP Receptor Protein

CTX = Chromosomal Integrating Vector

cyaA/B = Adenylate Cyclase A/B

DAN = 2,3-Diaminonaphthalene

DAF-2 DA = Diaminofluorescein-2 Diacetate

DGC = Diguanylate Cyclase

DNR = Dissimilative Nitrate Respiration Regulator

ECF = Extra-Cytoplasmic Function Sigma Factor

eDNA = Extracellular DNA

ELISA = Enzyme-Linked Immunosorbent Assay

EPS = Extracellular Polymeric Substances

FleQ = Flagellar Master Regulator

GacA/S = Global Activator Sensor/Regulator

GAP = GTPase-Activating Protein

Gm^r = Gentamicin Resistance

GMP = Guanosine Monophosphate

Hcp = Haemolysin-Coregulated Protein

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HHK = Hybrid Histidine Kinase

Km^r = Kanamycin Resistance

LB = Luria-Bertani

LPS = Lipopolysaccharide

NO = Nitric Oxide

NO₂⁻ = Nitrite

NO₃⁻ = Nitrate

N₂O = Nitrous Oxide

OD₆₀₀ = Optical Density at 600 nm

PAAR = Proline-Alanine-Alanine-Arginine Protein

PBS = Phosphate-Buffered Saline

PCR = Polymerase Chain Reaction

PCN = Pyocyanin

PDE = Phosphodiesterase

PIA = *Pseudomonas* Isolation Agar

PldA/B = Phospholipase D Effectors

PNAG = Poly-N-Acetylglucosamine

PQS = *Pseudomonas* Quinolone Signal

ppGpp = Guanosine Tetraphosphate

ppm / pppGpp = Guanosine Pentaphosphate

PAO1 = *Pseudomonas aeruginosa* Reference Strain

RetS = Hybrid Sensor Kinase

RLU = Relative Luminescence Units

SacB = Sucrose Sensitivity Gene

SEM = Standard Error of Mean

SiaD = Diguanylate Cyclase for Biofilm

SNP = Sodium Nitroprusside

SOD1 = Superoxide Dismutase

T1SS = Type I Secretion System

T2SS = Type II Secretion System

T3SS = Type III Secretion System

T5SS = Type V Secretion System

T6SS = Type VI Secretion System

Tle = Type VI Lipase Effector

Tmpr = Trimethoprim Resistance

TssA-M = T6SS Structural Proteins

Vfr = Virulence Factor Regulator

VgrG = Valine-Glycine Repeat Protein

VipA/B = T6SS Sheath Proteins (TssB/C)

Δ = Gene Deletion Marker

Chapter 1

1. Introduction

Microorganisms rarely live in isolation but rather in communities where individual microbes constantly engage in intercellular interactions. Such interactions affect the gene expression, biological activities, and survival of individual microbes and shape the structure and function of the microbial community (Aziz et al., 2015; Jakubovics, 2015). Bacteria can sense and react to the neighboring cells, and engage in cell-cell communication and social interactions with the adjacent cells (Henke & Bassler, 2004). Quorum sensing (QS) enables bacteria to communicate by secreting signaling molecules. Chemical signals, such as autoinducers, are produced to mediate communication between bacterial cells. There are two N-acyl-homoserine lactone based QS systems, known as rhl and las systems, present in *P. aeruginosa*. Another QS system, which is based on quinolone, also known as the PQS system (pqs), exists in *P. aeruginosa*. By modulating gene expression, bacterial intercellular interactions give rise to altered pathogenicity, changed susceptibility to antibiotics, and switched lifestyles (Waters & Bassler, 2005). Investigating the molecular basis and regulatory pathways of interspecies and intercellular interactions is essential to understanding and controlling bacterial activities and microbial communities.

1.1 Characteristics of the bacterium *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative pathogen that plays a major role in chronic polymicrobial lung infections in cystic fibrosis (CF) patients (Nakatsuka, Matsumoto, Inohara, & Nunez, 2023). It is also a common cause of pneumonia, urinary tract infections, and burn wound infections. Antimicrobial-resistant carbapenem *P. aeruginosa* has been recognized by the World Health Organization (WHO) as a high-priority pathogen that poses serious risks to global public health (WHO bacterial priority pathogens list, 2024). It forms biofilms, develops resistance to antibiotics, and produces an arsenal of virulence factors that make *P. aeruginosa* a major life-

threatening pathogen. Several QS system have been found in this bacterium that regulate gene expressions related to virulence and biofilm formation. Currently, four QS systems, namely *pqs*, *rhl*, *las*, and *iqs* are involved to regulate the expression of more than 300 genes connected with the virulence factors in *P. aeruginosa* (Smith & Iglewski, 2003). These systems are interconnected with each other and specific to *P. aeruginosa*. Among these, the *rhl* and *las* systems occupy the highest level of the hierarchy. The signaling network of *P. aeruginosa* is one of the most complex and diverse systems known among all microorganisms. Thus, the signaling molecules and their relevance to the QS system in signal integration, adaptation response, and virulence deserve further investigation for understanding the global virulence network of *P. aeruginosa*.

1.2 Key virulence factors of *P. aeruginosa*

P. aeruginosa harbors a plethora of virulence factors that contribute to its ability to cause successful infection in the host (Qin et al., 2022). The factors can be classified as cell-associated (pili, flagellum, lipopolysaccharide [LPS], alginate, other adhesins) and extracellular virulence factors (proteases, exotoxin A, exoenzyme S, hemolysins, phospholipases C, pyocyanin, pyoverdine) (Van Delden & Iglewski, 1998). Additionally, the bacteria use six types of secretion systems to inject bacterial toxins into host cells, causing host damage and helping them evade immune defenses. Flagellum, pili (type IV pili), and other types of adhesins contribute to increased antibiotic resistance and the development of persistent, potentially life-threatening nosocomial infections (L. Chen & Wen, 2011; Ozer et al., 2021). Type IV pili also contribute to the twitching motility of *P. aeruginosa* on solid surfaces or interfaces (Rashid & Kornberg, 2000). For *P. aeruginosa*, motility is an important aspect of pathogenicity, as it helps the

bacterium colonize different environments, attach to surfaces, and thus promotes the formation of biofilms (G. A. O'Toole & Kolter, 1998). Various forms of motility, including swimming, swarming, twitching, and sliding motility, are performed by *P. aeruginosa*. Swarming motility is prevalent in the lung environment, which resembles the mucous layers with epithelial surfaces at the top, a common site where *P. aeruginosa* commonly establishes infection in patients with CF (Hutchison & Govan, 1999). In the pathogenic process of *P. aeruginosa*, LPS interacts with host receptors and inhibits host defense systems. Specifically, the lipid A in LPS enables attachment, promotes tissue damage, and enhances recognition by host receptors (Park et al., 2022). Also, LPS contributes to the formation of biofilms and mediates resistance to antibiotics (Qin et al., 2022). Alginate is a polysaccharide that is important for the formation of biofilm (Mann & Wozniak, 2012) and is commonly associated with the mucoid phenotype observed in the strains isolated from CF patients (Gellatly & Hancock, 2013). Proteases are extracellular virulence factors that are responsible for tissue damage in the host as well as interfere with host antibacterial defense mechanisms (Oldak & Trafny, 2005). Added to that, proteases inactivate the components of the immune system. Alkaline protease (AprA) in *P. aeruginosa* has been associated with its virulence, evades the host immune system by escaping phagocytosis, and inhibits neutrophil-mediated killing (Laarman et al., 2012).

P. aeruginosa produces a variety of extracellular toxins, with exotoxin A and exoenzyme S being the most extensively studied and recognized for their roles in virulence. Exotoxin A has structural similarity with diphtheria toxin, which inhibits protein biosynthesis and causes cell death (Wick, Hamood, & Iglewski, 1990), whereas exoenzyme S is responsible for multiple deleterious effects in the host, leading to cell death (Hauser, 2009; Shaver & Hauser, 2004).

Sometimes, hemolysin, phospholipases, and rhamnolipid act synergistically to degrade lipids and lecithin. Among the phospholipases, *P. aeruginosa* produces both hemolytic phospholipase and non-hemolytic phospholipase, which have a homologous structure. These extracellular enzymes are necessary for twitching motility to promote tissue damage (Barker et al., 2004) and hydrolyze lung surfactant molecules (Lopez et al., 2011). *P. aeruginosa* produces pathogenic pigments like pyocyanin (blue-green) and pyoverdine (yellow-green). Pyocyanin is a phenazine exotoxin that imparts toxicity through the generation of reactive oxygen species by inactivating glutathione reductase in cystic fibrosis (Kasozi et al., 2013) and induces neutrophil apoptosis in the host (Allen et al., 2005). In patients with bronchiectasis, PCN has been shown to damage lung tissue during chronic infection, as demonstrated by *in vivo* studies (Gao et al., 2016). The key virulence factors of *P. aeruginosa* are summarized in **Figure 1.1**.

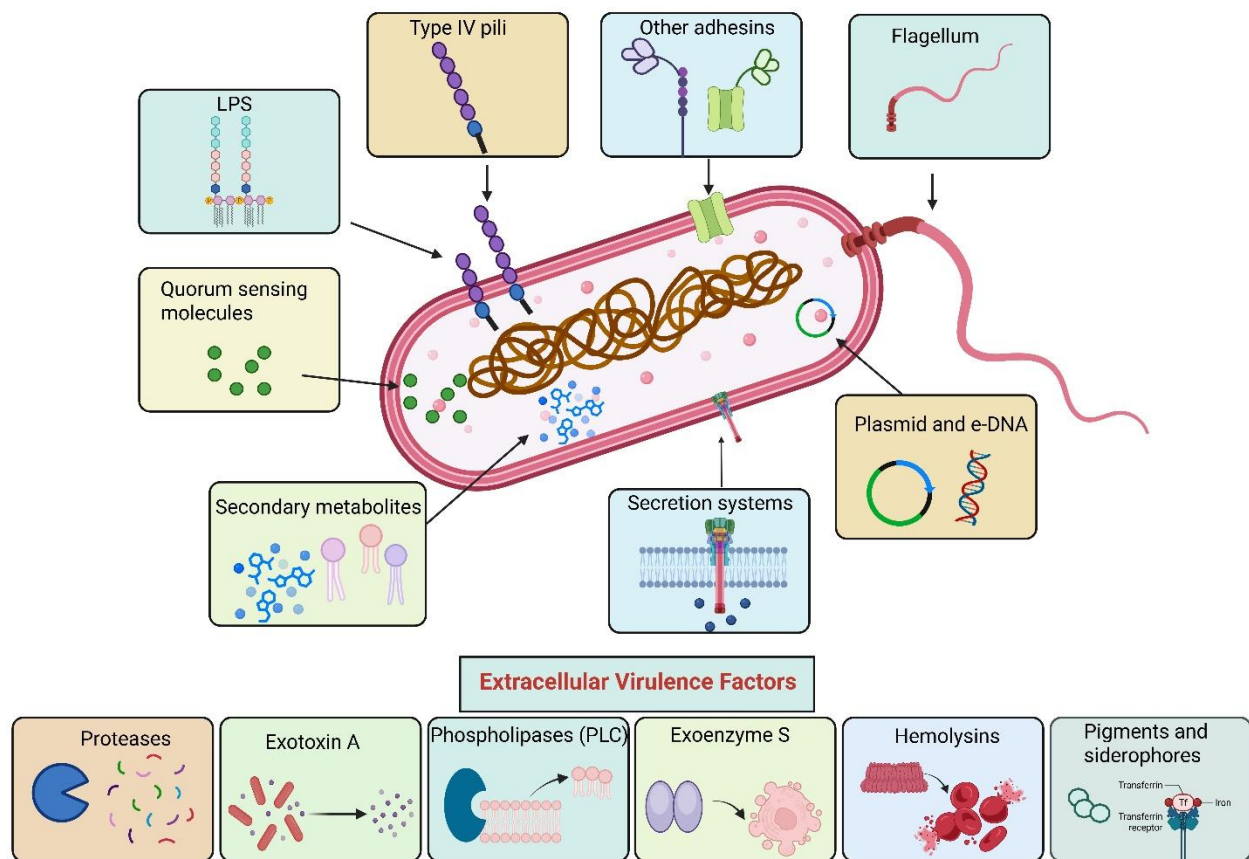


Figure 1.1 Key virulence factors associated with *P. aeruginosa* pathogenesis. *P.*

aeruginosa possesses a variety of virulence factors that contribute to its pathogenesis. Among these, cell-associated components alginate, flagellum, type IV pili, LPS, and adhesins are the key virulence determinants. Toxins, enzymes, and protein secretion systems collectively mediate host damage and facilitate immune evasion. Additionally, motility and biofilm formation enhance bacterial colonization, persistence, and chronic infection. This illustration was prepared using BioRender.

1.3 Biofilm formation

Bacteria can sense and respond to environmental changes through a sensory system. By responding to environmental cues, bacteria can alter their metabolic pathways and physiological adaptations (C. D. Boyd & O'Toole, 2012). Biofilm formation is an adaptation that plays a vital role in bacterial pathogenesis. Biofilms are commonly described as surface-attached bacterial communities enclosed in a polymeric matrix. The matrix of these biofilms is usually comprised of the extracellular polymeric substances (EPS), which retards the diffusion of potential antibiotics into bacterial cells (Walsh, 2020). Bacteria can be thousands of times more resistant when in biofilm form than in planktonic form (Donlan, 2000). Biofilm formation is a developmental process that involves cell-surface attachment through both reversible and irreversible stages, microcolony formation, maturation of biofilm structure, and dispersion (Chua et al., 2014; G. O'Toole, Kaplan, & Kolter, 2000). According to the National Institute of Health, bacterial infections (up to 80%) are linked to the development of biofilms (Romling & Balsalobre, 2012). A proper understanding of controlling biofilm formation is needed for developing an *in vivo* model for biofilm studies and developing novel treatment strategies for biofilm-related infections caused by *P. aeruginosa*. Second messengers play key role in biofilm regulation and, consequently, influence biofilm-associated infections. Second messengers include c-di-GMP, c-di-AMP, c-AMP, and (p)ppGpp, altogether regulate major regulatory pathways involved in biofilm development. Second messengers modulate biofilm determinants by flagellar rotation, production of exopolysaccharide, and other stress responses, such as biofilm dispersion and production of secondary metabolites (Romling, Galperin, & Gomelsky, 2013).

1.3.1 Role of second messengers in biofilm regulation

1.3.1.1 c-di-GMP

Bis-(3'-5')-cyclic dimeric guanosine monophosphate is a soluble second messenger molecule (c-di-GMP) that modulates cell cycle, biofilm formation, biosynthesis of surface adhesins, production of exopolysaccharides, and regulates different types of motilities in a range of bacteria (C. D. Boyd & O'Toole, 2012). In addition, this intracellular molecule coordinates the change of lifestyle in bacteria from their motile planktonic to a sedentary immobile form (Romling et al., 2013).

1.3.1.1.1 Synthesis and breakdown of c-di-GMP

Phosphodiesterases (PDEs) and diguanylate cyclases (DGCs) are two types of enzymes which control c-di-GMP levels. The GGDEF domain present in DGCs combines two GTP molecules to produce c-di-GMP (Simm et al., 2004). In contrast, Phosphodiesterases break down c-di-GMP into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) and/or GMP (R. Paul et al., 2004). Amino acid motifs such as the EAL domain of PDE (Christen, Christen, Folcher, Schauerte, & Jenal, 2005) hydrolyze and linearize the second messenger molecule cyclic di-GMP to pGpG (**Figure 1.2**). On the contrary, the second messenger is converted to guanosine monophosphate by PDEs with the HD-GYP motif. This second messenger interacts with several effectors in the cell to conduct a variety of regulatory functions. DGC and PDE modulate c-di-GMP through their catalytic action, while effectors that interact with c-di-GMP govern its production. They convert high or low concentrations of the molecule into distinct phenotypes or functional changes.

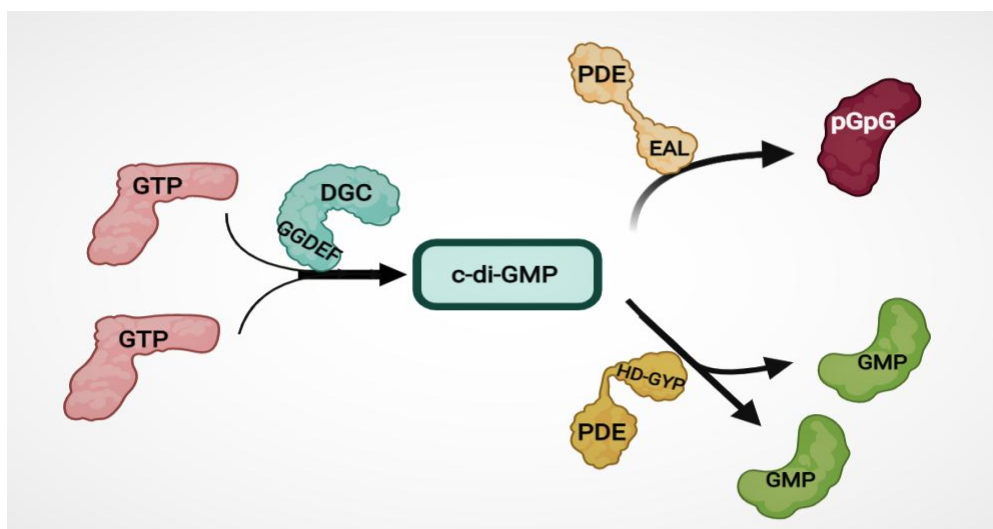


Figure 1.2 Biosynthesis and degradation of c-di-GMP. Two GTP units are combined to generate c-di-GMP by the GGDEF domain found in DGCs. Phosphodiesterases degrade c-di-GMP to produce GMP and/or 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG). Amino acid motifs that linearize the second messenger molecule to pGpG include the EAL domain of PDE. PDEs with the HD-GYP motif convert the second messenger to guanosine monophosphate.

1.3.1.1.2 Role of c-di-GMP in biofilm regulation in *P. aeruginosa*

Biofilms are microbial communities attached to living or non-living surfaces where cells are covered with a polymeric matrix by the bacteria (Costerton, Stewart, & Greenberg, 1999). Though bacteria prefer to be in such a community to survive harsh environments, biofilm formation has been mostly associated with notorious chronic infections to date. Added to that, microbes gain increased tolerance against antibiotics, which renders these microbial infections hard to treat (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995). Hence, characterization of c-di-GMP is important to find out regulatory pathways involved in biofilm formation. High levels of this second messenger have been linked to the formation of biofilm,

whereas low levels affect motility and planktonic formation (Hengge, 2009).

A few developmental processes are involved in the formation of microbial biofilm, and these steps are extensively studied in the model organism *P. aeruginosa*. First, the bacteria attach to the surface, then form microcolonies and eventually macrocolonies containing thick layers of bacteria, and finally disperse to continue planktonic life (Ha & O'Toole, 2015). In the attachment stage, *P. aeruginosa* first attaches reversibly on contact with a surface by utilizing flagellar-mediated motility (Y. Liang, Hilal, Langston, & Starov, 2007). When c-di-GMP is released in higher amounts, it creates a complex with FleQ, a transcriptional regulator of flagella machinery. This complex, known as FleQ-c-di-GMP, represses the genes essential for the assembly of flagella (Baraquet & Harwood, 2013). Another protein named YcgR binds with c-di-GMP, also interacts with both FliG and MotA, a motor and stator protein, respectively, and reduces motility (K. Paul, Nieto, Carlquist, Blair, & Harshey, 2010). Consequently, c-di-GMP levels are inversely connected with motility.

Following this step, where bacteria attach irreversibly to the surface, the second messenger regulates several pathways that help curb twitching and swarming motility. Moreover, studies indicate the involvement of extracellular polysaccharide (EPS) and extracellular DNA (eDNA) in this attachment process, which is regulated by c-di-GMP (Ha & O'Toole, 2015). After attachment, *P. aeruginosa* tends to form microcolonies and macrocolonies where Pel and Psl sugars play critical roles by forming EPS. WspR, a modulator of EPS production, plays a role in time-dependent expression of the Pel/Psl system by triggering c-di-GMP production through increasing DGC activity, which is evident by “wrinkly colony” phenotype (Huangyutitham, Guvener, & Harwood, 2013). As mentioned, this second messenger boosts the efficiency of Psl.

and the resultant EPS then accelerates the production of this second messenger, catalyzed by the enzymes named SiaD and SadC, forming positive feedback (Irie et al., 2012). Meanwhile, the biofilm matures, Psl expression is enhanced in the periphery of microcolonies and harnesses the EPS with the help of CdrA in a process that is c-di-GMP-based (Borlee et al., 2010). CdrA maintains the structure of macrocolonies and strengthens the attachment of biofilm to the surface. In the final stage, dispersal of bacteria occurs in a restricted manner to resume their planktonic form. In *P. aeruginosa*, DGC activity controls c-di-GMP, leading to the breakdown of BdlA, which promotes bacterial dispersal. Additionally, DipA, a protein expressed in the late stage of mature biofilm, modulates the expression of c-di-GMP, thus promoting the dispersal mechanism (Petrova & Sauer, 2012).

1.3.1.2 cAMP

Another important second messenger named cAMP, found in different bacterial species, essential for the modulation of biofilm with the transcriptional receptor protein called CRP. This molecule (cAMP-CRP) works as a catabolic repressor of carbon and modulates the expression of genes when carbohydrates become available.

In *E. coli*, biofilm formation is modulated by various carbon sources (C. Liu, Sun, Zhu, Liu, & Liu, 2020). cAMP-CRP auxiliary functions to regulate biofilm by three regulatory pathways (**Figure 1.3**). Firstly, it regulates the synthesis of an extracellular matrix known as curli fiber that helps in the process of surface attachment during biofilm formation. cAMP-CRP can turn on the transcriptional pathway of *csgD*, an important regulator that controls the operon responsible for curli fiber production (Hufnagel et al., 2016). Secondly, this second messenger can modulate the expression of the flagellum, which helps in the process of motility and the initial biofilm

attachment stage. cAMP-CRP works as a positive regulator for *flhDC*, which controls the motility and thus helps in biofilm formation (Guttenplan & Kearns, 2013; Soutourina et al., 1999). Lastly, the formation of biofilm is inhibited by *rpoS*. cAMP-CRP actively inhibits the transcriptional pathway of *rpoS*, and as a result, biofilm formation is restored (Corona-Izquierdo & Membrillo-Hernandez, 2002).

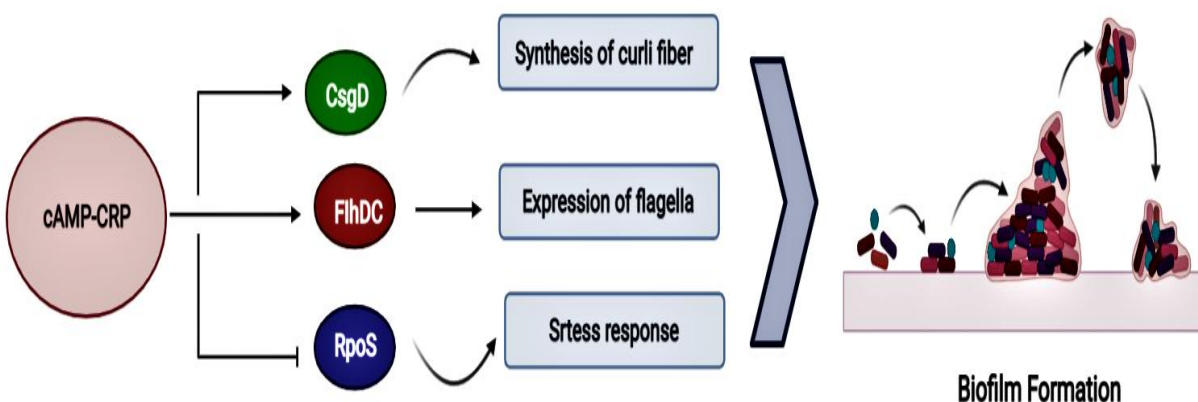


Figure 1.3 Regulation of biofilm through cAMP-CRP. Different carbon sources influence the production of biofilms, and this regulation is further coordinated by auxiliary cAMP-CRP activities, which control biofilm formation through three mechanisms. Specifically, cAMP-CRP modulates biofilm development via three transcriptional pathways: CsgD, FlhDC, and RpoS. The figure was prepared using BioRender.

In *P. aeruginosa*, the association of cAMP and biofilm formation is well-developed. Acetate or intermediates of the TCA pathway are used as a carbon source, which differs from *E. coli*. Vfr, also known as virulence factor regulator, is one of the allosteric catalyzers for cAMP, and the role of cAMP-Vfr is associated with biofilm surface attachment (Gosset, Zhang, Nayyar, Cuevas, & Saier, 2004). The biofilm regulation by cAMP is governed in the following way. First, flagella

help bacteria attach to the surface, and after a certain time, when the flagellar load reaches a higher concentration, it increases interaction between two molecules known as FimV and FlhF (Schniederberend et al., 2019). This causes an increase in cAMP level by activating CyaA and CyaB. Following that, cAMP-Vfr can negatively modulate the expression of the regulator responsible for flagella synthesis, and thus, flagellar biosynthesis is inhibited (Wolfgang, Lee, Gilmore, & Lory, 2003). In the meantime, cAMP-Vfr positively regulates the synthesis of type IV pili, which in turn activate CyaB to produce supplementary cAMP for *P. aeruginosa* (Persat, Inclan, Engel, Stone, & Gitai, 2015). Furthermore, cAMP-Vfr promotes the biosynthesis of Type IV pili genes and other virulence-controlling genes responsible for the formation of biofilm. In addition, environmental clues can also instigate the biofilm-forming pathway by attaching to a solid surface, which serves as a primary signal (Belas, 2014).

1.3.1.3 c-di-AMP

Nucleotide second messenger is cyclic dimeric adenosine 3'–5'-monophosphate known as c-di-AMP, which plays pivotal role in bacterial physiology and metabolism, cell cycle regulation, transcriptional pathway, and biofilm formation. The biosynthesis and breakdown pathway of this second messenger is governed by diadenylate cyclases (DC) alongside phosphodiesterases (PDEs) (Commichau, Dickmanns, Gundlach, Ficner, & Stulke, 2015). It has been found in different bacterial species, including *S. mutans*, *B. subtilis*, *S. pneumoniae*, *L. monocytogenes*, *L. lactis*, and *S. aureus*.

During bacterial cell growth and metabolism, polymeric sugar residues are released, which are known as exopolysaccharides (EPSs). EPSs can determine the regulatory pathway of biofilm formation. Studies have shown that EPSs are modulated by c-di-AMP and thus control biofilm

formation (Xiong et al., 2020). In *Streptococcus mutans*, biofilm formation is inhibited when GdpP (domain of DAC) is released in low concentration. c-di-AMP can also modulate the backbone protein responsible for biofilm formation. In *B. subtilis*, EPSs can be produced by a c-di-AMP-linked protein called CabpA, which can participate in biofilm formation (Peng, Li, & Xu, 2017).

Another important EPS is poly-N-acetyl glucosamine, commonly known as PNAG, which is essential for the biofilm regulatory pathway. The biosynthesis of EPS is dependent on an operon system called *epsA-O*. This second messenger can control the expression of this operon, and at the same time, it can also modulate SinR, which is a transcriptional regulator. The expression of the gene is suppressed when SinR attaches to the promoter region of *epsA-O* (Gundlach, Rath, Herzberg, Mader, & Stulke, 2016). Thus, SinR works here as a suppressive regulator. SinR contains DNA-binding capacity, which is inhibited by SlrR, and thus, the gene responsible for autolysin and motility is also repressed by this phenomenon. In *B. subtilis*, c-di-AMP negatively regulates biofilm formation steps. The production of *slrR*, as well as the *epsA-O* operon, is significantly reduced when the cell stores c-di-AMP, and as a result, biofilm formation is hindered (Gundlach et al., 2016). Nonetheless, the underlying mechanisms by which c-di-AMP influences the modulation of SinR are still not clear.

1.3.1.4 (p)ppGpp

(p)ppGpp is a nucleotide second messenger initially found in *E. coli* and consists of two modified nucleic acids, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), combinedly and known as (p)ppGpp. (p)ppGpp regulates bacterial physiology by altering cellular processes (transcription, translation) and virulence by modulating signaling

pathways. Biofilm formation can be regulated in different bacterial species by (p)ppGpp, which includes *S. mutans*, *V. cholerae*, *L. monocytogenes*, and *E. coli* (Romling & Balsalobre, 2012). Most of the studies indicate a significant reduction in biofilm formation when the cell does not have (p)ppGpp. In *E. coli*, Type I fimbriae promote biofilm formation, which in turn is directly regulated by (p)ppGpp (Aberg, Shingler, & Balsalobre, 2006). Indirect regulation can be seen for other bacterial species for controlling the gene responsible for biofilm formation. Two enzymes are responsible for regulating the activity of (p)ppGpp. Those enzymes include RelA and SpoT. Repression of *relA* in *S. mutans* showed a clear reduction in biofilm by downregulation of the quorum-sensing *lux* gene (Lemos, Brown, & Burne, 2004). Studies showed that the CsrA pathway plays a negative role in biofilm formation. In *E. coli*, (p)ppGpp can control the expression of RNAs that can restrain the functional activity of CsrA, thus affecting the biofilm-forming property (Dalebroux & Swanson, 2012). In *P. aeruginosa*, (p)ppGpp contributes to biofilm development and antibiotic resistance, in part by limiting the release of quinolone compounds (Nguyen et al., 2011). Hence, (p)ppGpp is omnipresent in different bacterial species and plays pivotal role in biofilm formation and bacterial virulence properties.

1.4 Protein secretion systems

Protein secretion systems, known as molecular nanomachines, are effective mechanisms for thriving within the environment (Green & Mecsas, 2016). In *P. aeruginosa*, the cell envelope of protein secretion systems is composed of two hydrophobic membranes, where the periplasm and the hydrophilic space serve as a separation barrier (Filloux, 2011). This complex and dynamic secretion system helps to deliver virulence factors by injecting them into the host cell membranes and cytoplasm (Galan & Waksman, 2018). Among the different protein secretion

systems, *P. aeruginosa* uses five major systems: T1SS, T2SS, T3SS, T5SS, and T6SS (Bleves et al., 2010). Each varied in their roles in protein transport, adaptability, and contributions to virulence under environmental conditions. In this study, I will be focusing on the two protein secretion systems, T3SS and T6SS, and their functions and regulations will be discussed in detail in the following sections of my thesis.

1.4.1 Type 3 secretion system in *P. aeruginosa*

P. aeruginosa utilizes a macromolecular structure, named the T3SS, which was first discovered in 1996 and structurally visualized in 2005 (Pastor, Chabert, Louwagie, Garin, & Attree, 2005). This syringe-like export machine enables bacteria to transport effector proteins directly into the cytosol of host cells in both animals and plants (Mattei et al., 2011). T3SS is widely conserved among Gram-negative bacterial species and is believed to have evolved from the bacterial flagellum machinery (Cornelis, 2006). Effector proteins are injected into a range of cells, including endothelial cells, epithelial cells, and immune cells, leading to host cell death and thus impeding bacterial clearance (Hauser, 2009).

1.4.1.1 Components of T3SS

Within the T3SS, two main components work in concert to deliver effector proteins into the host cytoplasm: the secretion apparatus, which mediates the transport of effectors across the bacterial membranes, and a translocon, which facilitates their entry into cell membrane of the host (Cornelis, 2006). In *P. aeruginosa*, the T3SS and its associated regulatory components are encoded by approximately 43 genes (Frank, 1997). These genes are located on five operons and collectively encode proteins involved in secretion, translocation, and regulation of T3SS function

in a coordinated manner.

The needle complex is composed of around 25 diverse proteins building the basal body, including a multi-ring and a protruding needle structure. The basal body anchors the secretion apparatus into the bacterial membrane (Cornelis, 2006) and includes an inner membrane ring that is formed by the lipoprotein PscJ (Burns, McDaniel-Craig, & Sukhan, 2008) and an outer membrane ring composed of secretin protein, PscC (Tosi et al., 2014). The needle itself is assembled through the polymerization of PscF subunits, forming a hollow shape through which effector proteins are translocated from the basal body. The needle structure also detects contact with the host cell, thereby triggering the secretion process (Pastor et al., 2005).

The translocation structure is mainly composed hydrophobic proteins namely, PopB and PopD, and PcrV, serves as a hydrophilic protein, all of which are secreted by T3SS. PopD and PopB act synergistically to promote membrane permeabilization (Mattei et al., 2011), facilitating the transportation of effector proteins across the host cell membrane via the needle complex and delivery of the effectors into the host cytosol. Studies have suggested that PcrV plays an important part in ensuring proper assembly and activity of the PopB/D translocon (Goure et al., 2004).

PscN, which is a cytoplasmic ATPase, enables the identification of effector/chaperone complexes and unfolds secreted proteins, preparing them for export. PscN binds T3SS substrates and hydrolyzes ATP to power the secretion process. ATP hydrolysis facilitates chaperone release and substrates unfolding, enabling their transportation through the needle apparatus (Ngo et al., 2020). In addition, PscN is responsible for substrate recognition and binding, thereby initiating ATP hydrolysis and regulating effector secretion. Protein secretion proceeds in an ordered

sequence, contributing to a hierarchical secretion mechanism. During T3SS assembly, bacteria first construct a needle-like apparatus, facilitate the formation of a translocation pore in the host cell membrane, and subsequently inject effector proteins. After the needle reaches to its expected length, secretion is regulated by the PopN complex, composed of Pcr1, Pcr2, PscB and PopN. The PopN complex interacts with Pcr1 and Pcr2 to repress PscN activity and modulate the ATPase's substrate specificity (Ngo et al., 2020). However, the role of PscN as an energy source for T3SS remains incompletely understood (Lee & Rietsch, 2015), and additional research is needed to determine its exact function.

1.4.1.2 Effectors of T3SS

To date, *P. aeruginosa* T3SS have been characterized by four effector proteins which include ExoS, ExoT, ExoU, and ExoY. While most *P. aeruginosa* strains contain the genes encoding the machinery of T3SS, they often do not have a complete set of all effector genes (Feltman et al., 2001; Fleiszig et al., 1997). Among the strains that cause acute infections, the *exoS* is detected in 58–72% of strains, the *exoU* in 28–42%, the *exoY* in approximately 89%, and the *exoT* in 92–100% of isolates. Typically, most strains express ExoT and ExoY, along with either ExoS or ExoU (Hauser, 2009). During infection, a strain's phenotype is usually determined by its secreted set of effector proteins (Schulert et al., 2003). For instance, strains expressing ExoS are associated with delayed host cell death exhibiting features of apoptosis, whereas ExoU-harboring strains induce rapid and extensive host cell lysis. The effector proteins of T3SS are shown in

Figure 1.4.

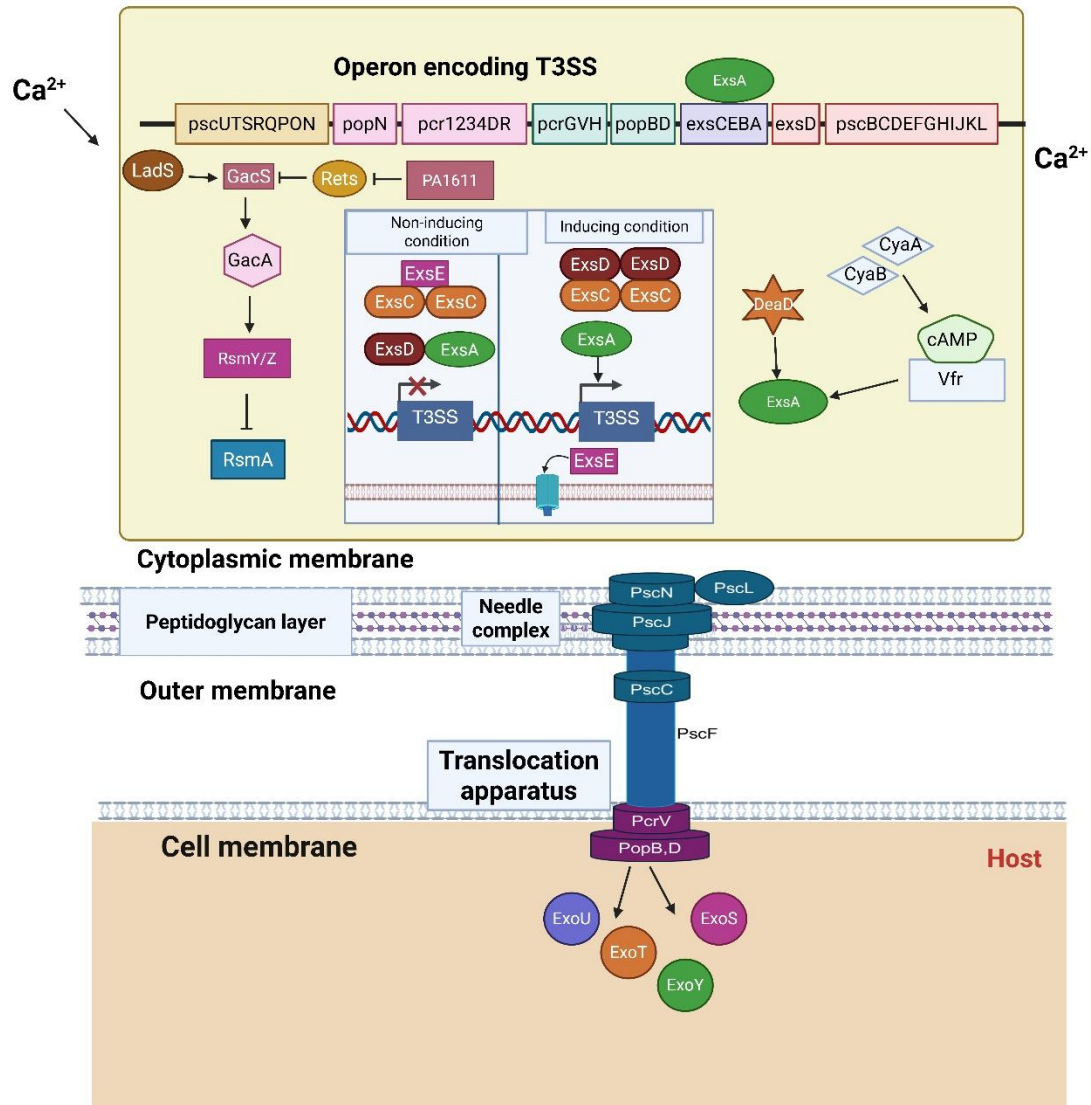


Figure 1.4 Schematic representation of the T3SS in *P. aeruginosa*. The structural components of T3SS include three major parts, namely a needle complex and translocation machinery that help to inject toxins into host cells. Four effectors are found in the T3SS, which include ExoS, ExoU, ExoT, and ExoY. The regulations of T3SS are governed by the master regulator ExsA. Three more proteins (ExsC, ExsD, and ExsE) are involved in a partner-switching system that controls ExsA itself. Other regulators of T3SS includes GacS/A-RsmA Pathway, where RetS, PA1611, and LadS play a significant role. The figure was prepared using BioRender.

ExoS and ExoT have shown amino acid similarity with each other with a percentage of approximately 76% and are classified as bifunctional toxins. They possess an N-terminal Rho GTPase activating protein (GAP) domain and a C-terminal ADP ribosyltransferase (ADPRT) domain (Hauser, 2009). These enzymes work cooperatively to interrupt the function of actin cytoskeleton in the host cell, inhibit the process of phagocytosis, and ultimately lead to cell death (J. T. Barbieri & Sun, 2004).

Both effectors contain several structural features in common, including an N-terminal secretion signal (1-15 residues in ExoS, 1-50 residues in ExoT), enzymatic domains (GAP and ADPRT), a membrane localization domain (51-72 residues in both ExoS and ExoT), a chaperone binding domain, and a binding site for cofactor. The function of the secretion signal is to direct effectors to the T3SS structure. The chaperone binding part of ExoS interacts with SpsA, which facilitates effector stability and secretion by maintaining a secretion-competent conformation and ensuring their appropriate localization to the host cell membrane (Shen et al., 2008). Following translocation, ExoS initially localizes to its plasma membrane before trafficking to intracellular compartments in the host cell, such as the Golgi/endoplasmic reticulum and endosomes. Its localization at the host cell membrane is required for its GAP activity, whereas its association with intracellular compartment membranes supports ADPRT activity (Anantharajah, Mingeot-Leclercq, & Van Bambeke, 2016).

Both ExoS and ExoT utilize their GAP activity to inactivate Rho family GTPases, including Rho, Rac, and Cdc42, thereby impairing phagocytosis (Frithz-Lindsten, Du, Rosqvist, & Forsberg, 1997). Their ADP-ribosyltransferase (ADPRT) requires interaction with the host cell cofactor 14-3-3 for activation (S. Liu, Yahr, Frank, & Barbieri, 1997). Despite their structural

similarity, ExoS and ExoT differ in substrate specificity and functional outcomes. ExoT targets a limited set of proteins in the host cell, including the CT10 regulator of kinase (CRK) I and CRKII adaptor proteins (Sun & Barbieri, 2003), leading to disruption in phagocytosis and induction of apoptosis and impairment of epithelial barriers, which facilitates bacterial dissemination. ExoS has broader substrate specificity, induces apoptosis more rapidly, inhibits DNA synthesis, and hinders vesicular trafficking (A. M. Barbieri, Sha, Bette-Bobillo, Stahl, & Vidal, 2001; Deng & Barbieri, 2008). Additionally, extracellular ExoS can promote T cell apoptosis and stimulate the release of proinflammatory cytokines from monocytes. Taken together, these activities shed light on the ability of T3SS effector proteins to manipulate cellular processes and factors to establish successful infection in the host.

ExoU is a highly potent cytotoxin that exhibits phospholipase A2 (PLA2) activity and rapidly causes host cell lysis and is associated with severe disease outcomes. It is a 687 amino acid protein that requires superoxide dismutase 1 (SOD1) from the host cell as a cofactor for activation (Anantharajah et al., 2016). ExoU contains a signal for secretion that directs it to the T3SS machinery (positioned within the first 15 residues), as well as a chaperon-binding domain (residue 3-123) that interacts with SpcU (Finck-Barbancon, Yahr, & Frank, 1998). Adjacent to this region is a patatin-like domain (107-357 residues) responsible for its PLA2 activity (Phillips, Six, Dennis, & Ghosh, 2003; Sato et al., 2003), followed by a membrane localization domain. ExoU localizes to the host cell plasma membrane as well, which is similar to ExoS (Veesenmeyer et al., 2010). Its lipase activity targets a broad range of substrates which includes neutral lipids, lysophospholipids, and phospholipids (Tamura et al., 2004).

Fractionation analysis of host cell extracts identified Cu^{2+} , Zn^{2+} superoxide dismutase (SOD1) as

a host cofactor and is required for ExoU activation (Sato, Feix, & Frank, 2006). Notably, the enzymatic activity of SOD1 is not required for this process, suggesting that ExoU activation occurs through alternative mechanisms, such as conformational changes or interactions that help association with other host cofactors or phospholipid substrates. However, the domain of ExoU that specifically interacts with SOD1 has not yet been characterized.

Upon entry into the host cell, ExoU undergoes ubiquitination by two ubiquitin moieties that are conjugated to its Lys178. However, this modification does not appear to affect its cytotoxic activity or subcellular localization (Stirling, Cuzick, Kelly, Oxley, & Evans, 2006). Overall, intoxication with ExoU results in the rapid death of the host cell. This host cell death is characterized by a rapid rupture of cell membrane integrity just within 1 to 2 hours, in contrast to the slower cytotoxic effects mediated by ExoS and ExoT (Finck-Barbancon et al., 1997; Hauser, Kang, & Engel, 1998). This rapid lytic activity targets both epithelial barriers and phagocytes, hence encouraging bacterial survival, dissemination, and persistence (M. H. Diaz et al., 2008; Kurahashi et al., 1999; Zaborina et al., 2006).

ExoY is a secreted adenylate cyclase that requires a eukaryotic cofactor for its activation, although the identity of the cofactor is not known to date. Structurally, ExoY consists of two domains that are similar to the adenylate cyclase toxins of *Bacillus anthracis* (edema factor) and *Bordetella pertussis* (CyaA) (Yahr, Vallis, Hancock, Barbieri, & Frank, 1998). More recent studies have demonstrated that ExoY preferentially produces cyclic nucleotides such as cGMP and cUMP (Beckert et al., 2014). The protein is composed of 378 amino acids and harbors two domains of adenylate cyclase that function together to bind ATP.

1.4.1.3 Regulation of T3SS

In response to both extracellular and intracellular cues, a complex signaling network regulates T3SS expression in *P. aeruginosa*. While several pathways regulate T3SS gene expression exclusively, other pathways act broadly through global regulatory mechanisms that coordinate the expression of several virulence factors (Horna & Ruiz, 2021; Yahr & Wolfgang, 2006). Regulation of T3SS is evident at different levels, including transcriptional, post-transcriptional, and post-translational stages in *P. aeruginosa*. Activation of the secretion process strongly induces the expression of T3SS genes (McCaw, Lykken, Singh, & Yahr, 2002; Yahr, Goranson, & Frank, 1996), leading to the high-level production of T3SS components, particularly following contact with host cells. This highlights the close coupling between T3SS transcription and its secretory activity.

The master transcriptional regulator ExsA operates within a complex regulatory cascade to control T3SS gene expression (M. R. Diaz, King, & Yahr, 2011). ExsA belongs to the class of the AraC family of transcriptional activators. It directly controls transcription of genes encoding T3SS structural components and effector proteins (Zheng et al., 2007). T3SS expression is influenced by environmental signals, such as Ca^{2+} depletion, the presence of serum, and contact with eukaryotic host cells (Anantharajah et al., 2016). ExsA binds to regions of promoter that control the expression of T3SS genes, where a conserved binding site (TNAAAANA) is positioned around 15 base pairs upstream of the -35 region. RNA polymerase binds that region, thereby facilitating transcriptional activation (Hovey & Frank, 1995; Yahr & Frank, 1994).

Three proteins, ExsC, ExsD, and ExsE, coordinate a “catch and release” mechanism that links ExsA-dependent transcription to T3SS secretion. ExsA is inhibited by the “anti-activator,” which

is ExsD, which represses ExsA-dependent transcription under non-secreting conditions (McCaw et al., 2002). On the contrary, ExsC functions as an “anti-anti-activator,” by attaching to and sequestering ExsD, thereby relieving ExsA inhibition (Dasgupta, Lykken, Wolfgang, & Yahr, 2004). However, this molecular interaction is regulated by ExsE, which has a higher binding affinity to ExsC and prevents it from interacting with ExsD (Rietsch, Vallet-Gely, Dove, & Mekalanos, 2005; Urbanowski, Lykken, & Yahr, 2005). As a result, ExsE acts as a negative regulator of ExsC.

Under conditions that stimulate secretion, intracellular levels of ExsE are reduced as it is secreted from the bacteria into either the extracellular environment or into host cell cytoplasm, thereby linking secretion with transcriptional regulation (Urbanowski, Brutinel, & Yahr, 2007). The depletion of ExsE allows ExsC to bind ExsD, releasing ExsA from inhibition, enabling continuous transcriptional activation of T3SS genes. Through this regulatory mechanism, production of T3SS components, which include needle structure and effector proteins, is maintained by low basal where inducing signal lacks. When bacteria are in contact with host cells, the secretion is triggered, leading to a rapid upregulation in expression of T3SS genes. Additionally, ExsA expression is modulated at the post-transcriptional level by the RNA helicase, DeaD. This helicase enhances ExsA production by resolving an inhibitory secondary structure in the mRNA of *exsA* that would otherwise block its translation (Intile, Balzer, Wolfgang, & Yahr, 2015).

GacS/A-RsmA pathway represents another key regulatory system controlling T3SS expression. It has been observed that elevated levels of RsmA, a post-transcriptional regulatory protein, promote T3SS gene expression. Whereas reduced levels of RsmA favor the production of

polysaccharides such as PSI, thus suppressing T3SS expression. The sensor kinases LadS and RetS relay external signals to the GacA/GacS two-component system, which in turn modulates downstream gene expression. RetS promotes T3SS gene expression and motility-associated functions while repressing genes that are involved in biofilm formation, in part by inhibiting the expression of RsmY/Z, favoring acute infection stages. In contrast, LadS acts oppositely, enhancing biofilm formation and repressing T3SS expression through RsmA during chronic infections (A. L. Goodman et al., 2004; Laskowski, Osborn, & Kazmierczak, 2004; Ventre et al., 2006; Zolfaghar et al., 2005). It was also evidenced through several studies that the T3SS regulation is performed by RetS/GacSA/LadS via the cAMP/Vfr pathway (M. R. Diaz et al., 2011). Collectively, these regulatory pathways are pivotal in modulating the switch between acute and chronic phase infections in bacteria.

The transition between chronic and acute infection states is further controlled by a hybrid sensor kinase, PA1611, through modulation of the RsmAYZ in *P. aeruginosa*. By interacting with RetS, PA1611 limits its activity on acute virulence factors under chronic conditions, thereby contributing to virulence regulation through the RetS-PA1611 and RetS-GacS signaling pathway (Kong et al., 2013). Moreover, PA1611 and RetS interaction has also been implicated in the type 3 secretion system regulation and expression (Bhagirath et al., 2017). Additionally, the Gac system is linked to cyclic-di-GMP (c-di-GMP) signaling, as evident by the high levels of c-di-GMP influence RsmY and RsmZ to regulate T3SS activity (Moscoso, Mikkelsen, Heeb, Williams, & Filloux, 2011).

The cAMP-dependent signaling pathway can also regulate T3SS. Under inducing conditions, the adenylate cyclases, CyaB and CyaA, are both activated, increasing intracellular cAMP levels

(Wolfgang et al., 2003). CyaB is an inner membrane adenylate cyclase, and CyaA is a cytoplasmic class I adenylate cyclase contributing to the production of cAMP. It is assumed that cAMP allosterically regulates Vfr, a cAMP-dependent transcription factor. Vfr plays an essential role in the regulation of an array of genes associated with type 3 secretion system virulence, such as type 4 pili (T4P), pyocyanin production, flagellar biogenesis, protease activity, and production of exotoxin A via the quorum-sensing system known as LasR/LasI. Furthermore, the cAMP-Vfr complex enhances *exsA* transcription from a newly recognized promoter, which is located immediately upstream of *ExsA* (Marsden et al., 2016).

1.4.2 Type 6 secretion system in *P. aeruginosa*

T6SS is widely distributed in Gram-negative bacteria such as *P. aeruginosa*. This system confers a survival advantage to its neighboring bacteria by injecting toxins into the host cell (L. Chen, Zou, She, & Wu, 2015). The effectors and regulators of T6SS provide mutual exclusivity among bacterial species (Habich, Chaves Vargas, Robinson, Allsopp, & Unterweger, 2025).

1.4.2.1 Components of T6SS

T6SS is a complex structure encoded by a cluster of contiguous genes. This molecular structure uses a contractile tail to puncture the target cell and inject deleterious toxins/effectors like bacteriophages. The core component of T6SS consists of thirteen essential genes named TssA to TssM, and these proteins are conserved in all type 6 secretion systems (Filloux et al., 2008; Boyer et al., 2009). Among these proteins, TssL and TssM are integral membrane proteins, and TssJ is a lipoprotein (Zoued et al., 2013). These proteins interact to make a membrane complex that acts as a platform to assemble the baseplate, serves as a docking site for the tail, and as a channel for the transit of the inner tube (Logger, Aschtgen, Guerin, Cascales, & Durand, 2016).

The inner tube comprises Hcp (Haemolysin coregulated protein) and VgrG (valine-glycine repeat G) proteins. Hcp proteins resemble phage tail protein λ , gpV, and form tubes as hexamers (L. Chen et al., 2015). The tip of the inner tube is formed by the VgrG protein trimers and is similar to the spike of bacteriophage T4, comprising the gp27/gp5 complex. This tip resembles a needle topped by a sharpening PAAR protein that can penetrate a target's cell membrane (Leiman et al., 2009; Shneider et al., 2013). The trimer of VgrG is also present in the baseplate used as a platform to assemble the tail (Logger et al., 2016). Moreover, other proteins present in the baseplate are TssE, TssF, and TssG subunits, which resemble the proteins of phage T4, namely gp25, gp6, and gp7, respectively. In addition, TssK is involved in connecting the baseplate to the membrane complex, and its function is still unknown. TssK subunit does not have many similarities to phage T4 proteins, namely gp8 or gp10 (Brunet, Zoued, Boyer, Douzi, & Cascales, 2015; Zoued et al., 2013). The interactions of TssE and TssK subunits with TssL, and of TssG and TssK subunits with TssM are needed for docking of the baseplate onto the membrane complex (Taylor et al., 2016). TssB and TssC subunits (also known as VipA/B components in *V. cholera*) are contractile proteins that form tube-like structures. Moreover, these subunits have much resemblance to the sheath protein of the bacteriophage, gp18. The sheath structure TssB/TssC contracts and provides energy to eject the Hcp/VgrG tail-spike structure to deliver harmful effectors, puncturing the target cell (Bonemann, Pietrosiuk, & Mogk, 2010). In connection to this, TssA links the structural sheath to the membrane complex by forming the structure of dodecamers (Planamente et al., 2016).

ClpV is known as a specialized AAA⁺ ATPase protein and plays essential role in the regulation and function of T6SS (Schlieker, Zentgraf, Dersch, & Mogk, 2005). It interacts through the N-

terminal domain with the complex (VipA/VipB), which is composed of two essential and conserved members of T6SS (Bonemann, Pietrosiuk, Diemand, Zentgraf, & Mogk, 2009). AAA⁺ proteins bind ATP through the conserved domain of AAA. This process converts ATP hydrolysis energy into mechanical force to secrete toxins and remodel substrates necessary for T6SS (Corbitt, Yeo, Davis, LeRoux, & Wiggins, 2018; Hanson & Whiteheart, 2005). ClpV, with the help of the TssC subunit, disassembles the sheath when contraction occurs in the sheath structure. Thus, TssC and TssB subunits can be recycled to be ready to translocate again. A study conducted by Planamente et al., 2016 showed the interaction between ClpV and TssA. This suggests the role of ClpV in disassembling the sheath structure and recycling other T6SS components. Protein repair, degradation, membrane fusion, and many other cellular processes are controlled by AAA⁺ proteins.

1.4.2.2 Effectors of T6SS

The proteins that are secreted by the secretion system are known as effectors. They can range from small proteins containing a single domain to large proteins containing multiple domains, comprising toxins and secretory components of T6SS (Russell, Peterson, & Mougous, 2014). Interestingly, these effectors strictly co-occur with cognate immunity proteins encoded by genes situated adjacent to the cognate effector genes. Effectors are inactivated by binding directly to the immunity proteins to avoid self-intoxication (Benz, Sendlmeier, Barends, & Meinhart, 2012). This co- occurrence of effector/immunity proteins upholds the ability of T6SS to assault both friend and enemy. The effectors of T6SS are illustrated in **Figure 1.5**.

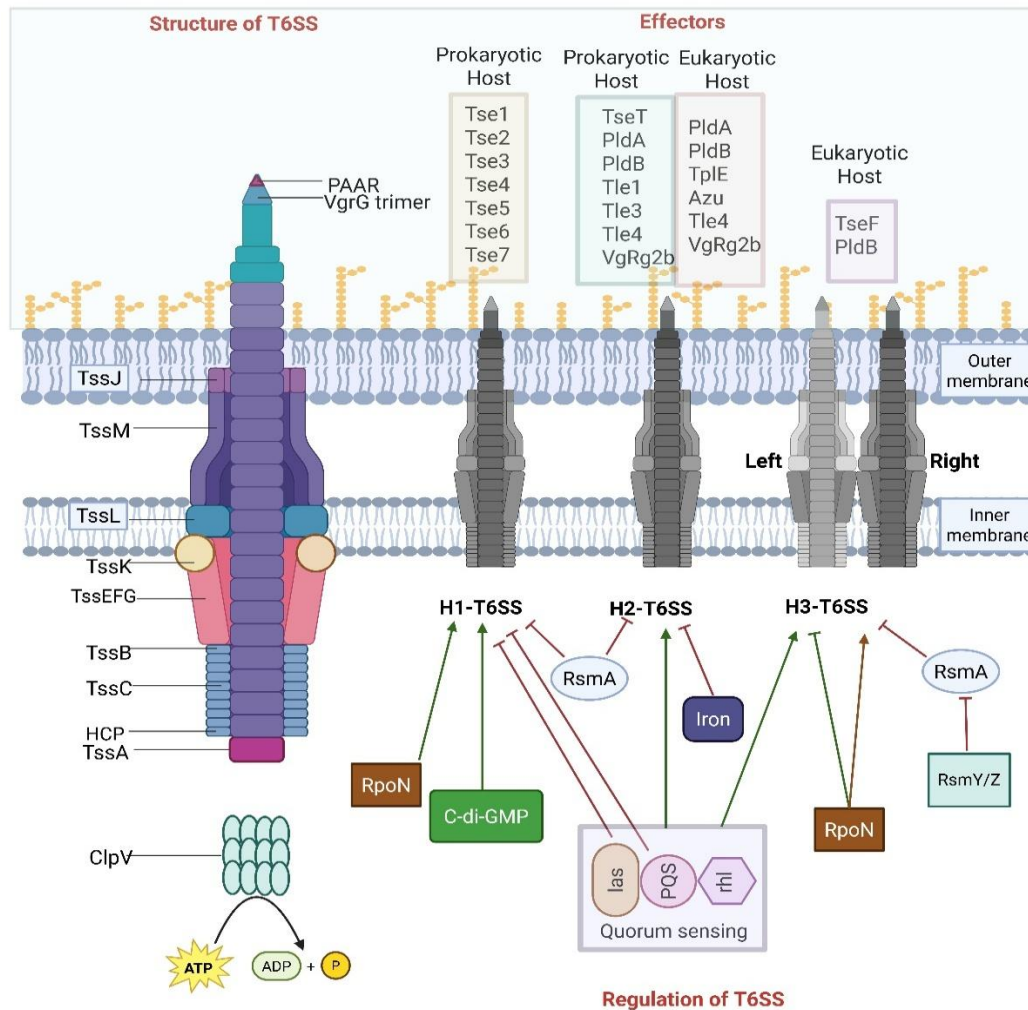


Figure 1.5 Schematic representation of the T6SS in *P. aeruginosa*. Three functionally different H1-T6SS, H2-T6SS, and H3-T6SS found in *P. aeruginosa*. The H1-T6SS is responsible for delivering Tse1-Tse7 toxins, into competing bacterial species and is primarily involved in interactions within the same species. In contrast, the H2-T6SS and H3-T6SS can act against both eukaryotic prokaryotic cells. The delivery of effectors is coordinated by H2-T6SS, including PldA, PldB, TplE and Azu, whereas the H3-T6SS contributes to iron acquisition through TseF. The major regulatory mechanisms controlling these T6SSs include QS molecules, the Gac-Rsm pathway, and the second messenger c-di-GMP. This figure was prepared using BioRender.

VgrG protein is the first identified effector (Pukatzki, Ma, Revel, Sturtevant, & Mekalanos, 2007).

The fold of a muramidase has been predicted in the C-terminal domain of VgrG3, which causes lysis when the effector is released in the periplasm and can degrade the cell wall peptidoglycan (Brooks, Unterweger, Bachmann, Kostiuk, & Pukatzki, 2013). A recent study observed that VgrG2b secreted from H2-T6SS can act as a trans-kingdom T6SS effector. Moreover, the C-terminal domain of VgrG2b effector represents a metallopeptidase that can elicit morphological abnormalities in bacterial periplasm, producing effects comparable to those of β -lactam antibiotics (Wood et al., 2019). Though Hcp is a secreted protein of T6SS, it works as a chaperone and important receptor for substrates of T6SS. Thus, Hcp acts as a gatekeeper protein of T6SS (Silverman et al., 2013).

Among the Tse effectors, Tse1 and Tse3 can cleave the peptidoglycan of the bacterial cell wall by lysing the target cells. Tse2 targets bacterial cell membranes and eliminates other bacteria, thereby providing *P. aeruginosa* with a fitness advantage (M. Li et al., 2012). *P. aeruginosa* produces immunity proteins called Tsi (Tse-specific immunity protein), which can diminish the toxicity of Tse effectors and save itself from Tse1-7 (Pissaridou et al., 2018; Russell et al., 2011). Among other effectors, H2-T6SS delivers PldA, PldB, Azu, Tle3, TplE, and H3-T6SS delivers TseF.

T6SS helps *P. aeruginosa* to deliver toxins to other competing bacteria and provides a survival advantage. Moreover, Hood et al. 2010 demonstrated that Tsi2 blocked the activity of Tse2 as part of the toxin element of the toxin immunity pair. This blocking process was done by direct or indirect protein-protein interaction to protect *Pseudomonads* from damage by Tse2 (Hood et al., 2010). In addition, when Tse2 was delivered into eukaryotic or prokaryotic cells in the

absence of Tsi2, it can halt the overall growth of host cells to protect *P. aeruginosa*. A study conducted by (Basler, Ho & Mekalanos, 2013) revealed that *P. aeruginosa* cannot effectively kill T6SS *Acinetobacter baylyi* or T6SS *V. cholera* but can attack these bacteria if they express functional T6SS activity. Therefore, an evolutionary strategy called “tit-for-tat” helps *P. aeruginosa* battle against different bacterial species that express T6SS, while *P. aeruginosa* does not cause any harm to the innocent bystanders co-existing in the same ecological niche.

T6SS plays important role in the virulence of *P. aeruginosa*. Experiments on *Caenorhabditis elegans* with $\Delta clpV2$ mutant and $\Delta clpV3$ mutant showed that H2- and H3-T6SS are crucial for maintaining the physiological properties of *P. aeruginosa*. Recent study showed that the impairment of H3-T6SS affected several virulence properties, including production of pyocyanin, formation of biofilm, proteolytic activity, and motility, which is found in the *clpV3* deletion mutant (Li, Chen, Zhang, Bhagirath, & Duan, 2020).

T6SS also has an essential role in biofilm formation. The expression level of Hcp proteins in *P. aeruginosa* PAO1 at the stage of biofilm maturation revealed that Hcp contributed to the formation of biofilm in *P. aeruginosa* (Southey-Pillig, Davies, & Sauer, 2005).

1.4.2.3 Regulations of T6SS

At the transcriptional level, the expressions of T6SS genes in *P. aeruginosa* are influenced by QS. Additionally, QS regulates three T6SSs of *P. aeruginosa* differentially (Lesic, Starkey, He, Hazan, & Rahme, 2009). Both pqs and las QS systems have been shown to modulate the expression of H1-T6SS negatively. On the other hand, las, rhl, and pqs have positively regulated

H2- and H3-T6SS expression.

The Gac-Rsm pathway is a central regulatory pathway that controls numerous traits, including production of QS signal molecules, production of enzymes, secretion of toxins, and motility in *Pseudomonas spp* (Bhagirath et al., 2017; Lapouge, Schubert, Allain, & Haas, 2008). It plays a critical role in regulating T6SS (Kusmieriek & Dersch, 2018; Vakulskas, Potts, Babitzke, Ahmer, & Romeo, 2015). RsmA is an RNA-binding posttranscriptional regulator that activates T3SS and inhibits all three gene clusters of T6SS in *P. aeruginosa* (Allsopp et al., 2017). RetS is a hybrid sensor kinase which inhibits the GacS/GacA two-component system, whereas active phosphorylated GacA is required for the transcription of the RsmA sequesters: small regulatory RNAs RsmZ/Y. Hence, RetS inhibits rsmZ/Y transcription and leads to activation of T3SS and repression of T6SS. On the other hand, another hybrid sensor named LadS suppresses the activity of T3SS and promotes biofilm formation, which also acts by altering the level of RsmZ (Ventre et al., 2006).

A study by Moscoso et al., 2011 reported that the formation of biofilm was induced, and high levels of c-di-GMP were found in the *retS* mutant. Moreover, the formation of biofilm is enhanced when the c-di-GMP concentration is increased, and it leads to chronic infection. By artificially modulating the levels of c-di-GMP, T3SS and T6SS production can be shifted. In this c-di-GMP-dependent switching, RsmY and RsmZ are needed, indicating a strong connection between the c-di-GMP and RetS/GacS pathways.

1.5 Hypothesis and objectives

1.5.1 Study rationale

One of the biggest problems with cystic fibrosis (CF) lung is *P. aeruginosa* infection. Examining changes in the pulmonary environment and physiological abnormalities in CF provides important information on the pathophysiology of the disease. A thorough understanding of CF lung illness requires an investigation of the CF airway microbiome, interactions between microbes, the host, environmental variables, and the protein secretion system. Developing novel strategies for infection control in CF requires a deeper understanding of the regulatory systems and bacterial pathogenic mechanisms, as well as the interaction between host responses and the human microbiome. *P. aeruginosa* possesses three functionally distinct T6SSs: H1-T6SS, H2-T6SS, and H3-T6SS. H1-T6SS is primarily involved in intraspecies competition and delivers at least seven identified toxins (Tse1-Tse7) into rival bacteria (George, Narayanan, Tejada-Arranz, Plack, & Basler, 2024; Hood et al., 2010). In contrast, H2-T6SS and H3-T6SS can target both prokaryotic and eukaryotic cells (Lin et al., 2017). Although these systems perform essential functions, the mechanisms regulating T6SS activity, especially those of H2-T6SS and H3-T6SS, are not yet fully understood.

Although substantial progress has been made in understanding the functions of T3SS and T6SS, insights into their regulatory mechanisms remain limited. It is unclear how the T3SS and T6SS are regulated and how they function differentially and are interconnected with each other in a microbial community. The ultimate goal of my thesis is to understand the molecular mechanisms and regulatory pathways of bacterial interspecies and intercellular interactions. My research will focus on the regulation and function of T3SS and T6SS in *P. aeruginosa*.

1.5.2 Hypothesis

T3SSs and T6SSs are crucial in *P. aeruginosa* for maintaining virulence and facilitating the acquisition of antibiotic resistance. Both secretion systems play significant role in bacterial pathogenicity and the structural and functional dynamics. Although the principal signaling pathways of *P. aeruginosa* have been identified, the global regulators and functions of T3SSs and T6SSs remain largely uncharacterized. Understanding these regulatory networks could reveal additional pathways and enhance our understanding of the diverse nature of bacterial pathogenesis. The hypothesis of my thesis is T6SS and T3SS are interconnected through distinct regulatory pathways that regulate bacterial physiology, metabolism, and adaptation *P. aeruginosa*.

1.5.3 Objectives

The four specific objectives of my thesis include:

1. Investigate the role of *norCBD* in H2-T6SS regulation and virulence in *P. aeruginosa*

To investigate the role of the *norCBD* operon in regulating the H2-T6SS in *P. aeruginosa*, and to determine how its disruption influences nitric oxide homeostasis, Dnr-dependent gene regulation, and virulence-associated traits, thereby contributing to bacterial pathogenicity.

2. Elucidate the roles and potential connection of *clpV3* and *pscN* in H3-T6SS and T3SS

To characterize the functional roles of the ATPases, PscN and ClpV3 in *P. aeruginosa*, and to investigate their contributions to the regulation and coordination of the T3SS and T6SS, as well as their potential impact on key virulence-associated traits and cellular energy dynamics.

3. Characterize the operon that is regulated by the Fem system

To investigate the functional relationship between the PA2294-PA2299 operon and the Fem regulatory system in *P. aeruginosa*, and to elucidate how Fem-mediated signaling controls the expression of this operon and its contribution to virulence-associated traits.

4. Examine the involvement of PA1611 in the mucin response of *P. aeruginosa*

To characterize PA1611 expression in *P. aeruginosa* isolates, to identify the environmental cues that regulate its activity, and to determine the role of mucin as a host-derived signal in modulating PA1611 expression and its contribution to virulence regulation.

Chapter 2

2. General materials and methods

2.1 Materials and equipment

General culture media and antibiotics used in this study were purchased from Fisher Scientific (Toronto, Canada) or Sigma Aldrich (St. Louis, MO). The compounds used: 2, 2'-bipyridyl (Cat # D216305-25G), Tetracycline (Cat # T7660), Gentamicin (Cat # 15750078), Chloramphenicol (Cat # C0378), Trimethoprim (Cat # T7883), Ciprofloxacin (Cat # 17850), Kanamycin (Cat # K1377), Moxifloxacin (Cat # SML1581), Tobramycin (Cat # T4014), Carbenicillin (Cat # 10177012), and mycobactin J (Allied Monitor, USA). Kits used: Cyclic AMP Select ELISA kit (Cayman Chemical, USA), Bacterial Genomic DNA kit (Geneaid, Cat# GBB300), Gel/PCR DNA fragments kit (Geneaid, Cat# DF300), and Presto mini plasmid kit (Geneaid, Cat# PDH300). For PCR, LifePro Thermal Cycler was purchased from Bioer Serves Life (Tokyo, Japan). For imaging purposes, Fusion FX Vilber Lourmat was purchased from MBI Lab Equipment (Montreal, Canada). For running electrophoresis, the system used consisted of a PowerPac™ Basic Power supply, a Mini-Sub® Cell GT Cell, and a Mini-PROTEAN® Tetra Cell, which were purchased from Bio-Rad (Mississauga, Canada). For centrifugation, a Sorvall ST 8R Benchtop Centrifuge was purchased from ThermoFisher Scientific (Toronto, Canada), and a SIGMA 1-14 microcentrifuge was purchased from MBI Lab Equipment (Montreal, Canada). Ultracentrifuge Optima XE-90 was purchased from Beckman Coulter (US). For ultrasonic cleaning purposes, Fisherbrand™ M Series Mechanical Ultrasonic Cleaning Bath (Cat 15-337-416) was purchased from Fisher Scientific (Ottawa, Canada). A polyscience general-purpose digital water bath was purchased from Scientific Ca (Canada). For electroporation, a Personal Electroporation Pak was purchased from BTX Harvard Apparatus (Holliston, US). For sterilization and autoclaving, LV 250 Laboratory Steam Sterilizer was purchased from Steris (Mississauga, Canada). For aseptic and safe working, a 1300 Series Class

II, Type A2 Biological safety Cabinet from Thermo Scientific (Ottawa, Canada) was used.

2.1.1 Media used for bacterial culture

All *P. aeruginosa* and *E. coli* strains, unless otherwise noted, were cultivated in Luria Bertani (LB) broth at 37 °C while being vigorously shaken at 220 rpm.

Luria-Bertani (LB) agar: Agar (15 g/l), NaCl (5 g/l), 5 g/l yeast extract, and 10 g/l tryptone

LB broth: 5 g/l yeast extract, 10 g/l tryptone, and 5 g/l NaCl (pH 7.0)

NaCl-free LB agar: Tryptone (10 g/l), yeast extract (5 g/l), and agar (15 g/l)

5X M9 Media salts: 2.5 g/l NaCl, 5 g/l NH₄Cl, 15 g/l KH₂PO₄, and 33.9 g/l Na₂HPO₄

M9 Minimal media: 5X M9 Media salts (20%), 0.4% glycerol, and 2 mM MgSO₄

SOC media: 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 g/l Tryptone, 5 g/l Yeast extract, and 20 mM Glucose (pH 7.0)

5X M63 media salts: 2.5 mg/l FeSO₄·7H₂O, 68 g/l KH₂PO₄, and 10 g/l (NH₄)₂SO₄

M63 Minimal media for quantifying biofilm development: 20% 5X M63 medium salts, 0.2% glucose, 0.5% casamino acids, and 1 mM MgSO₄

Proteolytic agar plate: Agar (15 g/l), 2% skim milk, 5 g/l yeast extract, 10 g/l tryptone, and 5 g/l NaCl

Pseudomonas Isolation Agar (PIA): 13.6 g/l Agar (pH 7.0), 20 g/l Peptone, 1.4 g/l MgCl₂, 10 g/l K₂SO₄, 25 mg/l IrgasanTM, and 2% Glycerol

2.1.2 Buffers and antibiotic solutions

2.1.2.1 Buffers

TBE 10X buffer: 20 mM EDTA, 1 M boric acid, and 1 M Tris base

TAE 50X buffer: 50 mM Ethylenediaminetetraacetic acid (EDTA) disodium salt, 1M Glacial

acetic acid, and 2 M Tris-base

Wash buffer: 25 mM imidazole (pH 7.4), 300 mM sodium chloride, and 20 mM sodium phosphate

Elution buffer: 10 mM Tris-HCl (pH 8.5)

PBS: 240 mg/l KH_2PO_4 , 1.44 g/l Na_2HPO_4 , 200 mg/l KCl, and 8 g/l NaCl (pH 7.4)

Agarose loading buffer: 150mM Tris 3.3ml, 25mg Bromophenol blue and Glycerol 6ml, Water up to 10 ml

Loading buffer (2X): 10% 2-mercaptoethanol, 20% glycerol, 4% SDS, and Tris-HCl (pH 6.8), 0.02% bromophenol blue at 125 mM

HEPES 1M stock: Water up to 1 L, 238.30 g HEPES, and 10 N NaOH for pH adjustment

2.1.2.2 Antibiotic solutions used as final concentration

Different concentrations of antibiotics were employed. The values for *Pseudomonas aeruginosa* were 70 µg/ml for tetracycline, 250 µg/ml for carbenicillin, and 300 µg/ml for trimethoprim. The antibiotic doses used for *E. coli* were tetracycline (12.0 µg/ml), ampicillin (100 µg/ml), and kanamycin (50 µg/ml). Tetracycline (300 µg/ml) was used to generate the deletion mutant of *P. aeruginosa*.

2.2 Molecular biology techniques

2.2.1 Isolation of genomic DNA

Genomic DNA was isolated using the Bacterial Genomic DNA kit from Geneaid as per the manufacturer's instructions. A single *P. aeruginosa* colony was inoculated into LB broth and shaken at 225 rpm at 37 °C. After overnight growth, 1ml liquid culture was transferred to a 1.5

ml microcentrifuge tube and centrifuged at $14,000 \times g$ for 1 minute at room temperature. After centrifugation, the cell pellet was suspended in 180 μ l of GT buffer. After adding 20 μ l of Proteinase K to the suspension, it was incubated for 10 min at 60°C. During this process, the tube was inverted slowly every 3 min. After adding 200 μ l of GB buffer, the sample was vortexed and incubated for 10 min at 70°C. 5 μ l of RNase A was added to the sample to avoid RNA contamination. For DNA binding, 200 μ l of absolute ethanol was added to the sample. After vigorous mixing, the sample was transferred to the GD column with the collection tube on it. After centrifugation at 16,000 xg for 2 min, the liquid was discarded. For washing purposes, 600 μ l of wash buffer was added to the column matrix and centrifuged at $14,000 \times g$ for 30 s. After removing the flow through additional centrifugation was required to dry the column matrix. After centrifugation, the GD column was placed into a new centrifuge tube, and 50 μ l pre-warmed elution buffer was added to the middle of the column matrix and centrifuged at 14,000 xg for 2 min. The Nanodrop machine was used to measure the quality and quantity of the genomic DNA. The genomic DNA was stored at -20°C for future use.

2.2.2 Isolation of plasmid DNA

Plasmid DNA was isolated using the Presto™ Mini Plasmid Kit (Geneaid). A single colony from the plasmid-containing strain was cultivated in LB broth supplemented with the requisite antibiotic(s) and incubated overnight at 37°C at 220 rpm. From the overnight culture, 1 mL of bacterial cells were transferred into a 1.5 ml microcentrifuge tube and centrifuged at $14,000 \times g$ for 1 min at room temperature. The cell pellet was then washed with 1X PBS and centrifuged to discard the supernatant. 200 μ l of PD1 buffer was added to the cell pellet and mixed well using a pipette. For cell lysis, 200 μ l of PD2 buffer was added and mixed gently by inverting the tube to

avoid shearing the genomic DNA. The mixture was then kept at room temperature for 2-3 minutes to ensure homogeneity. For neutralization, 300 µl of PD3 buffer was added, and the tube was immediately inverted 10 times and centrifuged at 14,000 xg for 3 min at room temperature. After centrifugation, all the supernatant was transferred carefully into a PDH column without disrupting the white precipitate. The PDH column was placed in a 2 ml collection tube after centrifugation at 14,000 xg for 30 s. For washing, 600 µl of wash buffer was added and centrifuged at 14,000 xg for 30 s at room temperature. After removing the flow through, the PDH column, along with the collection tube, was allowed to be centrifuged at 14,000 xg for 3 min to dry the column. To elute the plasmid DNA, 30 µl of elution buffer was added to the middle of the PDH column matrix and centrifuged at 14,000 xg for 2 min. The quantity and quality of the plasmid DNA were measured using a Nanodrop machine and stored at -20°C.

2.2.3 Preparation of *P. aeruginosa* competent cells

Competent cells of *P. aeruginosa* were prepared using methods as described, with some modifications (Choi, Kumar, & Schweizer, 2006). In brief, a single colony of *P. aeruginosa* was inoculated into LB broth and allowed for overnight growth at 37°C. The cultured cell pellet was suspended in 1 ml of 0.3 M sucrose and allowed to centrifuge at 6,000 xg for 3 min (room temperature). After centrifugation, the supernatant was discarded, and the wash was repeated 3 times. Then, the cell was washed with 10% glycerol and resuspended in 10% glycerol. 100µl of aliquoted competent cells were stored at -80°C for future use.

2.2.4 Preparation of *E. coli* competent cells for chemical transformation

A single colony of *E. coli* DH5α was inoculated in 3ml LB broth and incubated at 220 rpm overnight. The following morning, from the grown culture, 1-3 ml was transferred to 250 ml

fresh LB broth and allowed to grow for another 3-4 hrs until they reached the exponential phase. The bacterial cells were harvested by centrifuging at 3,000 xg for 15 min at 4°C and kept on ice for 30 min. After centrifugation, the supernatant was discarded, and 100 ml of 100mM cold MgCl₂ was added, and the cells were suspended gently. After centrifugation, the cell pellet was washed with 200ml of 85 mM CaCl₂ and washed with ice-cold 15% glycerol followed by incubation on ice for 20 min. After incubation, the cells were centrifuged at 3,000 xg for 15 min at 4°C. The washing steps with MgCl₂, CaCl₂, and 15% glycerol were repeated. Finally, the cell pellets were gently aliquoted into 50µl competent cells after resuspension in 2ml of ice-cold 85mM CaCl₂ and 15% glycerol. The competent cells were then stored at -80°C.

2.2.5 Preparation of *E. coli* competent cells for electroporation

For preparing *E. coli* competent cells for electroporation, *E. coli* DH5α was grown overnight at 37°C in LB broth and shaken at 220 rpm. From an overnight culture, 1-3ml of the culture was subcultured into 250 ml LB broth and allowed to grow another 3-4 h until they reach an OD of 0.2-0.3. After centrifugation at 3,000 xg for 15 min at 4°C, the cell pellet was kept on ice. The cell pellet was suspended in 10mM HEPES and resuspended gently by pipetting and kept on ice for 20 min. After incubation on ice, the cells were centrifuged again at 3,000 xg for 15 min (4°C). After removing the supernatant, the washing process was repeated. The cell pellet was further washed with 50 ml of 10% glycerol and centrifuged at 3,000 xg for 15 min at 4°C. These washing steps helped to remove the presence of additional HEPES. Finally, the cell pellets were resuspended in 10% glycerol, and a 50µl aliquot of competent cells was transferred into a 1 ml microcentrifuge tube for electroporation and storage at -80°C.

2.2.6 Chemical transformation

E. coli DH5 α competent cells were thawed on ice, and 5 μ l of ligase mixture was added gently to the competent cells and incubated on ice for 30 min. The tube containing the competent cells and ligase was then allowed to undergo heat shock for 30-40 seconds at 42°C in a water bath. After the heat shock, the tube was immediately placed on ice and incubated for 2 min. Pre-warmed 900 μ l of SOC medium was added to the tube and incubated at 37°C for 1 h with agitation at 220 rpm. After incubation, 200 μ l was spread on LB medium with appropriate antibiotic and incubated overnight at 37°C.

2.2.7 Electroporation

Electrocompetent *P. aeruginosa* or *E. coli* cells were thawed, and electroporation cuvettes were kept on ice beforehand. 2-3 μ l of plasmid was added gently to the electrocompetent cells by flicking the tube. To prevent the production of bubbles, the entire mixture was carefully moved to the middle of the cuvette using a broad tip. Electroporation was carried out using a voltage setting of 2250 V for *P. aeruginosa* or 1250 V for *E. coli* after the cuvette was put in a Personal Electroporation Pak chamber. After carefully moving the mixture to a microcentrifuge tube, the entire suspension was incubated for 1 h at 37°C at 220 rpm. Following incubation, 200 μ l was spread out on LB medium with the proper antibiotic and allowed to incubate at 37°C overnight. The transformation mixture of cells that remained was kept at 4°C.

2.2.8 Construction of promoter-reporter-based gene expression detecting systems

Plasmid pMS402, which contains a promoterless *luxCDABE* reporter gene cluster, was utilized to create a promoter-*luxCDABE* reporter using the procedure outlined (Duan, Dammel, Stein, Rabin, & Surette, 2003). The target promoter region was introduced into the plasmid pMS402's

promoter cloning site (BamHI-XhoI) after being amplified by PCR with primers. PCR and agarose gel electrophoresis were used to confirm the resulting plasmid, which is known as pKDp-reporter. To create a chromosomally integrated reporter, the PacI-digested pKDp fragment was cloned into the digested integration plasmid CTX6.1, which was derived from mini-CTX-lux. To create the reporter strain, the CTX-promoter was transferred to *E. coli* SM10- λ pir, and biparental mating between *E. coli* SM10- λ pir and *P. aeruginosa* was carried out. Plasmids CTX-*H2-T6SS*, CTX-*H3-T6SS*, CTX-*PA2296*, CTX-*PA2297*, and CTX-*PA2299* were constructed by using the same methods as described above.

2.2.9 Measurement of promoter activities

Gene expression was measured using Lux-based reporter assays in a Synergy H4 Multimode Microplate Reader (BioTek, USA) using the procedures outlined (Duan et al., 2003). To put it briefly, the bacteria were cultivated in LB broth for the entire night and then subcultured into new LB broth the following morning until they reached the exponential phase of growth. The inoculum was prepared for this experiment following two hours of further growth. 95 μ l of fresh media and 5 μ l of inoculum culture were combined in a 96-well flat-bottom plate. The mixture was topped with a 50 μ l coating of filter-sterilized mineral oil from Sigma Aldrich. During the experiment, the oil keeps things from evaporating. For 24 h, the gene expression level and bacterial growth (OD₆₀₀) were continually measured every 30 min.

2.2.10 Bi-parental mating

A *P. aeruginosa* lux-based integration reporter was created through bi-parental mating with minor modifications (H. Liang, Li, Dong, Surette, & Duan, 2008). The *E. coli* strain SM10 λ pir was utilized for conjugation with *P. aeruginosa* that carried a mobilizing element. Plasmid mini-

CTX-lux was the source of a clone of integration plasmid CTX6.1, which was then transferred to the SM10λpir strain with the appropriate insert. *P. aeruginosa* and *E. coli* were both cultured overnight at 37°C with shaking at 220 rpm. After being centrifuged for three minutes at 8,000 rpm, the bacterial cells were collected and washed twice with 1X PBS. The cells were then re-suspended by SOC media, and the final suspension concentrations for *P. aeruginosa* and *E. coli* were 250 µg/µl and 500 µg/µl, respectively. On an LB plate, the cell suspensions were spotted after being combined in equal amounts (50 µl). The cells were gathered after overnight incubation, diluted with PBS, and then spread on selective plates. Promoter-reporter strains were confirmed by PCR and agarose gel electrophoresis. Promoter-reporter strains CTX-*H2-T6SS* in PAO1, CTX-*H2-T6SS* in PAO1(Δ*norCBD*), CTX-*H2-T6SS* in PAO1(Δ*norCBD*)-*norCBD* in PAK1900, CTX-*H3-T6SS* in PAO1, CTX-*H3-T6SS* in PAO1(Δ*pscN*), CTX-*H3-T6SS* in PAO1(Δ*clpV3*), CTX-*exoS* in PAO1, CTX-*exoS* in PAO1(Δ*pscN*), CTX-*PA2296* in PAO1, CTX-*PA2297* in PAO1, CTX-*PA2296* in PAO1(Δ*FemA*) and CTX-*PA2297* in PAO1(Δ*FemA*) were constructed using the same methods as mentioned above.

2.2.11 Tri-parental mating

The Sucrose counter-selection technique was utilized to construct deletion mutants, where the allelic exchange approach was used, and pEX18Tc was used as the vector (Hmelo et al., 2015). The knockout mutant was obtained through tri-parental mating. The helper strain PRK2013, *E. coli*, which comprises the recipient strain in addition to the donor strains of pEX18Tc-up+down and the PAO1 (the recipient strain), was employed in tri-parental mating techniques. Initially, 1X PBS buffer was used to wash the cultures of the donor, helper, and recipient strains after they had been cultivated overnight. After mixing and spotting bacterial cultures (ratio, helper: donor:

recipient, 2:2:1) onto LB agar, they were incubated for 12 h at 37°C. The cells were scraped, reconstituted in LB broth, and plated on *Pseudomonas* isolation agar with 300 µg/ml of tetracycline in order to select the merodiploid. This procedure was known as the first crossover. After the initial crossover, the colony was streaked in LB agar with 10% sucrose and no NaCl in order to choose a second crossover. Using the confirmation primers, the deletion mutant was confirmed by polymerase chain reaction.

2.2.12 Characterization of phenotypic properties

2.2.12.1 Biofilm formation quantification

We used the previously outlined established procedure for the measurement of biofilm (G. A. O'Toole, 2011). We employed M63 minimum media for this test, along with supplementation that contained MgSO₄ (1 mM), glucose (20%), and casamino acids (0.5%). The overnight culture was incubated for 24 h at 37°C in 96-well microtiter plates. To get rid of planktonic cells, the cells were rinsed three times with 1X PBS following the incubation period. For staining, 100 µl of crystal violet solution was added, and the mixture was left to incubate at room temperature for 15 min. To get rid of extra dye, all the wells were rinsed with distilled water. The leftover crystal violet on the 96-well plate was dissolved using 125 µL of acetic acid (30%). 100 µl of the solution was moved into a fresh 96-well plate after 10 min of incubation, and absorbance at 550 nm was recorded with a plate reader.

2.2.12.2 Measurement of pyocyanin production

Pyocyanin production was evaluated and quantified using the previously mentioned techniques (Guo et al., 2016). In detail, 18-h overnight cultures were collected and centrifuged at 14,000 xg.

A 5 ml supernatant was then combined with 3 ml of chloroform. After adding 1 ml of 0.2 N HCl to the chloroform layer, the mixture was centrifuged at 4,500 xg for 10 min. The pink layer (top) was then moved to the cuvette after centrifugation, and absorbance at 520 nm was measured. The pyocyanin concentration was calculated by multiplying the extinction coefficient of 17.072 at 520 nm by the number of micrograms created per milliliter of culture supernatant.

2.2.12.3 Motility assay

Assays for bacterial motility were conducted using the previously outlined procedures (Rashid & Kornberg, 2000). Agar (0.3%), NaCl (5 g/L), and tryptone (10 g/L) were present in the swimming motility medium, whereas the swarming motility medium contained glucose (5 g/L), agar (0.5%), and nutritional broth (8 g/L). For the swarming and swimming motility tests, 2 µl of the overnight bacterial culture aliquot was spotted onto the suitable medium as mentioned above. For swimming and swarming motility, the incubation conditions were 15 h at 37°C and 8 h at room temperature, respectively, followed by 15 h at 37°C. After incubation, all of the photos were captured using a Vilber Lourmat Imager (Fusion FX7).

2.2.13 Statistical analysis

All experimental findings were presented using the mean $\pm$ standard error of the mean (SEM) from three different studies. Analysis of variance (ANOVA) was used when comparing more than three groups. An unpaired, two-tailed Student's t-test was used to compare the outcomes of the two groups. The following significance levels are shown where appropriate: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns (not significant) $p > 0.05$

Chapter 3

Investigate the role of *norCBD* in H2-T6SS regulation and virulence in *P. aeruginosa*

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3.1 Introduction

The pathogenic success of *P. aeruginosa* is largely attributed to its arsenal of virulence factors (Qin et al., 2022). In particular, it employs specialized secretion systems to deliver toxins into host cells and competing microbes. Among these, the Type VI Secretion Systems (T6SSs) act as contractile nanomachines that mediate intercellular interactions by injecting toxic effectors into neighboring cells (Gallique, Bouteiller, & Merieau, 2017). T6SSs have been implicated not only in interbacterial competition but also in metal ion acquisition (W. J. Chen et al., 2016; Cianfanelli, Monlezun, & Coulthurst, 2016; Wang et al., 2015) and biofilm formation (Alteri et al., 2013).

To identify genes involved in H2-T6SS regulation, we employed transposon mutagenesis using a CTX-H2-T6SS reporter system (Badr, 2016). This screen revealed that disruption of the *norCBD* operon significantly decreased H2-T6SS reporter activity. The *norCBD* operon encodes the nitric oxide reductase complex (NorCBD), which catalyzes the reduction of toxic nitric oxide (NO) to nitrous oxide (N₂O), a key step in anaerobic respiration.

The NorCBD complex is a cytochrome bc-type nitric oxide reductase. NorB forms the membrane-integrated catalytic subunit, NorC functions as a cytochrome c electron carrier, and NorD is thought to facilitate proper assembly and stabilization of the enzyme complex (Gao et al., 2016). Expression of the *norCBD* operon is activated by the nitric oxide-responsive transcription factor Dnr, which is itself regulated by Anr, the master transcriptional regulator that responds to low oxygen tension (Arai, Kodama, & Igarashi, 1997). Recent studies have shown that Dnr also controls the expression of the H2-type VI secretion system (H2-T6SS) (Dang, Wang, Wen, & Liang, 2022), which contributes to metal ion acquisition, interbacterial

competition, and biofilm fitness in low-oxygen environments (Han et al., 2019; T. Wang et al., 2021).

Although Dnr activation is NO-dependent, the optimal intracellular concentration of NO for Dnr function is poorly defined. Excessive NO may impair Dnr activity or other NO-sensitive regulators, potentially through disruption of iron-sulfur clusters or heme groups (Crack, Green, Hutchings, Thomson, & Le Brun, 2012; Crack, Stapleton, Green, Thomson, & Le Brun, 2013; Fontenot, Cheng, & Ding, 2022). Based on this rationale and the observation of disrupted expression of H2-T6SS in *norCBD* mutant, we investigated, in this study, whether *norCBD* disruption might impair Dnr-mediated gene expression by allowing toxic NO accumulation, thereby altering regulatory outputs, including the activation of H2-T6SS.

While the role of NorCBD in NO detoxification has been well documented (Zhou et al., 2019), its potential regulatory influence via modulation of intracellular NO levels and the resulting phenotypical changes have not been explored. We report the data suggesting that NorCBD not only facilitates anaerobic respiration but also contributes to the proper regulation of Dnr-dependent genes by maintaining intracellular NO balance that supports optimal Dnr activity.

3.2 Materials and Methods

3.2.1 Bacterial plasmids and strains

The bacterial strains and plasmids used in all experiments are listed in **Table 3.1**. *E. coli* and *P. aeruginosa* were grown on Luria-Bertani (LB) agar and broth at 37°C. Different concentrations of antibiotics were applied. For *P. aeruginosa*, these concentrations were carbenicillin (250 µg/ml), trimethoprim (300 µg/ml), and tetracycline (70 µg/ml). The antibiotic concentrations used for *E. coli* were kanamycin (50 µg/ml), ampicillin (100 µg/ml), and tetracycline (12.0 µg/ml). Tetracycline (300 µg/ml) was used for constructing *P. aeruginosa* mutant PAO1(*AnorCBD*), which was plated on a *Pseudomonas* isolation agar (PIA) plate.

Table 3.1 List of bacterial strains and plasmids used.

Bacterial strains and plasmids	Characteristics	Sources/ References
<i>P. aeruginosa</i> strains		
PAO1	Wild-type <i>P. aeruginosa</i>	Duan Lab
PAO1(Δ <i>norCBD</i>)	<i>norCBD</i> knockout mutant of PAO1	This study
PAO1(Δ <i>clpV3</i>)	<i>clpV3</i> knockout mutant of PAO1	(Y. Li et al., 2020)
<i>E. coli</i> strains		
DH5 α	F $^-$ Φ 80lacZ Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rK $^-$, mK $^+$) <i>phoA supE44</i> λ^- thi-1 <i>gyrA96 relA1</i>	Invitrogen
SM10- λ pir	Mobilizing strain, RP4 integrated into the chromosome; Km r	Duan Lab
Plasmids		
pEX18Tc	<i>oriT</i> $^+$ <i>sacB</i> $^+$ gene replacement vector with multiple-cloning site from pUC18; Tc r	(Hoang, Karkhoff-Schweizer, Kutchma, & Schweizer, 1998)
pBT20	Mini-TnM delivery vector; Gm	(Kulasekara et al., 2005)
pAK1900	<i>E. coli-P. aeruginosa</i> shuttle cloning vector, Amp r	(Sharp, Jansons, Gertman, & Kropinski, 1996)
pMS402	Expression reporter plasmid carrying the promoterless <i>luxCDABE</i> ; Km r Tmp r	(Duan et al., 2003)
CTX-6.1	Integration plasmid origins of plasmid mini-CTX- <i>lux</i> ; Tc r	(Kong et al., 2013)

pEX18Tc- <i>norC</i> up	pEX18Tc carrying the upstream fragment of <i>norC</i>	This study
pEX18Tc- <i>norC</i> up+dw	pEX18Tc carrying the upstream and downstream fragment of <i>norC</i>	This study
pEX18Tc- <i>norD</i> up	pEX18Tc carrying the upstream fragment of <i>norD</i>	This study
pEX18Tc- <i>norD</i> up+dw	pEX18Tc carrying the upstream and downstream fragments of <i>norD</i>	This study
pAK- <i>norCBD</i>	pAK1900 with a 2874 bp fragment of <i>norCBD</i> between <i>KpnI</i> and <i>HindIII</i> ; Amp ^r , Cb ^r	This study
CTX- <i>H2-T6SS</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>hsiA2</i> containing <i>H2</i> promoter (pMS402 containing <i>hsiA2</i> promoter region) region and <i>luxCDABE</i> gene; Kn ^r , Tmp ^r , Tc ^r	This study
CTX- <i>H2-T6SS</i> in PAO1(Δ <i>norCBD</i>)	Integration plasmid, CTX6.1 with a fragment of pKD- <i>H2-T6SS</i> -PAO1(Δ <i>norCBD</i>) containing <i>H2</i> promoter region and <i>luxCDABE</i> gene; Kn ^r , Tmp ^r , Tc ^r	This study
CTX- <i>H2-T6SS</i> in PAO1(Δ <i>norCBD</i>)- <i>norCBD</i> in PAK1900	Integration plasmid, CTX6.1 with a fragment of pKD- <i>H2-T6SS</i> -PAO1(Δ <i>norCBD</i>)- <i>norCBD</i> in PAK1900 containing <i>H2</i> promoter region and <i>luxCDABE</i> gene; Kn ^r , Tmp ^r , Tc ^r	This study

3.2.2 Generation of a *norCBD* deletion mutant PAO1 and a complementation strain

A deletion mutant of *norCBD* was generated in *P. aeruginosa* PAO1 using plasmid pEX18Tc (sucrose counter-selection system (Hmelo et al., 2015; Hoang et al., 1998)). In brief, both up and downstream fragments of the gene of interest (*norC*) were amplified by polymerase chain reaction using the designed primers shown in **Table 3.2**. Then, amplified fragments of up and downstream were cloned into a pEX18Tc vector that contains a counter-selectable *sacB* gene. After the double restriction enzyme digestion, both upstream and downstream products were ligated. PAO1(Δ *norC*) was obtained by tri-parental mating. Triparental mating was performed using the *E. coli* (helper strain) that contains PRK 2013, the donor strain *E. coli* had pEX18Tc-*norC* (up and down), and the recipient strain used for this purpose was PAO1. Overnight cultures of the donor, helper, and recipient strains were resuspended in a phosphate buffer saline (PBS). The bacteria were spotted on LB agar by mixing at a ratio of 2:2:1. An overnight culture was taken, and the developed pellets were resuspended in LB. The diluted bacteria suspension was spread on a PIA plate (containing tetracycline, 300 μ g/ml) for the selection of merodiploid. For selecting a double crossover, the colony was streaked in LB agar containing 15% sucrose and no NaCl after the first crossover. Deletion mutants were verified by PCR using the confirmation primers (**Table 3.2**). Finally, PAO1(Δ *norCBD*) was constructed using the same method as PAO1(Δ *norD*) as a background and was confirmed by PCR.

Shuttle vector pAK1900 was used for the complementation of *norCBD* knockout mutants. The gene of interest (*norCBD*) was amplified using designed primers (pAK-*norCBD*-F and pAK-*norCBD*-R) (**Table 3.2**). Then, the amplified product was cloned into a vector, pAK1900. Finally, the pAK-*norCBD* plasmid was transformed into PAO1(Δ *norCBD*) by electroporation.

Table 3.2 List of primers used.

Primer	Sequence	Restriction site
<i>norC</i> -up-S	TACGAATTCTGTTATACGCCGCAGGCTTG	<i>EcoRI</i>
<i>norC</i> -UP-AS	AAGGGATCCGCAGGATGAAGAACACACTC	<i>BamHI</i>
<i>norC</i> -dw-S	AAGGGATCCATCACCATCGACAAGGAGG	<i>BamHI</i>
<i>norC</i> -dw-AS	TCTAAGCTTGCGAAATCAGCATCCTCGAC	<i>HindIII</i>
<i>norD</i> -up-S	AAGGAATTCGCGGATGCTGAGCCTG	<i>EcoRI</i>
<i>norD</i> -up-AS	ATGGGATCCCGCTTCTTCTCCAGGTCG	<i>BamHI</i>
<i>norD</i> -dw-S	ATGGGATCCCGCTTCATCGACCTGGAG	<i>BamHI</i>
<i>norD</i> -dw-AS	ATGAAGCTTTCCCGAGTTGATCTGC	<i>HindIII</i>
pAK- <i>norCBD</i> -S	ATAGGTACCGTGCCGATGCGAACCCCTGAC	<i>KpnI</i>
pAK- <i>norCBD</i> -AS	TATAAGCTTGCATGATGCTCTGGTTGCCG	<i>HindIII</i>
P- <i>norCBD</i> -F	GATCTCGAGTGGTCTACGTCCTGCAATGAG	<i>XhoI</i>
P- <i>norCBD</i> -R	CATGGATCCCAGGATGAAGAACACACTCC	<i>BamHI</i>
H2-T6SS-promoter-S	CGCCTCGAGCTATTACGCTCTAAATCAGC	<i>XhoI</i>
H2-T6SS-promoter-AS	TGAGGATCCCCTAACCCCTCAATGCACAC	<i>BamHI</i>

3.2.3 Measurement of promoter activities with the addition of SNP and CPTIO

Gene expression was measured by the lux-based reporter assays using a synergy plate reader (Biotek, USA) using the method as described (Duan et al., 2003) with the addition of Sodium nitroprusside (SNP) and carboxy-phenyl-tetramethylimidazolineoxyl (CPTIO). SNP stock solutions were prepared in PBS (pH 7.4). SNP was prepared and kept in the dark (Cai & Webb, 2020). The stock solution of SNP (7.5 mM) was filter sterilized (0.22 μ m) and diluted into fresh M9 media with supplements of 20% glucose, 1M MgSO₄, 1M CaCl₂, and 0.5% casamino acid. During experimental procedures, SNP stocks were freshly prepared, placed on ice, and kept in dark conditions before use. The CPTIO stock solution was freshly prepared in HEPES buffer (0.4 mg/mL). The stock solution of CPTIO (100 mM) was also sterilized with a syringe filter and kept in ice and dark conditions (before use) due to light sensitivity. The strain containing the reporter was cultivated overnight in an M9 broth and then diluted to an OD₆₀₀ nm of 0.2. After another 3 h of incubation, 5 μ L of the bacterial cultures were inoculated into the parallel wells on a 96-well flat-bottom plate containing 95 μ L of M9 medium and 10 μ L SNP and CPTIO at different concentrations (Gao et al., 2022). In the test of the effect of NO donor and NO scavenger on H2-T6SS expression, NO donor, SNP (Sigma-Aldrich), was added at different concentrations (10 μ M, 25 μ M, and 50 μ M) in the media, and NO scavenger, CPTIO (Cayman Chemical), was added at the concentrations of 2 mM, 5 mM, and 10 mM.

3.2.4 Measurement of intracellular nitric oxide levels

The NO detection reagent diaminofluorescein-2 diacetate (DAF-2 DA) (Sigma-Aldrich, USA) was used to quantify the cellular NO levels of *P. aeruginosa* bacterial cells using a previously established method (Su et al., 2014). In detail, to reach the exponential growth phase (OD₆₀₀ =

0.2), the bacterial strain was subcultured for 3.5-4 h after being incubated overnight at 37°C. Anaerobic bacterial growth was accomplished using the BD GasPak EZ Anaerobe Pouch System. The LB broth was supplemented with 50 mM KNO₃ to promote anaerobic growth. The intracellular NO level was also measured under anaerobic conditions upon addition of 25µM SNP to the LB broth. We also measured the intracellular NO concentrations under normoxic conditions, where strains were cultured in a 96-well plate. In brief, when the OD₆₀₀ nm reached approximately 0.2, a 96-well plate with a flat bottom was filled with 180 µL of fresh M9 minimal media supplemented with 20 µL of bacterial culture and 50 µL of filtered (0.22 µm) mineral oil and then allowed to grow inside the plate reader without or with the addition of SNP (25µM). Following anaerobic and normoxic growth (at 5h), 1 ml of shaking culture was incubated with 10 µM DAF-2 DA for 1 h at 37°C. After incubation, the cell was washed with 1X PBS. Following washing, the fluorescence level of the reaction product DAF-2 T was measured (excitation wavelength, 495 nm; emission wavelength, 515 nm), normalized to OD₆₀₀ using the FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA).

3.2.5 Measurement of nitrite

The level of nitrite was measured using a nitrate/nitrite fluorescent assay kit (Cayman Chemical, catalog no. 780051) with some modifications. In detail, the bacterial strain was incubated overnight at 37°C, followed by the subculture of 3.5-4 h required to reach the exponential phase. 30µl of bacterial culture sample was added to the wells, and the volume was adjusted to 100µl with assay buffer. 10µl of DAN (2,3-diaminonaphthalene) reagent was added to the well and allowed to incubate for 10 min. Finally, 20µl of NaOH was added, and the

fluorescence level was measured (an excitation wavelength of 360-365 nm and an emission wavelength of 450 nm) with the FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA).

3.2.6 Detection of proteolytic activity

Proteolytic activity was measured using the method known as agar plate assay, as mentioned in the earlier studies, with some modifications (Gupta, Gobble, & Schuster, 2009). 2 μ L of cells from the overnight culture were plated on LB agar with 2% skim milk and incubated at 37°C overnight. The presence of clear zones around the bacterial colonies suggests proteolytic activity, and the diameters of these zones were recorded.

3.2.7 Intracellular cAMP levels quantification

The intracellular cAMP levels were quantified using the cAMP Select ELISA kit (Cayman Chemical, California, US). The cells from an overnight culture (OD_{600} was adjusted to 8) were collected by centrifugation, and 5 ml of culture was mixed with 500 μ L of 0.1N HCl. The cells were allowed to undergo 30 s sonication (120 V). The bacterial cells were then centrifuged at 1,000 $\times g$ for 10 min. After centrifugation, the supernatant was collected. The bacterial sample and standard were prepared according to the manufacturer's protocol and loaded into a 96-well plate supplied by the manufacturer. The 96-well plate was then covered with a plastic film and placed in an orbital shaker at 150 rpm under dark conditions (for 90 min). Using a plate reader, the absorbance was read at around 405 and 420 nm. The optical density of the bacterial sample concentration was determined from the standard curve plot using the equation as per the manufacturer's guidelines.

3.3 Results

3.3.1 *norCBD* disruption affects the expression of H2-T6SS

To verify the effect of *norCBD* disruption on the H2-T6SS expression, we constructed a *norCBD* deletion mutant PAO1(Δ *norCBD*) and compared the promoter activities of H2-T6SS in PAO1 and in the mutant. Same as the PAO1 harboring the CTX-H2-T6SS-*lux* reporter, the CTX-H2-T6SS-*lux* reporter was also chromosomally integrated in PAO1(Δ *norCBD*). A complementation strain of the mutant carrying the entire *norCBD* on a plasmid was also constructed. The transcriptional activities of H2-T6SS were measured and compared in these strains by measuring luminescence. Our results showed that the deletion of *norCBD* significantly reduced the CTX-H2-T6SS transcriptional activity (**Figure 3.1**), confirming the involvement of *norCBD* in the regulation of H2-T6SS. The maximal promoter activity of H2-T6SS at 8 h was around 8-fold lower in PAO1(Δ *norCBD*) compared with that in the wild-type PAO1. The complementation of *norCBD* restored the expression of H2-T6SS to the wild-type PAO1 level.

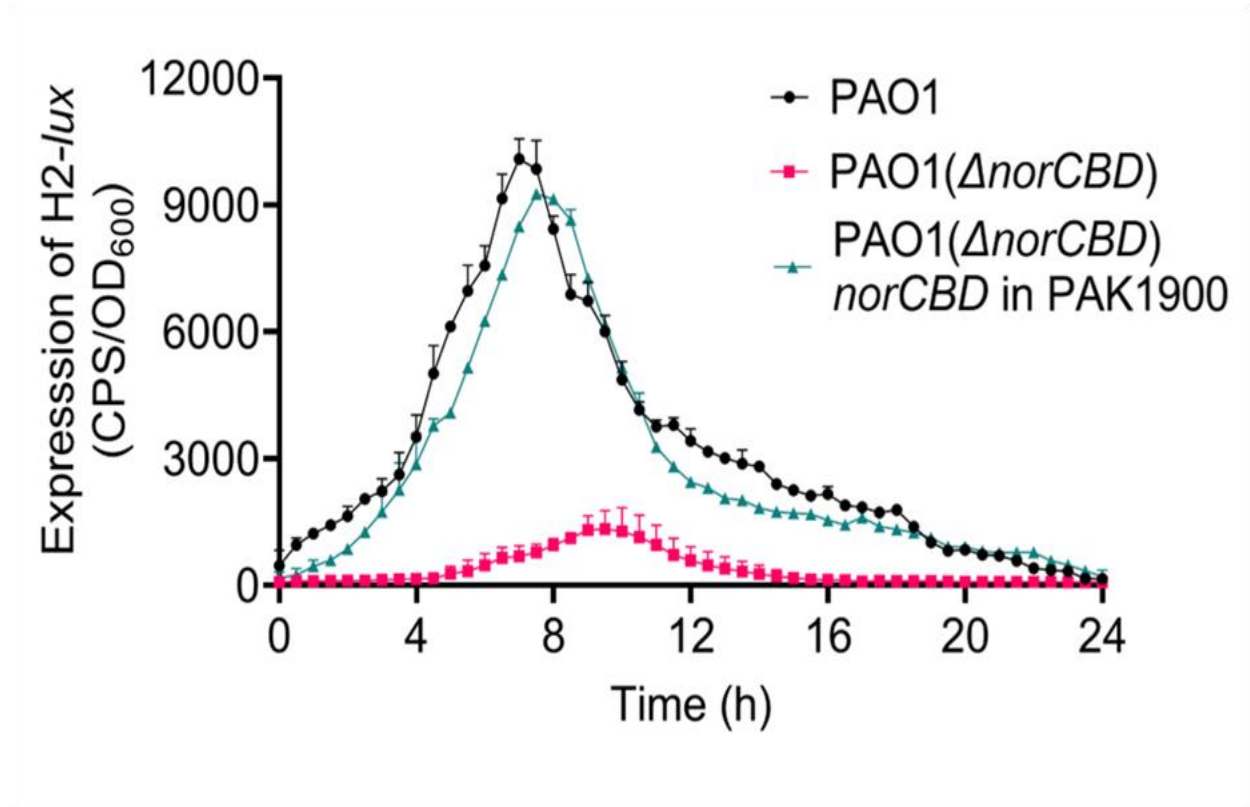


Figure 3.1 Deletion of *norCBD* resulted in decreased expression of H2-T6SS. The CTX-H2-T6SS reporter fusion integrated on the chromosome was used to measure the promoter activity of the H2-T6SS gene in PAO1(Δ *norCBD*). Compared to that in the wild-type PAO1, the promoter activity of H2-T6SS in PAO1(Δ *norCBD*) was significantly lower. The experiments were performed under normoxic conditions. The values represent the average of the triplicate experiments, and the error bars indicate standard deviations.

3.3.2 Effect of NO donor on the H2-T6SS expression

It is known that H2-T6SS is activated by Dnr in response to NO (Dang et al., 2022). It is expected that disruption of *norCBD* could increase the level of NO, which in turn would increase H2-T6SS expression. However, our results showed the opposite effect, namely, that potentially increasing NO in the mutant repressed H2-T6SS expression.

To test the possibility that the altered NO levels caused by the disruption of *norCBD* somehow contributed to the observed decrease of H2-T6SS expression in the *norCBD* mutant, we examined the expression of H2-T6SS in PAO1, PAO1(Δ *norCBD*), and the complementation strain in the presence and absence of NO donor, SNP. As shown, the H2-T6SS expression was significantly changed following the addition of the SNP. However, opposite changes were observed in the *norCBD* mutant compared to the wild-type and the complementation strain. The expression of H2-T6SS was significantly increased in wild-type PAO1 compared to that in the absence of SNP (**Figure 3.2**). The complementation strain of *norCBD* showed similar results to wild-type PAO1 (**Figure 3.4**). In the *norCBD* deletion mutant, PAO1(Δ *norCBD*), a significant reduction in H2-T6SS expression was observed in the presence of SNP (**Figure 3.3**). This observation contrasts with the increased H2-T6SS expression observed in the wild-type and complementation strains. These results appear to support our hypothesis that altered NO caused by *norCBD* disruption contributes to the changed H2-T6SS expression. In the wild type and the complementation strain, the increased H2-T6SS expression is probably a result of elevated Dnr activation in response to the increased NO level in the presence of SNP. In the *norCBD* mutant, however, further increase of NO on the already accumulated level potentially further impaired the Dnr-mediated H2-T6SS activation.

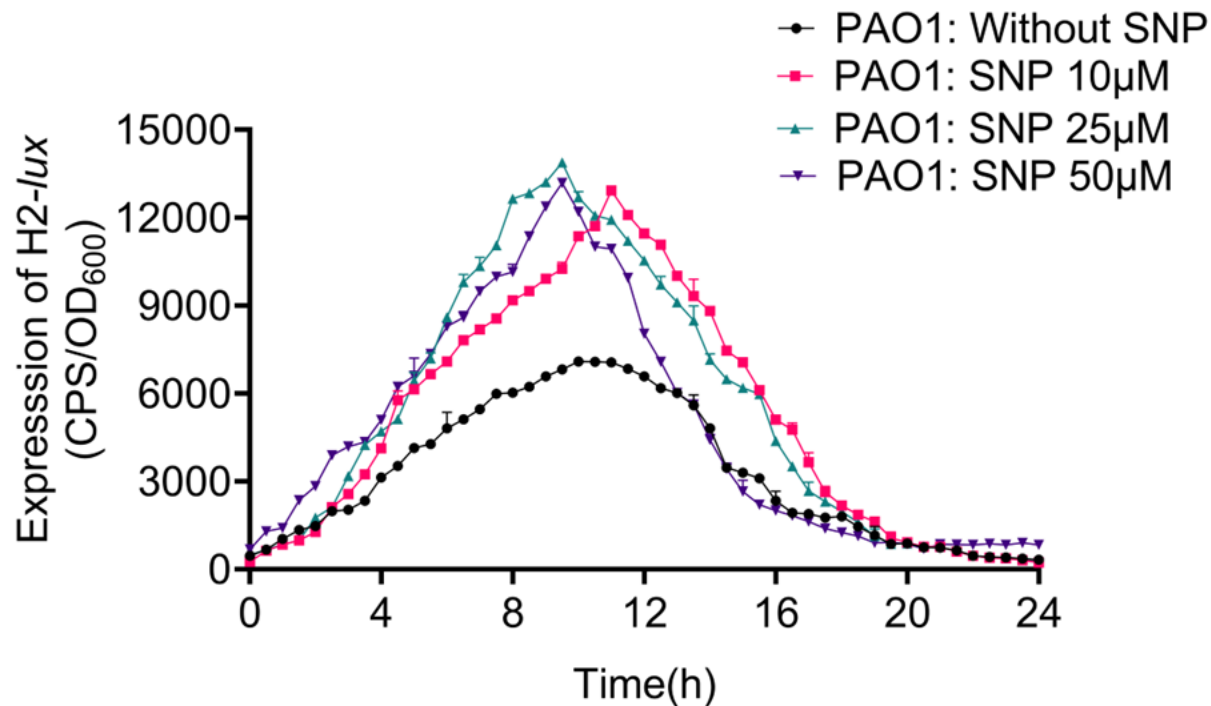


Figure 3.2 Effect of SNP on H2-T6SS expression in PAO1. For the wild-type PAO1 strain, different concentrations of SNP (10 µM, 25 µM, and 50 µM) were added to the medium. The experiments were performed under normoxic conditions. Our findings from this 24 h experiment demonstrated that the highest expression of H2-T6SS in PAO1 was achieved after 10 h, with the highest expression of the SNP at a dose of 25 µM, indicating the use of NO. The values represent the average of the triplicate experiments, and the error bars indicate standard deviations.

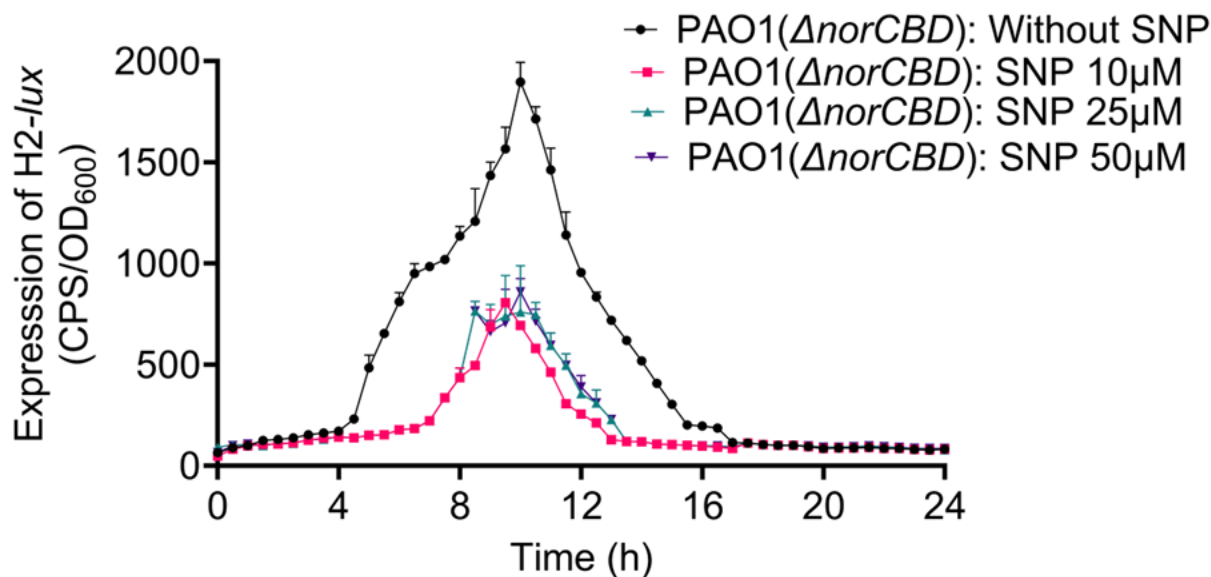


Figure 3.3 Effect of SNP on H2-T6SS expression in PAO1($\Delta norCBD$). For the deletion strain, PAO1($\Delta norCBD$), different concentrations of SNP (10 μ M, 25 μ M, and 50 μ M) were added to the medium. When compared to the expression level without SNP, H2-T6SS expression was considerably lower for the PAO1($\Delta norCBD$) strain. In comparison to the control, the lowest expression level was attained at a concentration of 50 μ M SNP after 10 h. The experiments were performed under normoxic conditions. The values represent the average of the triplicate experiments, and the error bars indicate standard deviations.

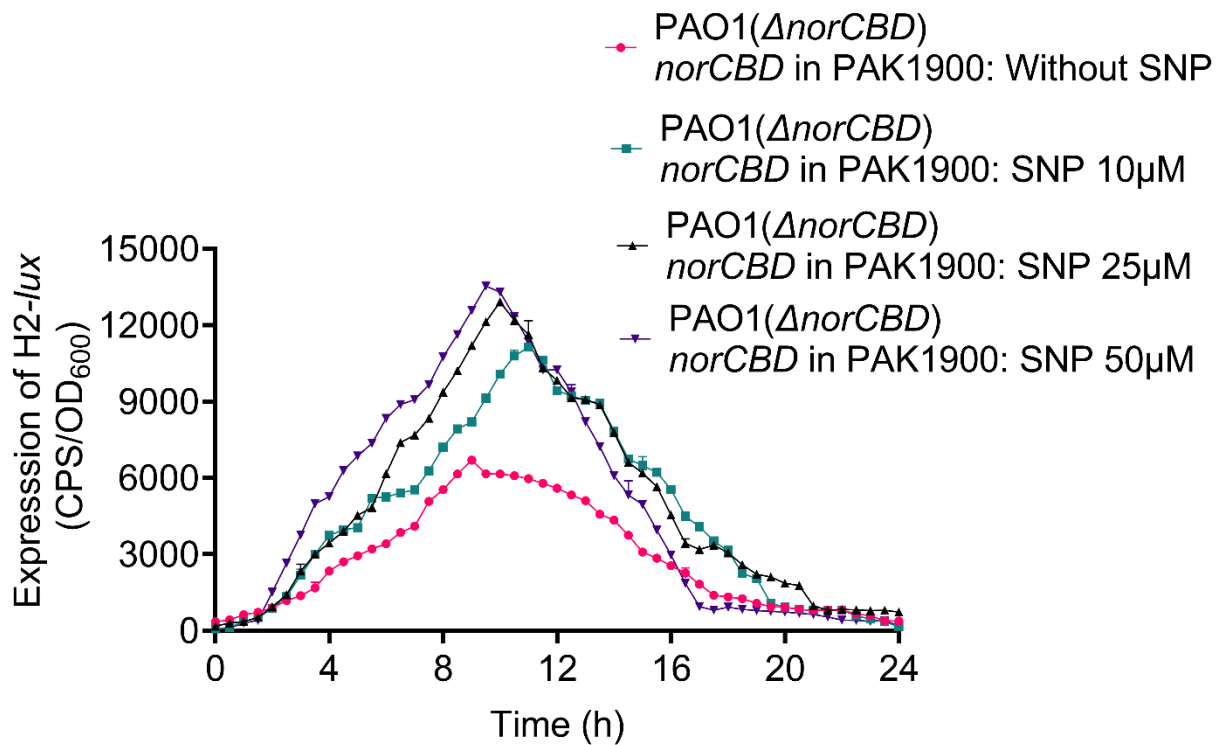


Figure 3.4 Effect of NO donor SNP on H2-T6SS expression in the complementation strain.

For the complementation strain, different concentrations of SNP (10 μ M, 25 μ M, and 50 μ M) were added to the medium. The experiments were performed under normoxic conditions. Similar outcomes to those of the wild-type PAO1 were likewise displayed by the complementation strain of *norCBD* combined with CTX-H2-T6SS. Additionally, the experiment demonstrated that at a concentration of 25 μ M SNP, the expression level peaked at 10 h. The values represent the average of the triplicate experiments, and the error bars indicate standard deviations.

3.3.3 Effect of NO scavenger on the H2-T6SS expression

To further pursue this line of inquiry, the effect of NO scavenger CPTIO on the H2-T6SS expression was also determined. The transcriptional activity of CTX-H2-T6SS was similarly assessed in the wild-type PAO1, PAO1($\Delta norCBD$), and complementation strains in the presence and absence of NO scavenger CPTIO. CPTIO reacts rapidly and stoichiometrically with NO to form nitrogen dioxide (NO₂), effectively removing NO from the cell (Goldstein, Russo, & Samuni, 2003; Lichtenberg et al., 2021). As shown, with CPTIO, the expression level of H2-T6SS was significantly reduced in wild-type PAO1 (**Figure 3.5**) and in the complementation strain of *norCBD* (**Figure 3.7**) in a dose-dependent manner. As anticipated, H2-T6SS expression was elevated in the PAO1($\Delta norCBD$) upon treatment with CPTIO (**Figure 3.6**), indicating that NO scavenging alleviates the inhibition of Dnr activation caused by NO accumulation in the mutant.

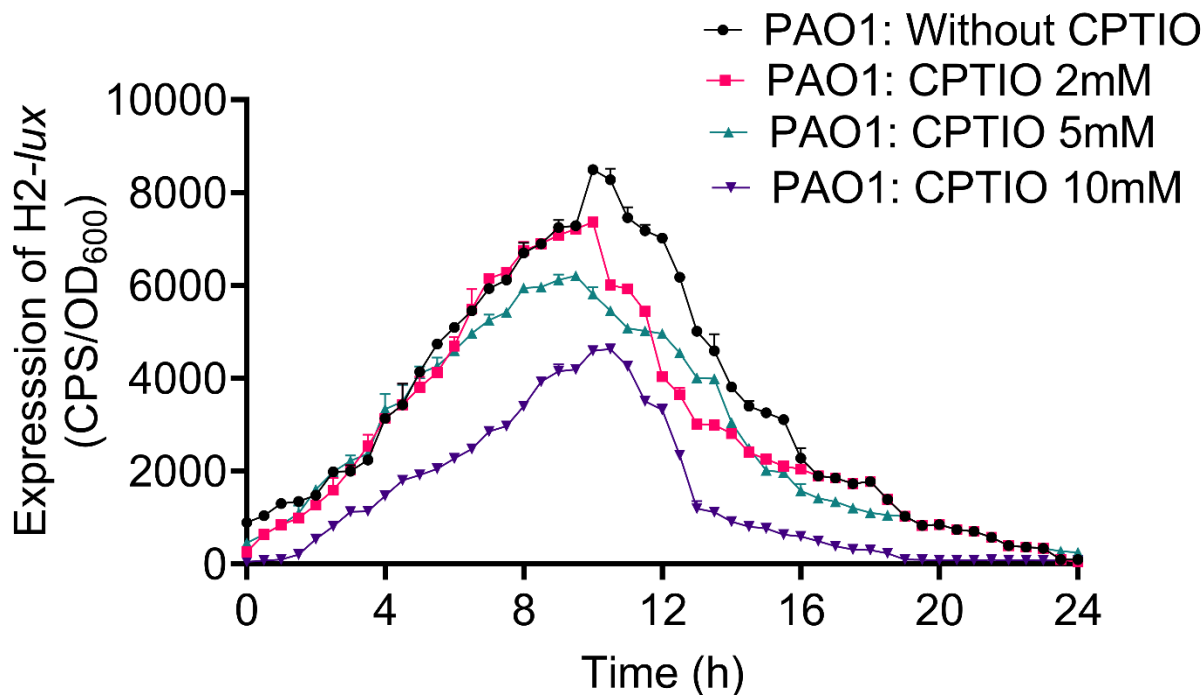


Figure 3.5 Effect of CPTIO on H2-T6SS expression in PAO1. For the wild-type PAO1 strain, Different concentrations of CPTIO (2 mM, 5 mM, and 10 mM) were added to the medium. The experiments were performed under normoxic conditions. Over time, the outcomes in wild-type PAO1 demonstrated a dose-dependent pattern. The expression of H2-T6SS gradually dropped when the concentration of CPTIO was raised to 5 mM and 10 mM, indicating that nitric oxide is being scavenged in the wild-type strains. When the H2-T6SS was examined, especially after 10 h, it was discovered that, in comparison to the control, the concentration of 10 mM revealed a low level of wild-type expression. The values represent the average of the triplicate experiments, and the error bars indicate standard deviations.

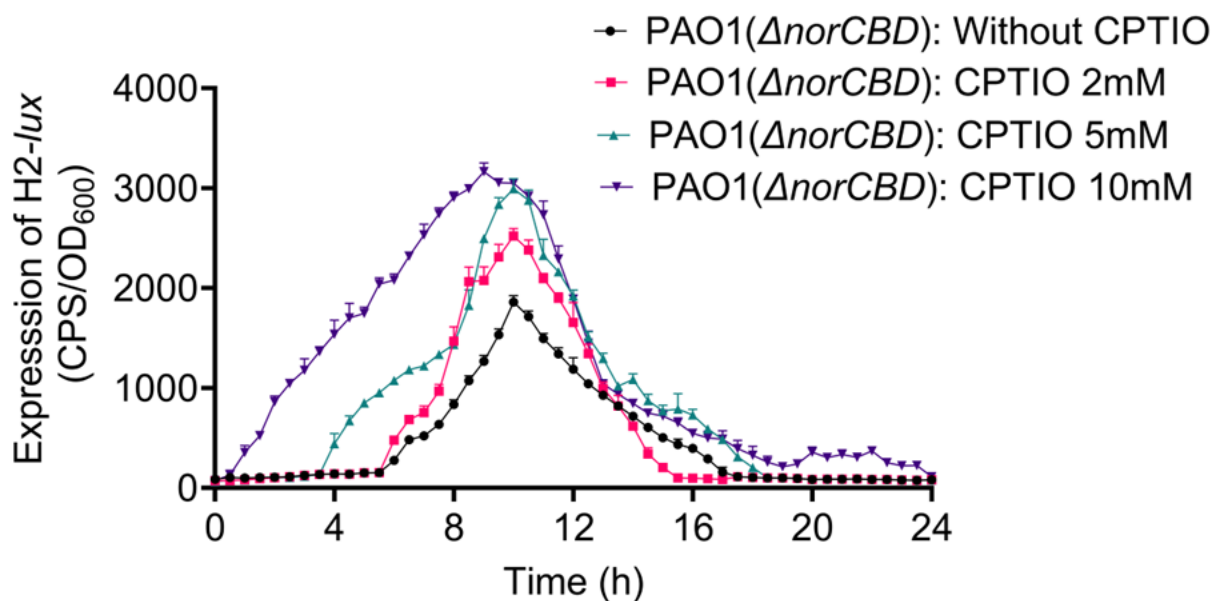


Figure 3.6 Effect of CPTIO on H2-T6SS expression in PAO1($\Delta norCBD$). For the deletion strain, PAO1($\Delta norCBD$), different concentrations of CPTIO (2 mM, 5 mM, and 10 mM) were added to the medium. In the PAO1($\Delta norCBD$), when we increased the CPTIO concentration, the expression level increased considerably. After 10 h, the maximum expression was observed at CPTIO concentrations of 5 mM and 10 mM. The experiments were performed under normoxic conditions. The values represent the average of the triplicate experiments, and the error bars indicate standard deviations.

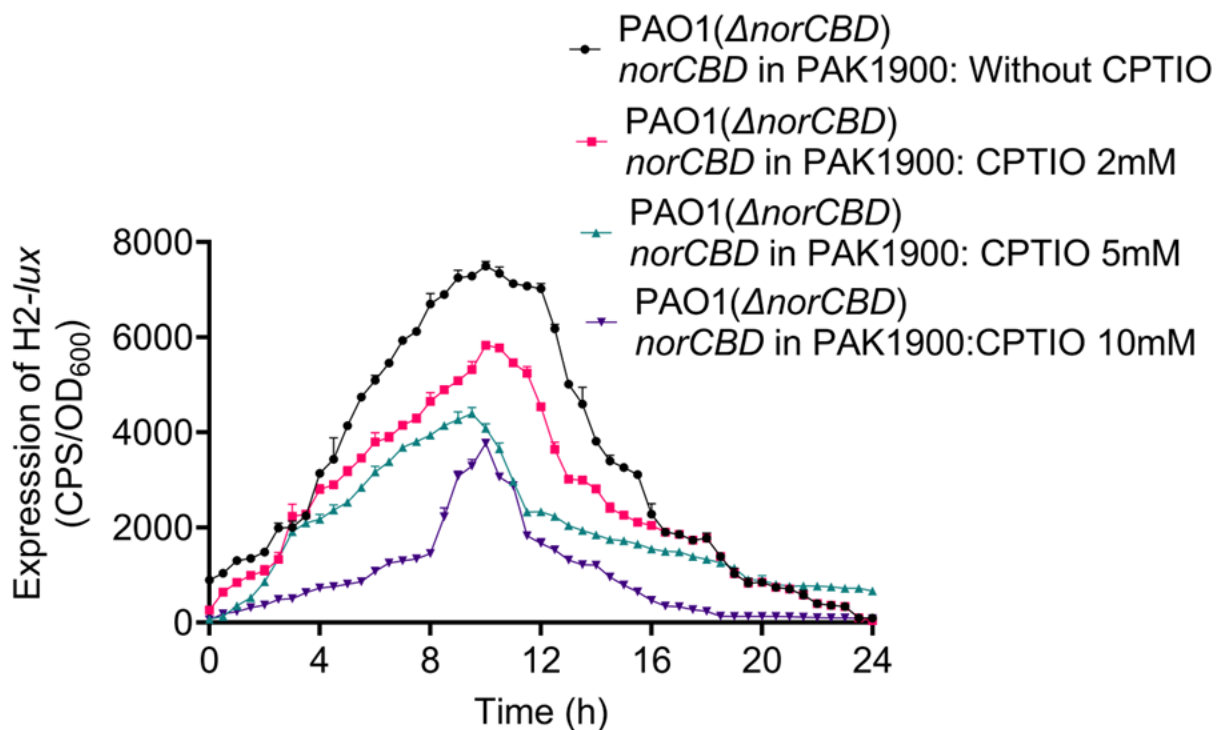


Figure 3.7 Effect of NO scavenger CPTIO on H2-T6SS expression in the complementation strain. For the complementation strain, different concentrations of CPTIO (2 mM, 5 mM, and 10 mM) were added to the medium. The experiments were performed under normoxic conditions. The complementation strain's H2-T6SS expression level coincides with that of the wild-type, PAO1. The values represent the average of the triplicate experiments, and the error bars indicate standard deviations.

3.3.4 Accumulation of NO in the *norCBD* deletion mutant

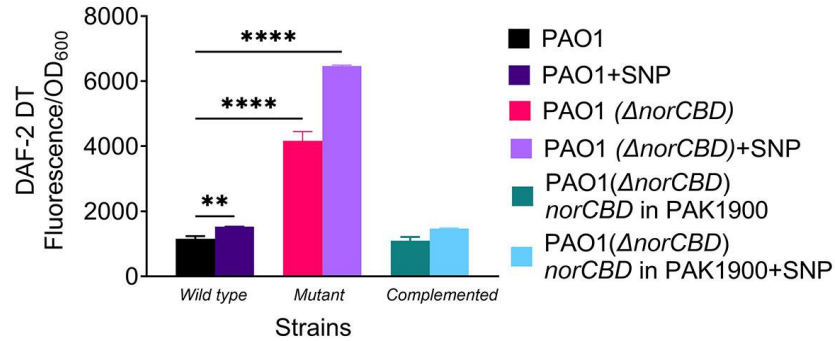
NO is a major signaling molecule in *P. aeruginosa*, but it is also toxic to the cells at high levels (Bogdan, Rollinghoff, & Diefenbach, 2000; Nisbett & Boon, 2016). To confirm the altered NO levels caused by the disruption of *norCBD*, we compared the level of intracellular NO in the wild type and the mutant strains, using diaminofluorescein-2 diacetate (DAF-2 DA), which is a cell-permeable fluorescent NO indicator converted to DAF-2 by the intracellular esterases. Upon reacting with NO, DAF-2 produces DAF-2 TA, a derivative that can be measured by fluorescence. The fluorescent intensity of DAF-2 TA gives a real-time measurement of NO. As expected, the *norCBD* deletion mutant showed significant accumulation of NO compared to the wild-type PAO1 (**Figure 3.8A**) under anaerobic conditions. At the early exponential phase (5 h), NO in the *norCBD* mutant was 3.60-fold higher than that in the wild-type PAO1. The complementation strains showed the accumulation of NO, which is close to wild-type PAO1.

As most of our H2-T6SS expression was measured in normoxic conditions, we also measured the intracellular NO concentrations in such conditions where the strains were cultured in a 96-well plate. Similarly, the results obtained (**Figure 3.8B**) showed that at 5 h, the intracellular NO level in the *norCBD* mutant was significantly elevated (2.78-fold) compared to the wild-type strain. The results confirm that NorCBD disruption causes an unchecked increase of intracellular NO, which likely results in dysregulation of H2-T6SS.

To compare how the wild-type and the mutant respond to NO disturbance, we also quantified intracellular NO levels following the addition of the NO donor, SNP. Final concentration of 25 μ M was added to the wild-type, Δ *norCBD* mutant, and complementation strains, and NO levels were measured at the 5 h time point. Under anaerobic conditions, SNP treatment resulted in a

1.31-fold change of NO in the wild-type relative to untreated control, whereas the *ΔnorCBD* mutant showed a higher fold change of 1.55 (**Figure 3.8A**). Similarly, under normoxic conditions, the wild type showed a 1.23-fold change, which was lower than the 1.54-fold change in the *ΔnorCBD* mutant (**Figure 3.8 B**). These results suggest that the presence of *norCBD* in wild-type effectively consumes excess NO, thereby preventing Dnr inactivation.

A: Anaerobic Conditions



B: Normoxic Conditions

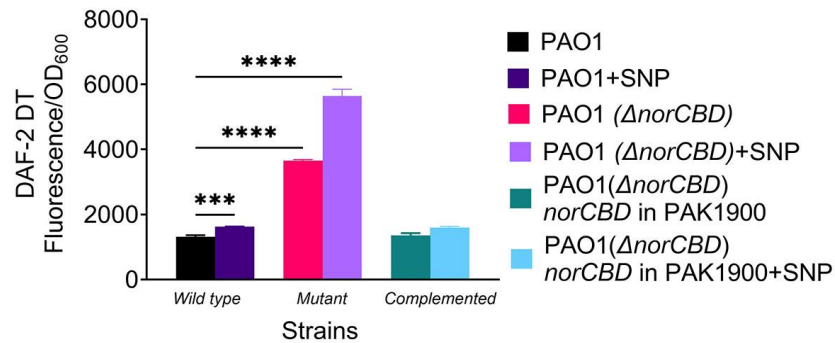


Figure 3.8 Comparison of intracellular nitric oxide levels. The level of intracellular nitric oxide in wild-type PAO1, PAO1($\Delta norCBD$) mutant, and the complemented strain was measured at the 5 h time point in the presence and absence of NO donor SNP. The NO levels reflected by the fluorescence intensity were normalized to OD₆₀₀. **(A)** Under anaerobic conditions, the intracellular NO in PAO1($\Delta norCBD$) was 3.60-fold higher compared with that in the wild-type PAO1. Compared to the untreated, SNP treatment generated a 1.31-fold increase of NO in the wild-type, and a 1.55-fold change in the $\Delta norCBD$ mutant. Complementation restored intracellular NO levels to values that were not statistically significantly different from those in wild-type PAO1. **(B)** Under normoxic conditions, the NO level was 2.78-fold higher in the

ΔnorCBD mutant compared to the wild-type. Upon SNP treatment, the wild type showed a 1.23-fold increase of NO, lower than the 1.54-fold increase in the *ΔnorCBD* mutant. Data was analyzed using one-way ANOVA. Error bars indicate standard deviations. ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3.3.5 An increased level of nitrite was observed upon deletion of *norCBD*

As nitrite plays a key role in the regulation of *P. aeruginosa*, we examined the potential regulatory effect of nitrite on *norCBD*, we measured the concentration of nitrite using a nitrate/nitrite fluorometric assay kit from Cayman Chemical. DAN was added, followed by the addition of NaOH, which allows the detection of a fluorescent compound known as 1 (H)-naphthotriazole. The concentration of nitrite (NO_2^-) was precisely determined by the measurement of fluorescence. In this experiment, we show that the level of fluorescence was significantly increased in the *norCBD* deletion mutant compared to wild-type PAO1. High fluorescence units indicate high nitrite levels. Whereas the level of fluorescence in the complement strain was restored to the level of wild-type PAO1 (**Figure 3.9**). These results indicate that nitrite is a central key player in nitrogen metabolism in *P. aeruginosa*, playing a significant role when reduced by the cytoplasmic nitrite reductase to produce NO.

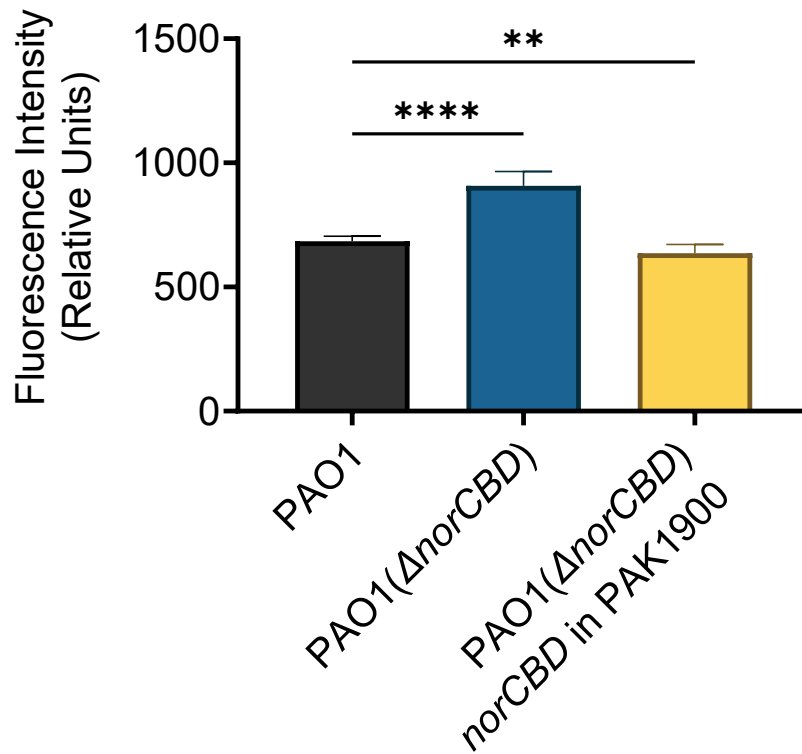


Figure 3.9 Measurement of nitrite level. Nitrite level was significantly increased in the *norCBD* deletion mutant. Nitrite level was measured in wild-type PAO1, PAO1(Δ norCBD) mutant, and its complemented strain using the nitrite fluorometric assay kit (Cayman Chemical). High fluorescence units indicate high nitrite levels. Data was analyzed using one-way ANOVA. Error bars indicate standard deviations. ** $p < 0.01$ and **** $p < 0.0001$.

3.3.6 Effect of *norCBD* disruption and NO accumulation on bacterial growth

It was noted that PAO1(Δ *norCBD*) exhibited slightly reduced growth compared to wild-type PAO1 and the complementation strain under anaerobic conditions (**Figure 3.10**). We reasoned that the accumulation of NO and disruption of anaerobic respiration in the *norCBD* mutant affected the growth.

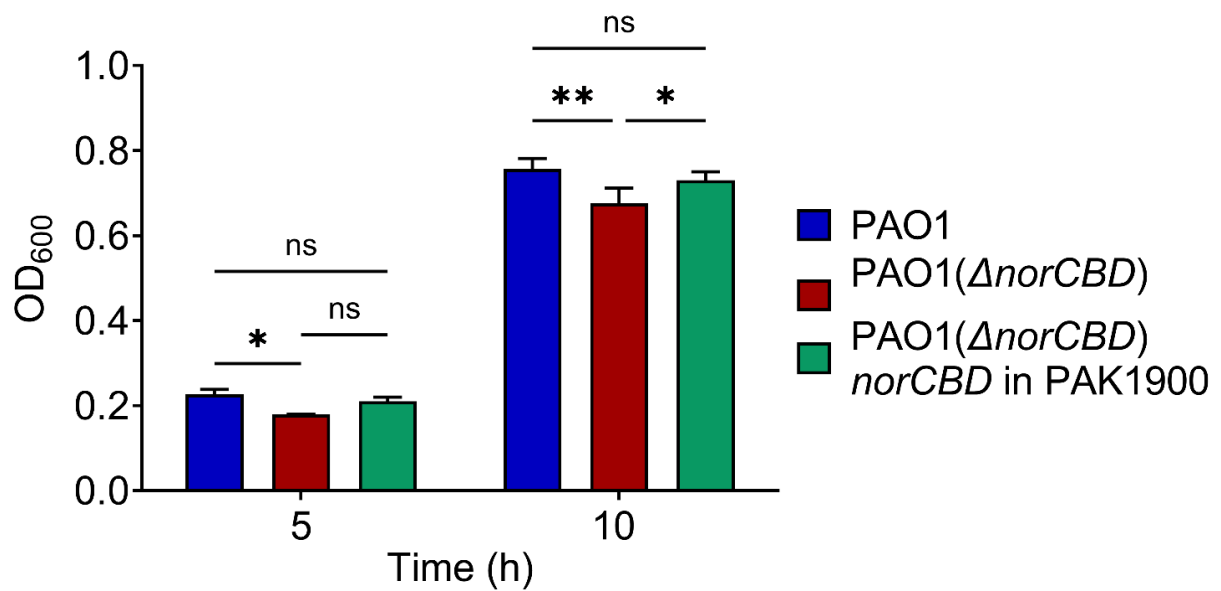


Figure 3.10 *P. aeruginosa* growth (OD_{600}) under anaerobic conditions. The growth of wild-type PAO1, PAO1(Δ *norCBD*), and complementation strains, PAO1(Δ *norCBD*)-*norCBD* in PAK1900, was measured at 5 h and 10 h under anaerobic conditions. Under anaerobic conditions, the growth is relatively slower in PAO1(Δ *norCBD*) than in the wild-type PAO1 and the complementation strain. Data were analyzed using two-way ANOVA and Tukey's multiple comparisons test. Error bars indicate standard deviations. ns (not significant, $p > 0.05$), * $p < 0.05$ and ** $p < 0.01$.

To further investigate the impact of NO accumulation and denitrification disruption on the growth of the *P. aeruginosa*, we compared the growth of the *norCBD* mutant with that of the wild-type and complementation strain under normoxic conditions (**Figure 3.11**).

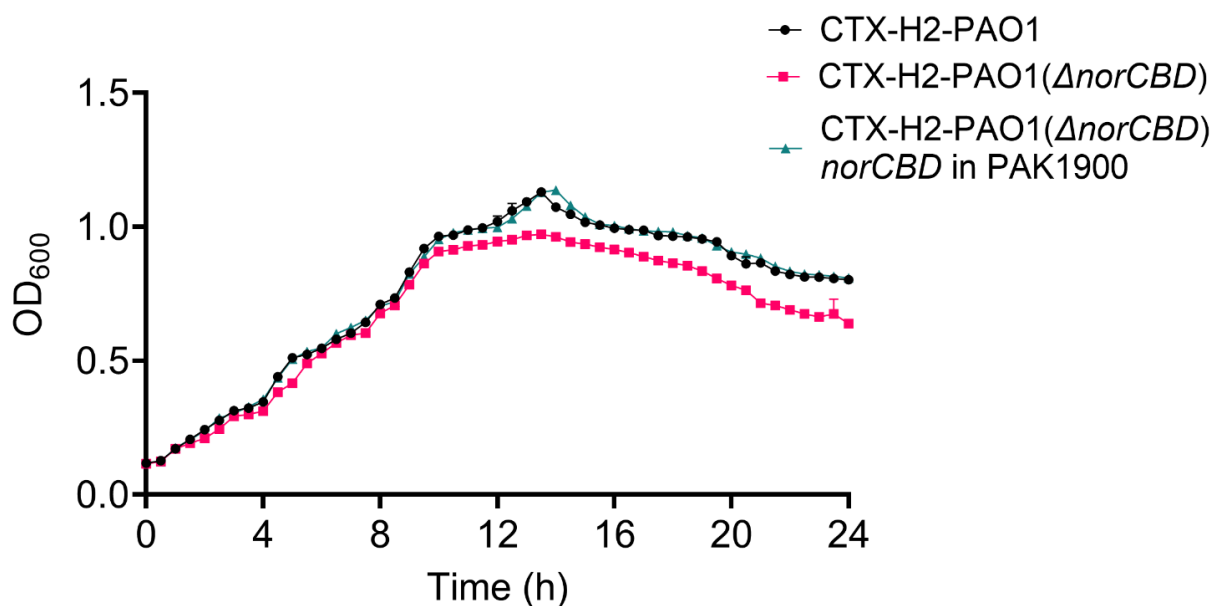


Figure 3.11 *P. aeruginosa* growth (OD₆₀₀) under normoxic conditions. OD₆₀₀ was measured in wild-type PAO1, PAO1(Δ *norCBD*), and complementation strains, PAO1(Δ *norCBD*)-*norCBD* in PAK1900, for 24 hours under normoxic conditions.

We also examined the effects of both an NO donor (**Figure 3.12**) and an NO scavenger (**Figure 3.13**) to determine whether modulation of NO levels could influence growth outcomes. Our results showed that there was no significant difference in growth between the wild-type and Δ *norCBD* mutant under normoxic conditions. A modest reduction of growth was only observed under anaerobic conditions.

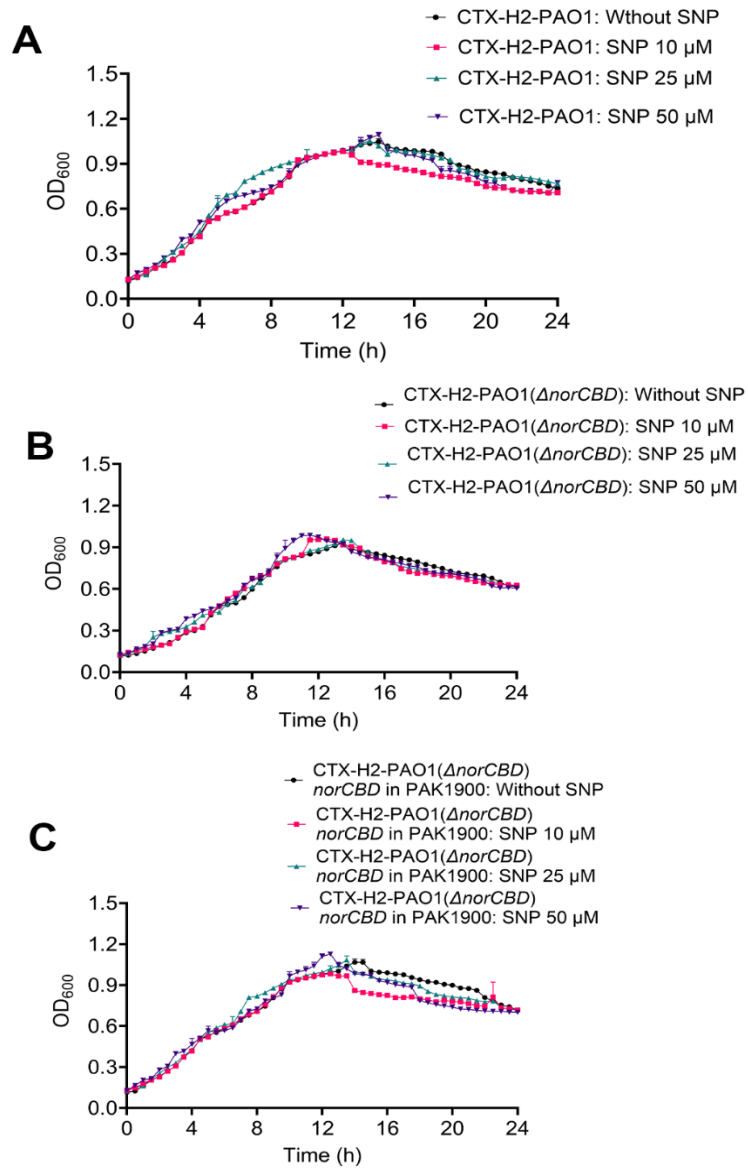


Figure 3.12 (A, B, C) *P. aeruginosa* growth (OD₆₀₀) under normoxic conditions with the addition of SNP. OD₆₀₀ was measured in wild-type PAO1, PAO1(Δ *norCBD*), and complementation strains, PAO1(Δ *norCBD*)-*norCBD* in PAK1900, for 24 hours, and was monitored with the addition of different concentrations of SNP (10 μ M, 25 μ M, and 50 μ M) under normoxic conditions.

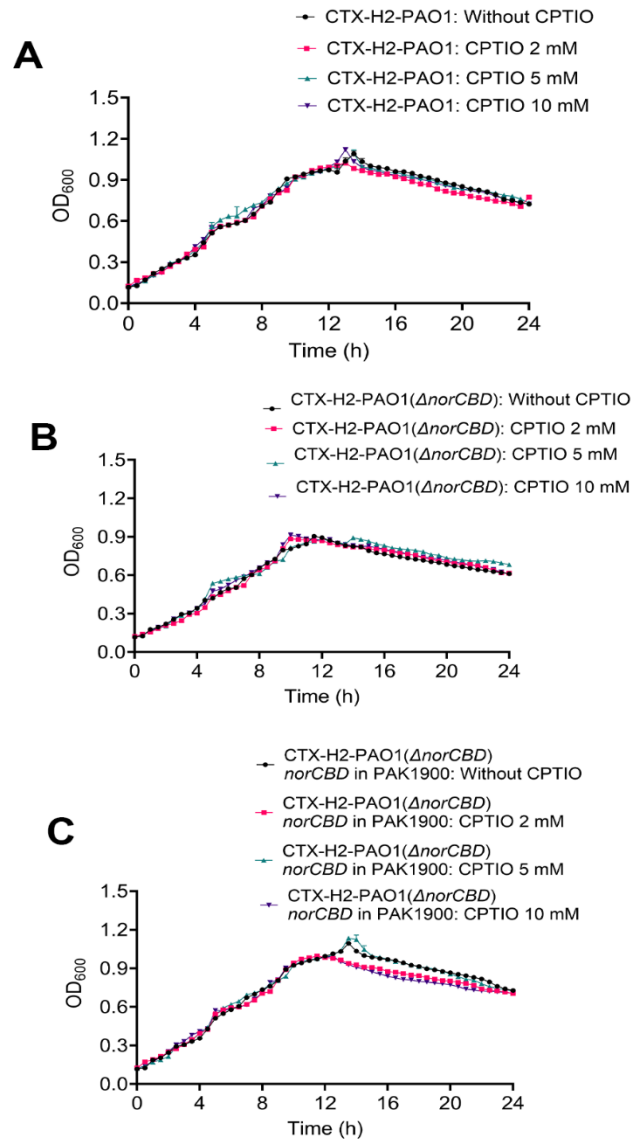


Figure 3.13 (A, B, C) *P. aeruginosa* growth (OD₆₀₀) under normoxic conditions with the addition of CPTIO. OD₆₀₀ was measured in wild-type PAO1, PAO1($\Delta norCBD$), and complementation strains, PAO1($\Delta norCBD$)-*norCBD* in PAK1900, for 24 hours, and was monitored with the addition of different concentrations of CPTIO (2 mM, 5 mM, and 10 mM) under normoxic conditions.

3.3.7 Altered virulence traits in the *norCBD* mutant

P. aeruginosa is widely distributed in the environment and used as a paradigmatic model microorganism for studying virulence properties (Krell & Matilla, 2024). NO serves as a signaling molecule in *P. aeruginosa* (Barraud, Schleheck, et al., 2009). It reduces intracellular levels of cyclic di-GMP, a second messenger that promotes biofilm formation and regulates bacterial motility and influences quorum-sensing systems, which are associated with proteases in *P. aeruginosa* (Barraud, Storey, et al., 2009; Cai et al., 2020). Pyocyanin is one of the major virulence factors in *P. aeruginosa* and plays a crucial role in the pathogenesis of CF-related infections that cause chronic infections in the lungs, where NO is significantly associated with the production of pyocyanin (Gao et al., 2016). NO is endogenously produced in the denitrification pathway, linking it to metabolic and environmental sensing functions. To assess the broader effect of *norCBD* disruption, we tested several phenotypes in the *norCBD* mutant strain PAO1(Δ *norCBD*), together with PAO1 and complementation strains. The phenotypes tested include pyocyanin production, biofilm formation, proteolytic activity, and motility.

As shown in **Figure 3.14**, when *norCBD* was inactivated, the level of pyocyanin was significantly reduced. The complementation of *norCBD* on a plasmid was able to restore the pyocyanin production to the wild-type level (**Figure 3.14A**). The biofilm formation assay showed that the PAO1(Δ *norCBD*) had a lower ability to form biofilms compared to the wild-type PAO1 (**Figure 3.14B**). Similarly, the *norCBD* mutant also showed reduced proteolytic activity (**Figure 3.14C**) and bacterial motility, especially the swimming motility and swarming motility (**Figure 3.14D**).

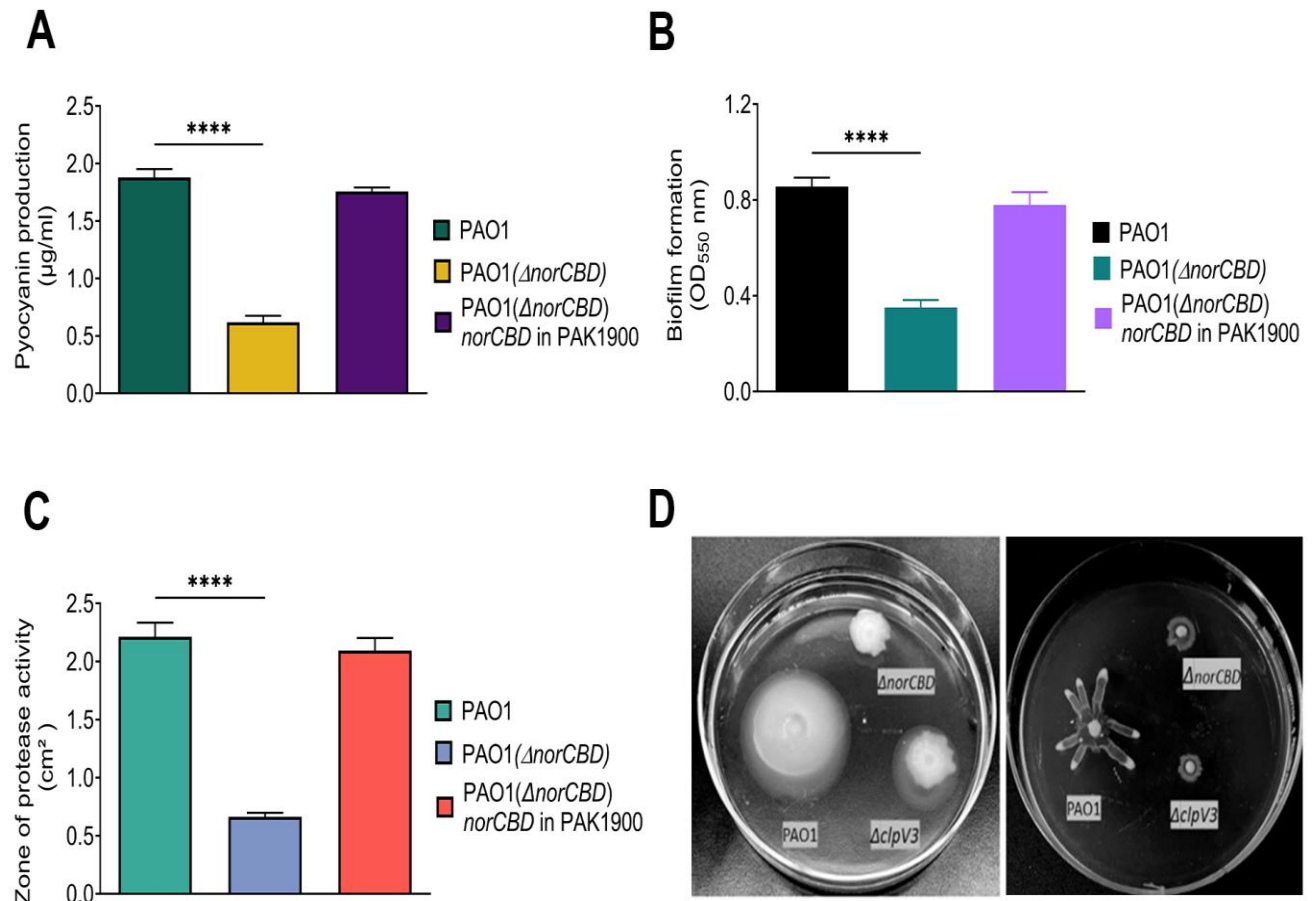


Figure 3.14 Multiple phenotypes of *P. aeruginosa* affected by the deletion of the *norCBD*

gene. (A, B, C) Pyocyanin production, biofilm formation, and proteolytic zone were significantly decreased in PAO1(Δ norCBD) compared with PAO1 or the complementation strain. **(D)** Inactivation of *norCBD* results in reduced swimming and swarming motility. Data was analyzed using one-way ANOVA. Error bars indicate standard deviations. **** $p < 0.0001$

These findings indicate that while NO is an important signal to activate multiple virulence-related phenotypes such as pyocyanin production, biofilm formation, proteolytic activity, and motility, the accumulation of NO in the PAO1($\Delta norCBD$) probably overrides its signaling function and results in the disruption of protease and pyocyanin production, biofilm formation, and motility. These results also indicate that increased intracellular NO and nitrosative stress might deactivate Dnr, which is a redox-sensitive regulator and interferes with signaling and quorum sensing, ultimately resulting in the regulation of these virulence traits.

3.3.8 Deletion of the *norCBD* gene reduced levels of the second messenger cAMP

It has been shown that the expression of H2-T6SS is positively regulated by NO-Dnr (Dang et al., 2022) but negatively modulated by cAMP-Vfr in *P. aeruginosa*. To investigate whether the observed phenotypical changes in the *norCBD* knockout mutant could be a result of altered cAMP levels, we compared the level of cAMP in the *norCBD* mutant with that of the wild-type and the complementation strains. Using the commercially available Cyclic AMP Select ELISA Kit, we assessed the intracellular concentrations of cAMP in PAO1 and PAO1($\Delta norCBD$), and the results obtained indicate that the disruption of *norCBD* reduced the cAMP level (**Figure 3.15**) when compared with the wild-type PAO1 and the complementation of *norCBD*.

Considering the previous report that the expression of T6SS inversely correlates with cAMP levels (Zhang et al., 2022), these results could not explain the decreased H2-T6SS expression observed in the *norCBD* mutant. It is possible that the effect of the NO imbalance on H2-T6SS overrode the cAMP effect in the mutant.

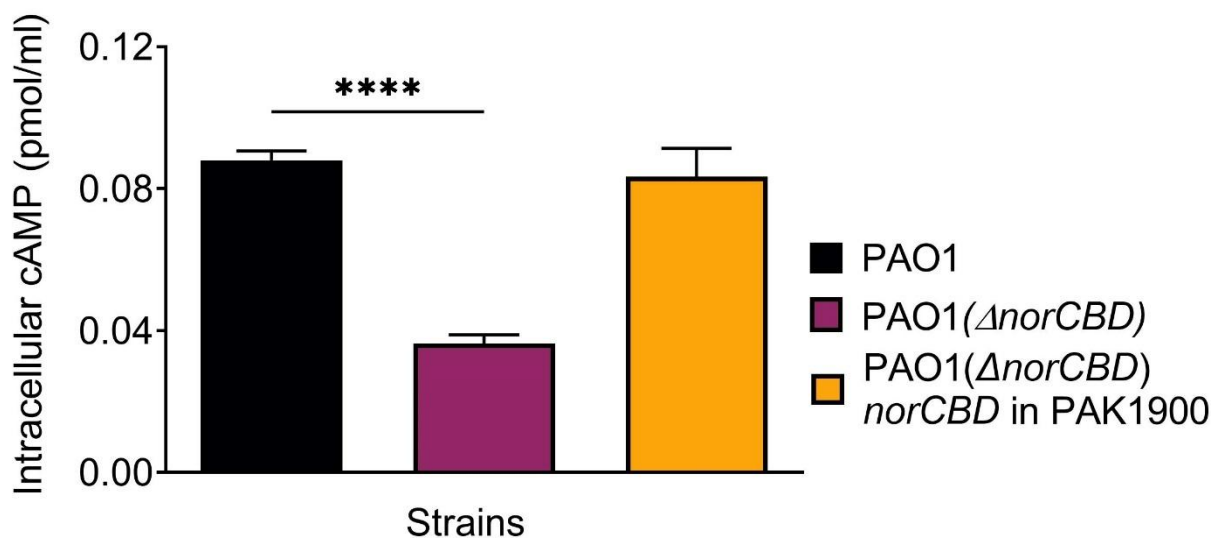


Figure 3.15 Reduced cAMP level in PAO1(Δ *norCBD*). cAMP concentration was quantified in wild-type PAO1, PAO1(Δ *norCBD*) mutant, and the complemented strain using the Cyclic AMP Select ELISA Kit. Data was analyzed using one-way ANOVA. Error bars indicate standard deviations. **** $p < 0.0001$.

3.4 Discussion

This study identifies the *norCBD* operon as a previously unrecognized component that positively influences H2-T6SS transcription in *P. aeruginosa*. Deletion of *norCBD* elevated intracellular NO yet paradoxically suppressed H2-T6SS promoter activity; the expression was restored by genetic complementation and by NO scavenging with CPTIO, and H2-T6SS expression was enhanced by an NO donor (SNP) only in wild-type and complemented strains.

The *norCBD* mutant showed a slight reduction in growth under anaerobic conditions, with no significant differences observed in other conditions. It exhibited broad defects in pyocyanin production, proteolysis, biofilm formation, and motility. Together, these observations support a

model in which NorCBD maintains an optimal intracellular NO range required for Dnr-dependent activation of H2-T6SS and associated virulence programs; excessive NO pushes cells outside this range, attenuating Dnr output and disrupting multiple traits.

The biphasic behavior of NO on H2-T6SS links two seemingly opposing effects: NO is the activating signal for the Anr-Dnr-H2-T6SS cascade (Luo et al., 2025; T. Wang et al., 2021), yet too much NO is inhibitory. In our data, moderate NO in the wild-type enhances H2-T6SS, whereas further NO elevation in the *norCBD* mutant depresses expression. Based on both the results observed and reports in the literature, we propose a potential model in which NorCBD in *P. aeruginosa* functions to maintain NO levels within a permissive range for Dnr function; without NorCBD, excessive NO would impair Dnr function.

This tentative model is supported by several lines of evidence. In the NO donor and scavenger experiments, we observed that the addition of donor SNP activated H2-T6SS expression, and the scavenger CPTIO decreased H2-T6SS expression in the wild-type. An activation of Dnr in response to NO (in turn, an activation of H2-T6SS) was expected when SNP was added, whereas CPTIO would result in decreased Dnr activity. However, in the *norCBD* mutant, opposite effects were observed, i.e., a decrease in H2-T6SS expression with SNP and an increase with CPTIO. With the proposed model, the impairment of Dnr function would only happen in the *norCBD* mutant but not in the wild type. In the wild-type, Dnr, in response to NO, induces *norCBD* expression, which in turn decreases NO accumulation, keeping the NO level in the Dnr functional range. The addition of the NO donor SNP in the wild type would increase NO level, allowing Dnr to activate H2-T6SS, and at the same time, Dnr induces *norCBD* expression, avoiding excessive NO accumulation. But in the *norCBD* mutant, the feedback loop is absent,

and the NO donor would make NO accumulation even worse, resulting in further impairment of Dnr function and further decrease in H2-T6SS expression. Likewise, CPTIO would alleviate the impairment of Dnr caused by the excessive NO, increasing H2-T6SS expression in the mutant. Hence, this model appears to be supported by the data obtained from our study.

A possible explanation for the harming effect of excessive NO is that high NO nitrosylates or damages NO-sensitive cofactors such as Fe-S clusters or heme groups in Dnr and other regulators (Crack et al., 2013; Mettert & Kiley, 2014), reducing their activity despite NO being the initial activating signal. Dnr binds NO via its heme cofactor, leading to conformational changes that activate transcription of downstream genes (Giardina et al., 2008). However, the study also notes that excessive NO levels can lead to the formation of inactive Dnr complexes, suggesting a threshold beyond which NO becomes inhibitory. Another study reported that while low to moderate NO levels activate Dnr-dependent transcription, higher NO concentrations fail to do so, also implying that excessive NO impairs Dnr function (Castiglione, Rinaldo, Giardina, & Cutruzzola, 2009). While NO is necessary for Dnr activation, an overabundance can lead to nitrosative stress, potentially modifying Dnr or its DNA-binding capability, thus inhibiting its function (Giardina, Castiglione, Caruso, Cutruzzola, & Rinaldo, 2011). All these findings or suggested mechanisms appear to be in agreement with our tentative model (**Figure 3.16**).

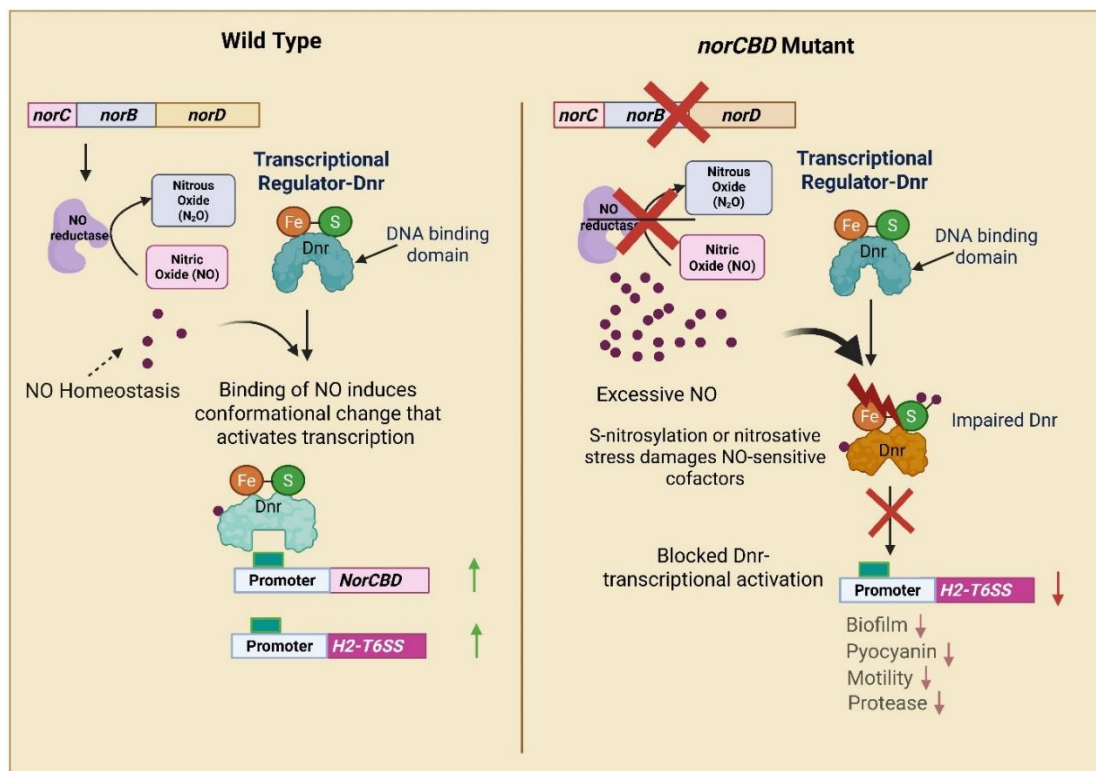


Figure 3.16 Schematic diagram outlining the role of *NorCBD* in H2-T6SS expression and multiple traits in *P. aeruginosa*. In the denitrification process under anaerobic conditions, nitric oxide reductase plays a key role. *norC*, *norB*, and *norD* are necessary for a functional nitric oxide reductase, which reduces NO to N₂O. This process is significant as it protects the bacteria from the toxic effects of excessive NO. The active expression of *norCBD* and H2-T6SS is positively controlled by Dnr in response to NO. However, excessive NO may destabilize iron-sulfur clusters or heme groups, potentially affecting Dnr function and other NO-sensitive regulators. We propose a model where *NorCBD* keeps NO level in a permissive range for Dnr function; without *NorCBD*, excessive NO would impair the function of Dnr. In the wild type, Dnr, in response to NO, induces *NorCBD* production, which in turn decreases NO accumulation. Hence, in conditions of increased NO, such as the addition of the NO donor SNP, an increase in *norCBD* expression and other Dnr-regulated genes would be observed. But in the *norCBD*

mutant, the feedback loop is absent, and NO accumulation would result in impairment of Dnr function and changed regulatory outputs, such as decreased H2-T6SS activation.

The growth defect of the *norCBD* knockout mutant under anaerobic conditions is consistent with the enzyme's canonical role as the terminal NO reductase of denitrification (Arai, 2011; Zumft, 1997); without NorCBD, NO accumulates, respiration is compromised, and the resulting stress spreads into regulatory circuits that shut down non-essential systems, including T6SS. To verify that disruption of *norCBD* alters intracellular NO levels, we quantified NO using diaminofluorescein-2 diacetate (DAF-2 DA) in the presence and absence of NO donor, SNP. As expected, the Δ *norCBD* mutant exhibited a markedly higher NO accumulation than the wild-type PAO1 both in anaerobic and normoxic conditions. These results indicate that increased intracellular accumulation of NO and/or disruption in NO homeostasis occurs when *norCBD* becomes non-functional. The wild type displayed a limited increase in intracellular NO relative to the Δ *norCBD* mutant under both conditions upon SNP treatment when compared with the untreated condition, supporting the proposed model that functional NorCBD in the wild type effectively consumes excess NO and protects Dnr from impairment by the excessive NO.

Prior works have established that Anr and Dnr activate H2-T6SS genes and that H2-T6SS-linked effectors promote fitness and competition in low-oxygen niches (Luo et al., 2025; T. Wang et al., 2021). Our results corroborate this Dnr-dependent logic but add an upstream physiological layer: endogenous NO homeostasis, maintained by NorCBD, is necessary for this pathway. The relevant variable appears to be not NO presence *per se*, but its intracellular range. This agrees with broader observations that redox and nitrosative states interact with multiple global regulators in *P. aeruginosa* (Barraud, Storey, et al., 2009; Korner, Sofia, & Zumft, 2003),

including those that control acute-chronic transitions and metal acquisition, and suggests that H2-T6SS integrates respiratory status more tightly than previously appreciated.

Our data also extend the role of NorCBD beyond classical anaerobic metabolism. A recent report by Stuut Balsam *et al.* (2025) demonstrated that Dnr contributes to fitness not only under strictly anoxic conditions but also under microoxic and even normoxic conditions in the presence of low micromolar nitrate, as is found in standard LB medium (Stuut Balsam, Conaway, Mould, Jean-Pierre, & Hogan, 2025). In that study, nitrate consumption and NO formation under microoxia, and in LasR⁻ strains even under normoxia, created a dependence on Dnr-regulated NorCBD for detoxification and survival. Another study showed that the oxygen-binding protein Mhr is elevated in response to enhanced Anr activity, giving lasR mutants an advantage in microoxic environments (Clay et al., 2020). Our findings align with this conclusion by showing that NorCBD is required to buffer NO and maintain optimal Dnr activity under both normoxic and oxygen-limited conditions. This positions NorCBD not only as a denitrification enzyme but also as a regulator of NO signaling in environments with fluctuating oxygen and nitrate availability, such as cystic fibrosis, where LasR⁻ strains are commonly encountered, and nitrate is abundant in sputum (Kolpen et al., 2014). Thus, NorCBD's influence extends into normoxic physiology, providing a mechanism that safeguards Dnr-dependent transcriptional programs against NO toxicity in diverse environmental contexts. Such a model is consistent with the fact that *norCBD* is under direct transcriptional control of Dnr in *P. aeruginosa*, further reinforcing its role as both a metabolic enzyme and a regulator of NO-dependent signaling.

The changes in virulence-related traits observed in our study support network-level coupling between denitrification physiology and global regulatory pathways. NO sensing in *P. aeruginosa*

causes c-di-GMP-degrading phosphodiesterase to become active, which lowers intracellular c-di-GMP and promotes biofilm dispersal (Cutruzzola & Frankenberg-Dinkel, 2016). At low concentrations, NO induces biofilm dispersal and promotes motility (Barraud et al., 2006; Howlin et al., 2017). As NO is known to reprogram second-messenger signaling, in particular, mis-timed or excessive NO can blunt c-di-GMP-driven switches that underlie biofilm formation and motility (Barraud, Storey, et al., 2009; Y. Li, Heine, Entian, Sauer, & Frankenberg-Dinkel, 2013). The potential connection of NO and Pyocyanin, a major virulence factor that is produced by 90-95% of the strains of *P. aeruginosa*. The observation of this study reported that in the *nor* deletion mutant level of pyocyanin was reduced by 86% when compared with the wild-type PAO1 (Gao et al., 2016). Our results also showed that the level of PCN was effectively inhibited when we knocked out the *norCBD* gene, which indicates that the virulence properties of *P. aeruginosa* were highly affected by nitric oxide. The activation of *norCBD* gene thus assists the bacteria in successfully colonizing and establishing infection by producing pyocyanin. In addition, the denitrification process in *P. aeruginosa* is controlled by the quorum-sensing networks that regulate pyocyanin and protease production (Toyofuku et al., 2007). Through redox and NO-dependent signaling, NO metabolism affects QS-regulated virulence features, such as protease synthesis (Iiyama et al., 2017). Therefore, the reduced virulence-associated phenotypes (pyocyanin, biofilm biomass, proteolytic activity, and motility) in the *ΔnorCBD* mutant are likely connected secondary consequences of impaired Dnr function and disrupted NO homeostasis.

We also observed a decrease in intracellular cAMP in the *norCBD* mutant. Although the cAMP-Vfr signaling axis coordinates diverse virulence-related phenotypes, including T6SS in *P.*

aeruginosa (Fuchs et al., 2010; Zhang et al., 2022), the reduction in cAMP observed in the *norCBD* mutant does not explain the decreased expression of H2-T6SS genes. One possibility is that the dysregulation of NO in the mutant exerts an overriding inhibitory effect on H2-T6SS expression, masking any contributions from lowered cAMP levels. The observed H2-T6SS expression in the mutant was a result of the combined effects of different regulatory factors. The overall outcome from the opposing effects of NO dysregulation and decreased cAMP was a decrease in H2-T6SS expression. As for the decreased cAMP level in the mutant, it is reported that NO can nitrosylate cysteine residues on CRP or associated proteins, potentially altering their DNA-binding affinity or interaction with RNA polymerase (Spiro, 2007). It is also possible that additional routes may exist in which the NO dysregulation can interfere with the cAMP-Vfr pathway. NO accumulation may interfere with the cAMP-Vfr pathway through an as-yet-unidentified mechanism, suggesting the existence of additional regulatory crosstalk between NO signaling and canonical virulence control systems. Nevertheless, future investigation is needed to fully understand the underlying mechanisms of the observed results.

In summary, this work identifies a regulatory layer upstream of Dnr, positioning NorCBD as a key component that enables Dnr-mediated activation of H2-T6SS across oxygen conditions. The results support a biphasic model in which H2-T6SS expression is maximal within an optimal NO window and falls when NO exceeds that range. Our findings broaden the scope of NO homeostasis from a purely anaerobic function to a principal factor that influences *P. aeruginosa* physiology across oxygen gradients, with NorCBD acting as an essential balancer for NO homeostasis.

Chapter 4

Elucidate the roles and potential connection of *clpV3* and *pscN* in H3-T6SS and T3SS

A manuscript for this section is under preparation, and a draft has been written. The manuscript will be authored by **Haque, M. M.**, and Duan, K. (2026), and the tentative title is “*pscN* and *clpV3* affect T3SS and H3-T6SS and multiple virulence factors in *Pseudomonas aeruginosa*”.

Author contributions: MMH and KD conceived and designed the study. MMH performed the experimentation and data generation. Data analysis and interpretation were performed by MMH and KD. Article writing was performed by MMH and KD.

4.1 Introduction

The Type III Secretion System (T3SS) of *P. aeruginosa* is a significant contributor to its virulence. T3SS is often referred to as a “molecular syringe” due to its needle-like structure, which injects proteins and effectors into the host cells (Horna & Ruiz, 2021). Within this secretion system, PscN serves as an ATPase, functioning as a core structural component. The structure of PscN, resembling the F1-ATPase, is categorized under the SctN family and features a distinctive hexameric ring (Ibuki et al., 2011). PscN consists of three domains: N-terminal, central, and C-terminal. The N-terminal domain flexibly repositions and recognizes other domains to assist in oligomerization and membrane association (Okabe, Minamino, Imada, Namba, & Kihara, 2009). Within this process, the N-terminal can strongly interact with PscL (the peripheral stalk) by forming a trimeric complex (Halder, Roy, & Datta, 2019). This complex inhibits ATPase activity by preventing its rotation. The central domain functions as a catalytic domain, with the ATP binding pocket formed by α/β Rossmann folding subunits (Ngo et al., 2020). The C-terminal domain consists of five α helices and primarily functions to recognize and sort the substrate (Allison et al., 2014). Meanwhile, PscO (central stalk) connects the C-terminal to the inner membrane, enhancing the efficiency of substrate translocation (Burkinshaw & Strynadka, 2014). Utilizing these domains, PscN recognizes and binds to T3SS substrates and hydrolyzes ATP. The energy from ATP hydrolysis is further used to release chaperones and unfold proteins for secretion through the needle.

PscN interacts with the needle complex protein, while PscF directs the assembly of the T3SS by ensuring that the needle structure is formed first. Once the needle reaches its full length, the system secretes translocators and effectors, which are mainly regulated by the PopN gatekeeper

(Ngo et al., 2020). The PopN gatekeeper complex consists of PopN, Pcr1, Pcr2, and PscB. PopN interacts with Pcr1 and Pcr2 to repress PscN and take control of the ATPase's substrate preference (Yang et al., 2007). This preference plays a significant role in the function of T3SS, as protein secretion should occur in a specific order to prevent system malfunction. Additionally, PscN interacts with PscL. In vitro, PscL strongly downregulates PscN at the post-translational level by forming a PscN-PscL heterotrimeric complex (Halder et al., 2019). Ultimately, this complex further impairs the T3SS. Given its regulation and interaction with many other genes, PscN could be a potential target for anti-virulence therapies in *P. aeruginosa*.

T6SS functions as a contractile nanomachine that mediates interbacterial competition and translocates toxic effectors into host cells. Among the major structural components, ClpV works as the T6SS system's ATPase. *P. aeruginosa* possesses three ClpV ATPases: ClpV1, ClpV2, and ClpV3. Each ATPase has different functions and interacts with a different T6SS cluster. ClpV1 is mainly involved in H1-T6SS, which primarily targets prokaryotic cells, and the ATPase resets this system after use (L. Chen, Zou, Kronfl, & Wu, 2020). ClpV2 works with H2-T6SS to acquire molybdate by secreting a molybdate-binding protein. This step essentially allows *P. aeruginosa* to survive under anaerobic conditions by enabling nitrate reduction (T. Wang et al., 2021). In H3-T6SS, ClpV3 significantly contributes to H₂O₂ stress resistance by decreasing intracellular free ferrous (Fe²⁺) levels, which can directly react with H₂O₂ to prevent the formation of reactive oxygen species (Lin et al., 2023). Consequently, ClpV activity enhances the bacterium's competitive fitness, as T6SS function is directly related to virulence.

As discussed above, PscN and ClpV3 play key functional roles as ATPases in the T3SS and T6SS, respectively. PscN controls effector secretion, while ClpV3 recycles the contracted H3-

T6SS sheath, allowing repeated rounds of firing. Therefore, further studies are needed to determine whether PscN and ClpV3 are functionally connected. Previous study from our lab demonstrated that deletion of *clpV3* reduces T3SS gene expression and alters several virulence-associated phenotypes (Li et al., 2020), suggesting a possible indirect link between the H3-T6SS and T3SS and a coordinated regulatory network between these two secretion systems. In this study, we have constructed the deletion mutant of *pscN* and *clpV3* in *P. aeruginosa* and report that the transcriptional activity of effector, exoS of T3SS and gene of H3-T6SS is functionally regulated by the knockdown of *pscN* and *clpV3*. We also found that *pscN* and *clpV3* contribute to virulence properties and can affect the ATP level. Overall, these findings highlight the potential role of ATPases in driving T3SS and T6SS functional connection through intracellular ATP utilization.

4.2 Materials and Methods

4.2.1 Bacterial strains and plasmids

All bacterial strains and plasmids used in the experiments are listed in **Table 4.1**. Luria-Bertani (LB) agar and broth were used for the growth media of *E. coli* and *P. aeruginosa*. *P. aeruginosa* knockout mutants PAO1($\Delta pscN$), PAO1 ($\Delta clpV3$), and PAO1 ($\Delta clpV3\Delta pscN$) were plated on the selective *Pseudomonas* isolation agar (PIA) plate. For *E. coli*, we used different antibiotic concentrations: ampicillin (100 $\mu\text{g/ml}$), tetracycline (12.0 $\mu\text{g/ml}$), and kanamycin (50 $\mu\text{g/ml}$). Carbenicillin (250 $\mu\text{g/ml}$) and tetracycline (70 $\mu\text{g/ml}$) were used for the *P. aeruginosa*. In addition, for constructing the deletion mutant, we used tetracycline with a concentration of 300 ($\mu\text{g/ml}$).

Table 4.1 List of bacterial strains and plasmids used.

Bacterial strains and plasmids	Relevant sequence/ characteristics	Source/ Reference
<i>P. aeruginosa</i> strains		
PAO1	Wild-type <i>P. aeruginosa</i>	Duan Lab
PAO1($\Delta pscN$)	<i>pscN</i> knockout mutant of PAO1	This study
PAO1($\Delta clpV3$)	<i>clpV3</i> knockout mutant of PAO1	(Y. Li et al., 2020)
PAO1($\Delta pscN\Delta clpV3$)	<i>pscNclpV3</i> knockout mutant of PAO1	This study
<i>E. coli</i> Stains		
DH5 α	F– Φ 80lacZ Δ M15 $\Delta(lacZYA-argF)$ U169 <i>recA1 endA1 hsdR17</i> (rK–, mK+) <i>phoA</i> <i>supE44</i> λ – <i>thi-1 gyrA96 relA1</i>	Invitrogen
SM10- λ pir	Mobilizing strain, RP4 integrated into the chromosome; Km ^r	Duan Lab
Plasmids		
pEX18Tc	<i>oriT</i> ⁺ <i>sacB</i> ⁺ gene replacement vector with multiple-cloning site from pUC18; Tc ^r	(Hoang et al., 1998)
pAK1900	<i>E. coli-P. aeruginosa</i> shuttle cloning vector, Amp ^r	(Sharp et al., 1996)
pRK2013	Broad-host-range helper vector; Tra ⁺ , Km ^r	(Ditta, Stanfield, Corbin, & Helinski, 1980)
CTX-6.1	Integration plasmid origins of plasmid mini-CTX- lux; Tc ^r	(Kong et al., 2013)
pEX18Tc- <i>pscN</i> up	pEX18Tc carrying the upstream fragment of <i>pscN</i>	This study

pEX18Tc- <i>pscN</i> _{up} +dw	pEX18Tc carrying the upstream and downstream fragments of <i>pscN</i>	This study
pEX18Tc- <i>clpV3</i> up	pEX18Tc carrying the upstream fragment of <i>clpV3</i>	This study
pEX18Tc- <i>clpV3</i> _{up} +dw	pEX18Tc carrying the upstream and downstream fragments of <i>clpV3</i>	This study
CTX- <i>H3-T6SS</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>H3-T6SS</i> containing <i>H3</i> promoter region and <i>luxCDABE</i> gene; <i>Kn^r</i> , <i>Tmp^r</i> , <i>Tc^r</i>	Duan Lab
CTX- <i>H3-T6SS</i> in PAO1(Δ <i>pscN</i>)	Integration plasmid, CTX6.1 with a fragment of pKD- <i>H3-T6SS</i> - PAO1(Δ <i>pscN</i>) containing <i>H3</i> promoter region and <i>luxCDABE</i> gene; <i>Kn^r</i> , <i>Tmp^r</i> , <i>Tc^r</i>	This study
CTX- <i>H3-T6SS</i> in PAO1(Δ <i>clpV3</i>)	Integration plasmid, CTX6.1 with a fragment of pKD- <i>H3-T6SS</i> - PAO1(Δ <i>clpV3</i>) containing <i>H3</i> promoter region and <i>luxCDABE</i> gene; <i>Kn^r</i> , <i>Tmp^r</i> , <i>Tc^r</i>	This Study
CTX- <i>H3-T6SS</i> in PAO1(Δ <i>pscN</i> Δ <i>clpV3</i>)	Integration plasmid, CTX6.1 with a fragment of pKD- <i>H3-T6SS</i> - PAO1(Δ <i>pscN</i> Δ <i>clpV3</i>) containing <i>H3</i> promoter region and <i>luxCDABE</i> gene; <i>Kn^r</i> , <i>Tmp^r</i> , <i>Tc^r</i>	This Study
CTX- <i>H3-T6SS</i> in PAO1(Δ <i>clpV3</i>)- <i>pscN</i> in PAK1900	Integration plasmid, CTX6.1 with a fragment of pKD- <i>H3-T6SS</i> - PAO1(Δ <i>clpV3</i>)- <i>pscN</i> in PAK1900 containing <i>H3</i> promoter region and <i>luxCDABE</i> gene; <i>Kn^r</i> , <i>Tmp^r</i> , <i>Tc^r</i>	This Study

CTX- <i>H3-T6SS</i> in PAO1($\Delta clpV3$)- <i>clpV3</i> in PAK1900	Integration plasmid, CTX6.1 with a fragment of pKD- <i>H3-T6SS</i> - PAO1($\Delta clpV3$)- <i>clpV3</i> in PAK1900 containing <i>H3</i> promoter region and <i>luxCDABE</i> gene; Km ^r , Tmp ^r , Tc ^r	This Study
CTX- <i>exoS</i> -PAO1	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoS</i> containing <i>exoS</i> promoter region and <i>luxCDABE</i> gene; Km ^r , Tmp ^r , Tc ^r	Duan Lab
CTX- <i>exoS</i> in PAO1($\Delta pscN$)	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoS</i> - PAO1($\Delta pscN$) containing <i>exoS</i> promoter region and <i>luxCDABE</i> gene; Km ^r , Tmp ^r , Tc ^r	This Study
CTX- <i>exoS</i> in PAO1($\Delta clpV3$)	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoS</i> - PAO1($\Delta clpV3$) containing <i>exoS</i> promoter region and <i>luxCDABE</i> gene; Km ^r , Tmp ^r , Tc ^r	This Study
CTX- <i>exoS</i> in PAO1($\Delta pscN\Delta clpV3$)	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoS</i> -PAO1($\Delta pscN\Delta clpV3$) containing <i>exoS</i> promoter region and <i>luxCDABE</i> gene; Km ^r , Tmp ^r , Tc ^r	This Study
CTX- <i>exoS</i> in PAO1($\Delta pscN$)- <i>pscN</i> in PAK1900	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoS</i> -PAO1($\Delta pscN$)- <i>pscN</i> in PAK1900 containing <i>exoS</i> promoter region and <i>luxCDABE</i> gene; Km ^r , Tmp ^r , Tc ^r	This Study
CTX- <i>exoS</i> in PAO1($\Delta pscN$)- <i>clpV3</i> in PAK1900	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoS</i> - PAO1($\Delta pscN$)- <i>clpV3</i> containing <i>exoS</i> promoter region and <i>luxCDABE</i> gene; Km ^r , Tmp ^r , Tc ^r	This Study

4.2.2 Construction of gene knockout strains

The Sucrose counter-selection system was used to generate deletion mutants, PAO1($\Delta pscN$), PAO1 ($\Delta clpV3$), and PAO1 ($\Delta clpV3\Delta pscN$), where the allelic exchange method was applied and pEX18Tc was used as a plasmid/vector (Hmelo et al., 2015). PAO1($\Delta pscN$) was constructed by the following methods. In detail, the primers (**Table 4.2**) were designed for the gene of interest ($pscN$). The upstream (0.5 kb) and downstream (0.6 kb) fragments of $pscN$ were amplified via PCR. The amplified fragments and plasmid were both digested with the appropriate restriction enzyme and ligated, and transformed into the vector, pEX18Tc, and generated pEX18Tc- $pscNup+down$. Tri-parental mating was used to obtain the knockout mutant, PAO1($\Delta pscN$). Tri-parental mating methods used the helper strain, *E. coli*, which contains the recipient strain along with the donor strains of pEX18Tc- $pscNup+down$ and the PAO1(the recipient strain). At first, all three of these strains (donor, helper, and recipient) were grown overnight, and the cultures were washed with 1X PBS buffer. Bacterial cultures were mixed and spotted (ratio, helper:donor: recipient, 2:2:1) onto the LB agar and incubated at 37°C for 12 hrs. For selection of merodiploid, the cells were scraped and resuspended in LB broth and plated on *Pseudomonas isolation agar* with tetracycline (300 µg/ml). This process is known as the first crossover. For selecting a second crossover, the colony was streaked in LB agar containing 10% sucrose and no NaCl after the first crossover. PAO1($\Delta pscN$) was verified by polymerase chain reaction using the confirmation primers (**Table 4.2**). Furthermore, PAO1 ($\Delta clpV3$) and PAO1 ($\Delta clpV3\Delta pscN$) were generated using the same methods as described above and were confirmed by PCR.

4.2.3 Construction of pscN and clpV3 complementation plasmids

E. coli-*P. aeruginosa* shuttle vector pAK1900 was used for the complementation of *pscN* and *clpV3* knockout mutants. First, the intact gene of interest (*pscN* and *clpV3*) was amplified from genomic DNA using designed primers (**Table 4.2**). Then the amplified product was cloned into the shuttle vector pAK1900. Finally, resultant plasmids were transformed into *P. aeruginosa* PAO1 by electroporation.

Table 4.2 List of primers used.

Primer	Sequence (5'→3')	Restriction Site
<i>pscN</i> -up-S	ATAGAATTCAGCTTCTGCTGGCTTTCCAGGT	<i>EcoRI</i>
<i>pscN</i> -UP-AS	TATGGATCCTTGAGCAGGGTTCCGGTGA	<i>BamHI</i>
<i>pscN</i> -dw-S	AATGGATCCTACCCACGAAACCAGCGATTA	<i>BamHI</i>
<i>pscN</i> -dw-AS	ATAAAGCTTCCGTATCCACGGCGTTCAGCTT	<i>HindIII</i>
C- <i>pscN</i> -S	CCATCCGCAGCAACGCCTGGTC	
C- <i>pscN</i> -AS	TCCGTCGTTTCGTCGCCCTTGTC	
<i>clpV3</i> -up-S	TAGGAATTCGCCCTATGCCTACCAGGAA	<i>EcoRI</i>
<i>clpV3</i> -up-AS	AAGGATCCGCAGGTGCTCGATCTCTACG	<i>BamHI</i>
<i>clpV3</i> -dw-S	TACGGATCCTGGTGGTGGACTTCAGGAAC	<i>BamHI</i>
<i>clpV3</i> -dw-AS	CCCAAGCTTTTGCTTTCTTCGCTTGTGAA	<i>HindIII</i>
C- <i>clpV3</i> -S	GGTGGAAAGCCTGCTCGACGAC	
C- <i>clpV3</i> -AS	GCGAGGATCCTTTGCCACTTGG	

4.2.4 Measurement of cellular ATP

The ATP levels of the exponential culture were measured using a BacTiter Glo kit (Promega) according to the manufacturer's instructions. In brief, the overnight culture of *P. aeruginosa* was subcultured the next morning until it reached the exponential phase ($OD=0.2$). For liquid culture media, M9 minimal media was used. Following this, 100 μ L ATP reagent and 100 μ L bacterial cells were mixed (1:1 ratio) in a 96-well plate designated for luminescence assay. Since the ATP reagent is light sensitive, all the reactions were set up in the dark. The reaction mixture was allowed to sit in an orbital shaker (2 min), followed by an incubation for 5 min at room temperature. The luminescence reading was taken in a plate reader. The bacterial cell was spread in LB agar in different dilutions to check the colony count. The results were expressed in luminescence (CPS/ OD_{600}). For generating an ATP standard curve, we prepared a concentration of 1 μ M ATP in M9 minimal medium. For this I prepared 1 μ M to 10pM of ATP in culture medium by serial dilution. Different concentration of ATP solution was added in 96 well plate with an equal (100 μ l) volume of BacTiter-Glo™ Reagent. The mixture was allowed to shake orbitally. After 1 min of incubation, the luminescence was recorded.

4.2.5 Bacterial competition assay

A bacterial competition assay was performed according to the previously described protocol with minor modifications (Zhao et al., 2023). In detail, *E. coli* DH5 α and *P. aeruginosa* were grown overnight and subcultured to fresh LB medium until they reached an OD_{600} of 0.2. Both bacterial cells were then washed with 1X PBS twice and mixed in an equal ratio of 1:1. The mixture (2 μ L) was allowed to be spotted onto the nitrocellulose membrane (pore size: 0.22 μ m) on an LB agar plate after concentrating into 20 μ L. Bacterial cells were incubated for 6 hrs at 37°C and plated on both PIA and LB agar plates with dilutions. The colony was counted after 20 hours of

incubation.

The competitive index was calculated as follows:

$$\text{Competitive index} = \frac{\text{CFU ratio of } P. \text{ aeruginosa (CFU after co-culture/Initial CFU)}}{\text{CFU ratio of } E. \text{ coli (CFU after co-culture/Initial CFU)}}$$

We also conducted a fluorometric competition assay using *E. coli* that contained the pilG-DsRed expressing vector. By emitting the red fluorescence, the assay confirms the survival of *E. coli* when they were co-incubated and sharing the same niche with *P. aeruginosa*. All the slides were prepared after co-incubation, and images were taken using a microscope (Nikon ECLIPSE Ti-E Inverted) at a magnification of 20X.

4.3 Results

4.3.1 Deletion of T3SS and T6SS ATPases leads to elevated intracellular ATP levels

P. aeruginosa relies on energy-demanding systems whose activity depends on the cellular ATP balance. We hypothesize that deletion of ATPases disrupts intracellular ATP homeostasis in *P. aeruginosa*. Given that the ATPases PscN and ClpV3 have not been fully characterized in *P. aeruginosa*, we sought to examine their functional roles by assessing intracellular ATP levels. This rationale is supported by recent findings showing that ClpV3-mediated H3-T6SS activity modulates T3SS expression and broader virulence-associated pathways, indicating a coordinated regulatory link between these secretion systems (Li et al., 2020). For this purpose, we constructed the deletion mutant of PAO1($\Delta clpV3$), PAO1($\Delta pscN$), and the double deletion mutant, PAO1($\Delta clpV3\Delta pscN$). We compared their energy level (ATP) with wild-type PAO1 by measuring the luminescence/OD₆₀₀ (ATP level) from the late exponential phase of growth. We have also constructed and tested the ATP level of different complementation strains, e.g., PAO1($\Delta clpV3$)-clpV3 in pAK1900, PAO1($\Delta pscN$)-pscN in pAK1900, PAO1($\Delta clpV3$)-pscN in pAK1900, and PAO1($\Delta pscN$)-clpV3 in pAK1900. The level of intracellular ATP was measured using a BacTiter Glo kit (Promega). The amount of ATP is directly proportional to the number of active cells present in the culture.

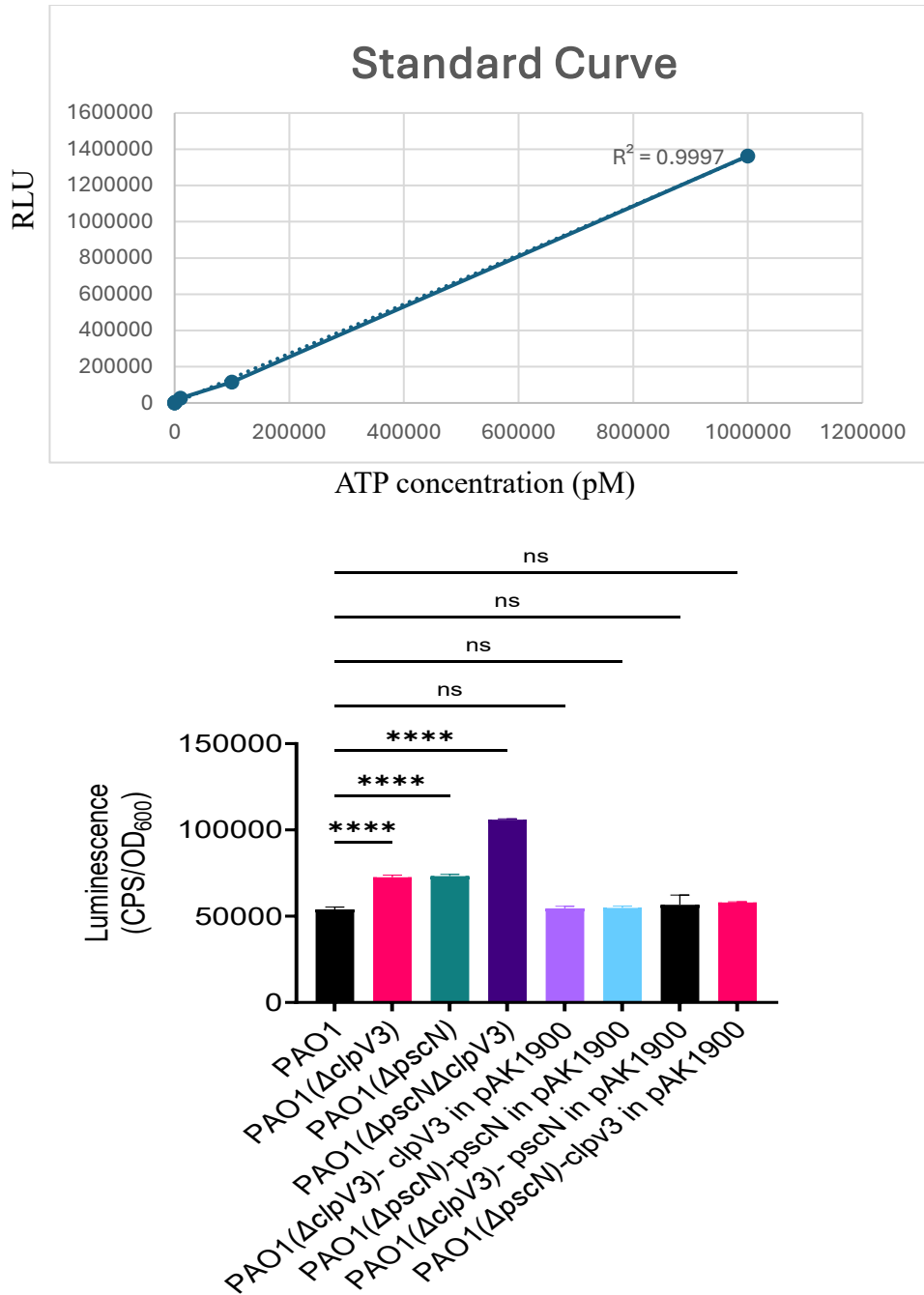


Figure 4.1 Accumulation of ATP upon deletion of ATPase. (A) Standard curve showed linearity ($R^2 = 0.9997$) (B) The ATP levels of the exponential culture were measured using a BacTiter Glo kit (Promega) according to the manufacturer's instructions. Tests of the ATP levels in PAO1(Δ pscN), PAO1(Δ clpV3), and the double mutant showed a higher ATP level than the

wild-type PAO1. The complementation of *pscN* and *clpV3* showed ATP levels closer to wild-type PAO1.

As shown in **Figure 4.1**, the ATP level increased significantly when we deleted ATPases *clpV3* and *pscN* compared to the wild-type PAO1. The ATP level in the knockout mutants of *clpV3* and *pscN* was 1.34- and 1.35-fold higher, respectively, than that in the wild-type PAO1.

Interestingly, our experiment indicates that the double knockout mutant PAO1(Δ *clpV3* Δ *pscN*) exhibited a 2-fold increase in ATP level compared to the wild-type PAO1 due to the absence of both ATPases, suggesting that the overall ATP pool is significantly impacted when both ATPases are knocked out simultaneously. The complementation strains of *clpV3* and *pscN* demonstrated that the level of cellular ATP was restored when compared with the wild-type PAO1. Moreover, our results also revealed that the ATP levels of the complementation strains PAO1(Δ *clpV3*)-*pscN* in pAK1900 and PAO1(Δ *pscN*)-*clpV3* in pAK1900 displayed similar trends, and these ATP levels are comparable to those of the wild type, indicating that ATPase, *clpV3*, and *pscN* support each other interchangeably. This is the very first report demonstrating that cellular energy levels were significantly affected when we knocked out the two major ATPases, *clpV3* and *pscN*. Therefore, further studies are required to elucidate the regulatory mechanisms and functional roles of protein secretion systems, as ClpV3 and PscN act as key ATPases driving T6SS and T3SS, respectively.

4.3.2 Influence of *pscN* and *clpV3* on the transcription of T3SS

In *P. aeruginosa*, the T3SS delivers various effectors to the host cell's cytoplasm, modulating cellular processes and contributing to disease development. Among these effectors, ExoS, ExoY, ExoT, and ExoU play significant roles in modulating host cell functions and promoting bacterial virulence (Kilmury, Graham, Lamers, MacNeil, & Burrows, 2025). To determine whether T3SS gene expression was affected by PAO1(Δ *pscN*) and PAO1(Δ *clpV3*), we constructed promoter reporter strains of CTX-exoS-PAO1(Δ *pscN*) and CTX-exoS-PAO1(Δ *clpV3*). We measured *exoS* promoter activity by analyzing luminescence relative to optical density (CPS/OD₆₀₀).

As shown in **Figure 4.2**, our study demonstrated that at 10 hrs, the transcriptional activity of the effector protein *exoS* was significantly reduced in the deletion mutants PAO1(Δ *pscN*) and PAO1(Δ *clpV3*) compared to the wild-type strain PAO1. This study provides the first evidence that the knockout mutants PAO1(Δ *pscN*) and PAO1(Δ *clpV3*) exhibited a lower expression of 5.44-fold and 4.75-fold, respectively, than PAO1. These results demonstrate a significant decrease in T3SS gene expression in the absence of *pscN* and *clpV3*. We checked the expression of *exoS* in the double deletion mutant and found that PAO1(Δ *clpV3* Δ *pscN*) showed a 12.77-fold lower expression compared to the wild-type PAO1 (**Figure 4.3**). To further assess T3SS promoter activity and validate the specificity of these effects, chromosomally integrated CTX-*exoS*-T3SS-lux reporters were introduced into the complementation strains, PAO1(Δ *pscN*)-*clpV3* in pAK1900, and PAO1(Δ *pscN*)-*pscN* in pAK1900. The complementation of the *pscN* mutant with either *PscN* or *ClpV3* can complement the mutant in T3SS transcriptional control, suggesting that the T6SS ATPase *ClpV3* can back up or compensate for the T3SS ATPase *PscN*.

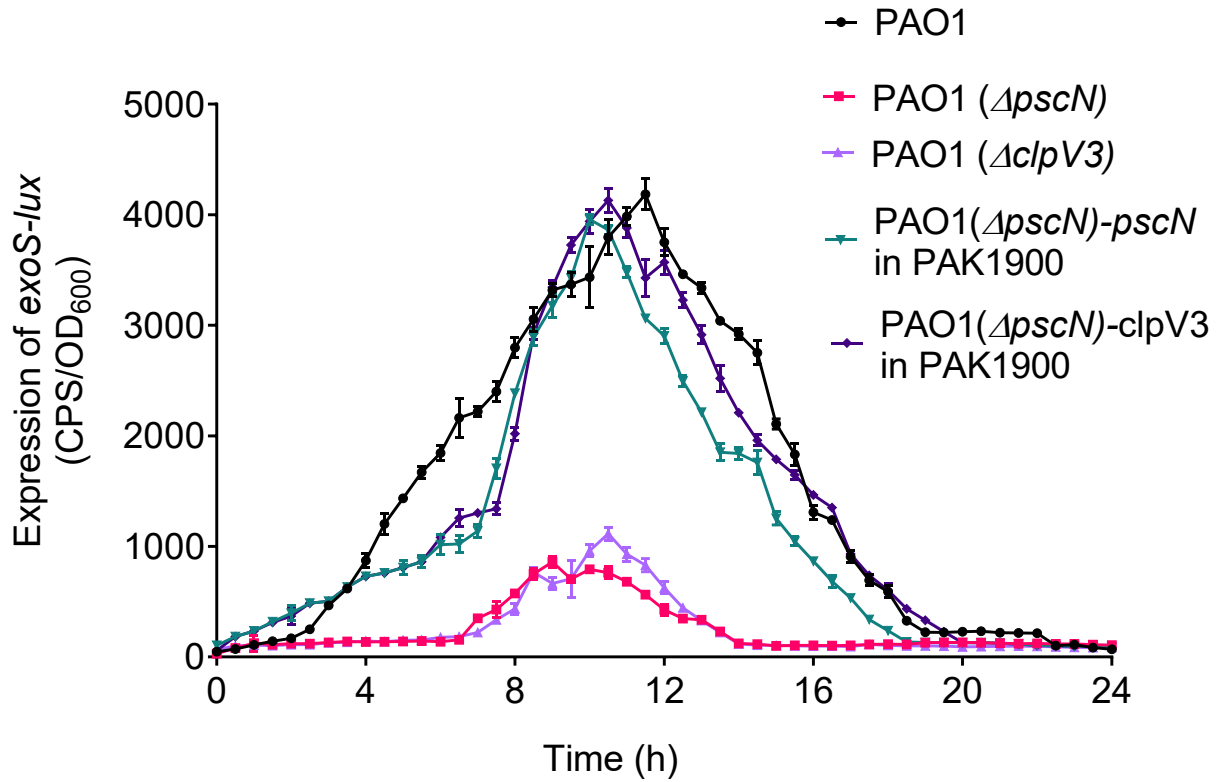


Figure 4.2 Deletion of *pscN* and *clpV3* resulted in decreased expression of *exoS*-T3SS. The CTX-*exoS*-T3SS reporter fusion integrated into the chromosome was used to measure the promoter activity of the *exoS*-T3SS gene in PAO1($\Delta pscN$), PAO1($\Delta clpV3$), and the complementation strains. The results represent the average of three independent experiments, and the error bars indicate standard deviations. The results showed that *exoS* reporter activity was reduced following deletion of *pscN* and *clpV3*.

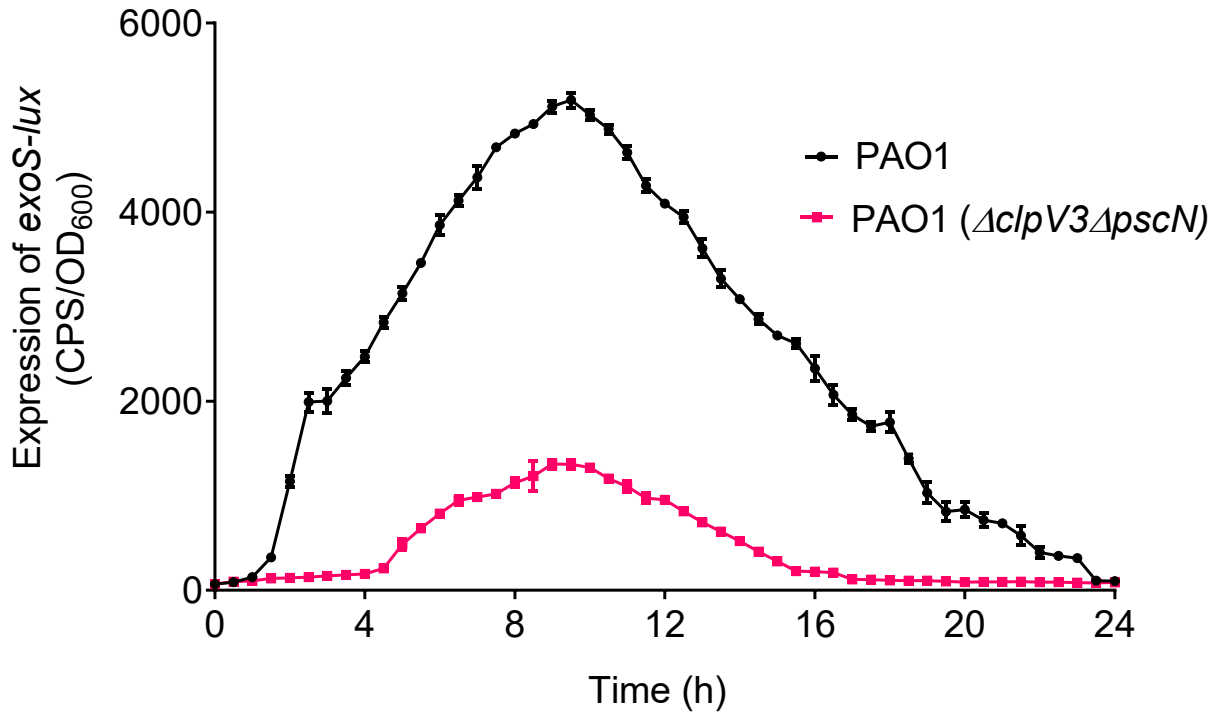


Figure 4.3 PAO1($\Delta clpV3\Delta pscN$) decreased the expression of exoS-T3SS. The CTX-exoS-T3SS reporter fusion integrated into the chromosome was used to measure the promoter activity of the exoS-T3SS gene in PAO1($\Delta clpV3\Delta pscN$). The results represent the average of three independent experiments, and the error bars indicate standard deviations. The findings showed decreased expression of the exoS reporter with the double deletion of pscN and clpV3 together.

4.3.3 The deletion mutants of *pscN* and *clpV3* decreased gene expression of H3-T6SS

Previous studies from our lab have shown that the major ATPase, *clpV3*, of the H3-T6SS can significantly affect the transcriptional expression of the T3SS. The promoter activities of *exoY* and *exsD* were significantly reduced in the PAO1 ($\Delta clpV3$) mutant compared to the wild-type PAO1. (Y. Li et al., 2020). To verify the effect on the transcriptional expression levels of H3-T6SS, we constructed a single-copy, chromosomally integrated CTX-H3-lux reporter in CTX-H3-lux-PAO1 ($\Delta clpV3$), CTX-H3-lux-PAO1 ($\Delta pscN$), and CTX-H3-lux-PAO1 ($\Delta clpV3\Delta pscN$)

by transforming the CTX-H3-PAO1 reporter into the chromosomes of PAO1 ($\Delta clpV3$), PAO1($\Delta pscN$), and PAO1($\Delta clpV3\Delta pscN$). We measured the gene expression by assessing the luminescence (CPS/OD₆₀₀).

As shown in **Figure 4.4**, at 10 hrs, the promoter activity of H3-T6SS was significantly reduced (7.7- and 6.8-fold, respectively, compared to PAO1) when *clpV3* and *pscN* were knocked out. At the same time, the gene expression of H3-T6SS was also significantly decreased by 8.85-fold compared to the wild-type PAO1 when we deleted both genes together (**Figure 4.5**).

Interestingly, in the complementation strains, the expression level of H3-T6SS was restored to that of the wild-type, PAO1 (**Figure 4.5**).

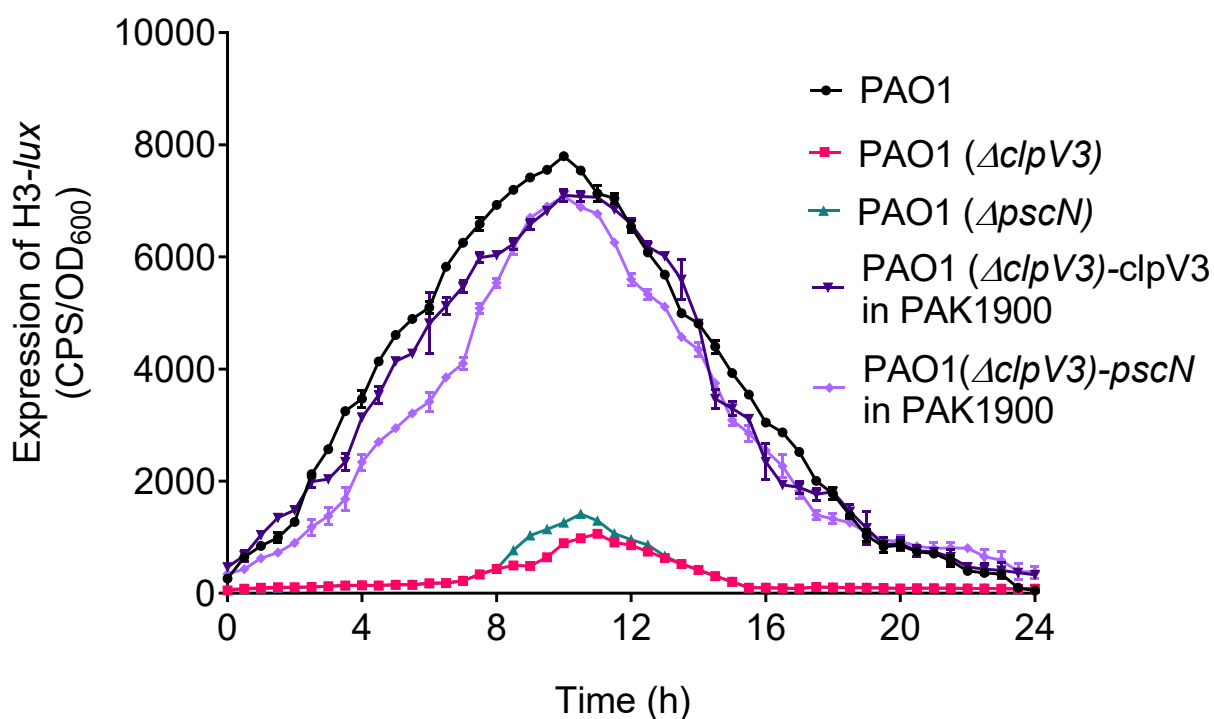


Figure 4.4 Deletion of *pscN* and *clpV3* resulted in decreased expression of H3-T6SS. The promoter activity of the H3-T6SS gene in PAO1($\Delta clpV3$), PAO1($\Delta pscN$), and the complementation strains was measured using the CTX-H3-T6SS reporter fusion inserted into the chromosome. The error bars show standard deviations, and the findings are the average of the three experiments. The results demonstrated that the deletion of *pscN* and *clpV3* resulted in decreased expression of the H3-T6SS reporter.

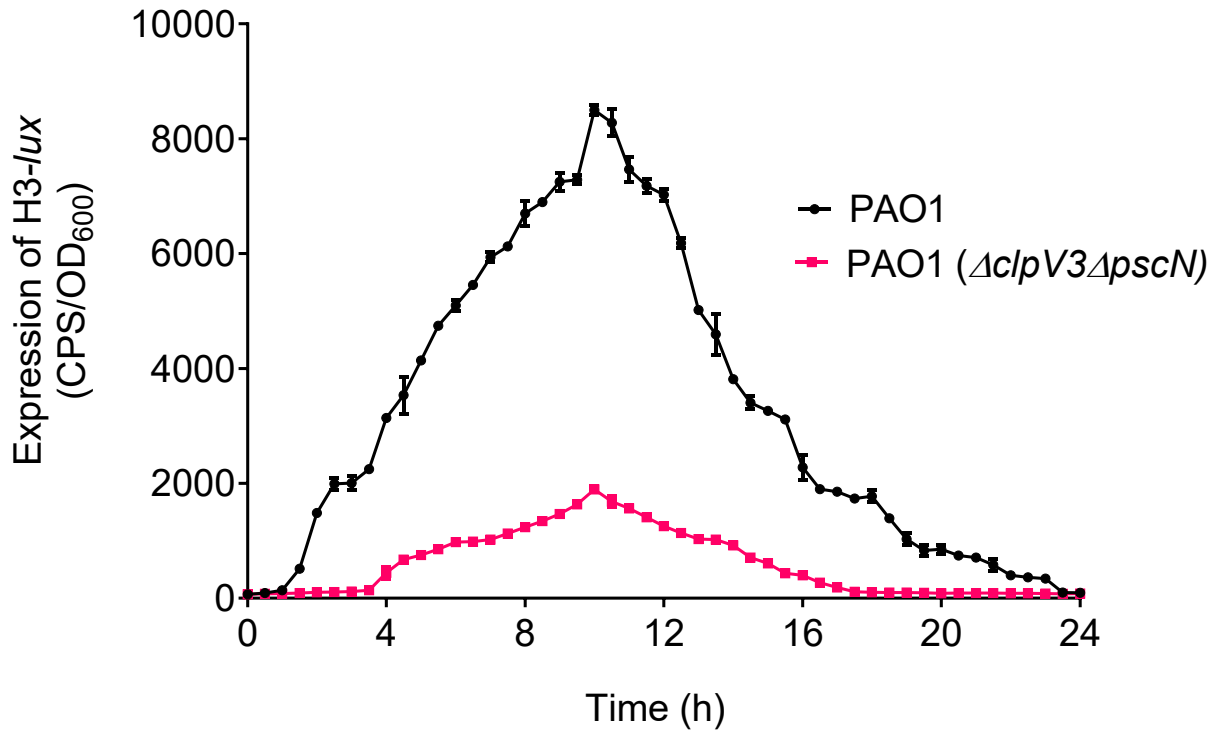


Figure 4.5 PAO1($\Delta clpV3\Delta pscN$) decreased the expression of H3-T6SS. The promoter activity of the H3-T6SS gene in PAO1($\Delta clpV3\Delta pscN$) was measured using the CTX-H3-T6SS reporter fusion inserted into the chromosome. The error bars show standard deviations, and the findings are the average of three experiments. The results demonstrated that the double deletion of *pscN* and *clpV3* together resulted in decreased expression of the H3-T6SS reporter.

4.3.4 H3-T6SS becomes non-functional and less competitive when ATPase *pscN* is deleted

To further confirm the heightened pathogenicity of H3-T6SS to prokaryotes, we have constructed a *pilG*-DsRed fluorescent reporter, where the vector was transformed into *E. coli* cells that harbors the reporter strain. We tested the fluorescence using the killing assay, known as the fluorometric killing assay. We checked the fluorescence of the knockout mutant PAO1($\Delta pscN$) and PAO1($\Delta pscN\Delta clpV3$) compared to the wild-type PAO1 and the

complementation strain, PAO1($\Delta pscN$)-clpV3 in pAK1900. We used PAO1 ($\Delta retS$) as a positive control for this experiment, as PAO1($\Delta retS$) increased T6SS-dependent killing of susceptible prey bacteria, making this strain a suitable benchmark for strong antibacterial competition. The viable number of *E. coli* cells is represented by the density of red fluorescence. As shown in **Figure 4.6**, the results of this study showed that PAO1($\Delta pscN$) exhibited a lower killing pattern in contrast to the wild-type PAO1 and $\Delta retS$. The complementation strains, PAO1($\Delta pscN$)-clpV3 in pAK1900, showed a similar trend in the killing assay compared to PAO1. The results suggested that H3-T6SS (clpV3) becomes non-functional in PAO1($\Delta pscN$); thus, it cannot suppress or eliminate *E. coli* cells more efficiently than in wild-type PAO1.

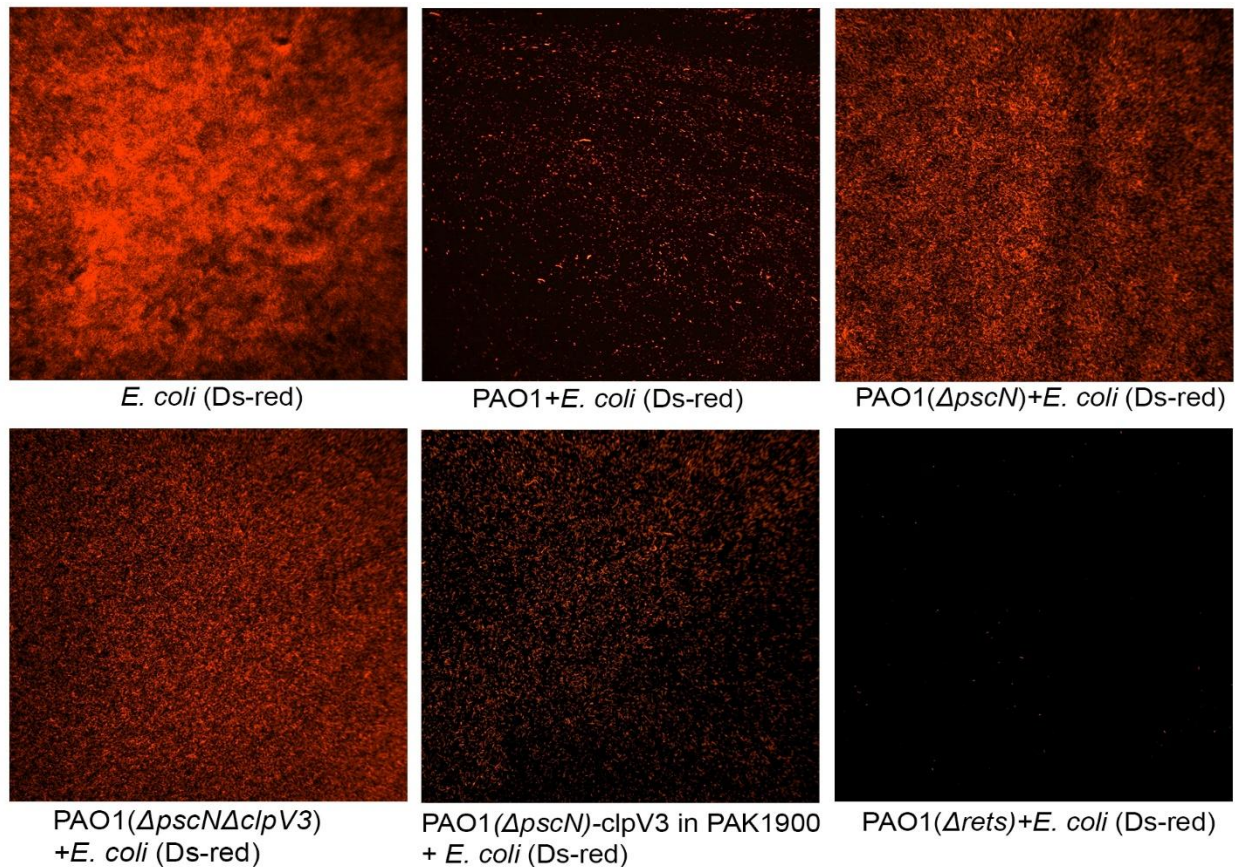


Figure 4.6 Competition assay between *P. aeruginosa* and *E. coli*. Fluorescence competition assay showing live *E. coli* harboring pilG-DsRed fluorescent reporter in red. The findings of this

study indicated that PAO1($\Delta pscN$) demonstrated a reduced killing effect compared to both the wild-type PAO1 and PAO1 ($\Delta retS$). All the slides were prepared following co-incubation of the strains of *P. aeruginosa* and *E. coli*, and images were captured using a microscope (Nikon ECLIPSE Ti-E Inverted) at a magnification of 20X.

H3-T6SS delivers toxins to target cells, conferring on *P. aeruginosa* an advantageous role in competition with other bacteria by occupying the same niche (Zhao et al., 2023). To confirm this, a competition assay was performed between *P. aeruginosa* and *E. coli*. The competitive index represents the ratio of CFU of *P. aeruginosa* strains to *E. coli*. We used a knock-out mutant, $\Delta retS$, for this competition assay as a positive control. Our results (**Figure 4.7**) demonstrated that PAO1 ($\Delta pscN$) showed lower killing activities against *E. coli*, as indicated by the low survival of the cells of PAO1 ($\Delta pscN$) when they grew together. Compared to that, $\Delta retS$ showed a highly competitive index, which was as expected for a positive control. The complementation and wild type strain, PAO1($\Delta pscN$)-clpV3 in pAK1900, and PAO1 showed a similar trend in terms of bacterial competitive index. These results reinforce that *pscN* controls the functional activities of H3-T6SS, and being an ATPase, the complementation of *clpV3* to some extent can provide support/backup for the ATPase, *pscN*.

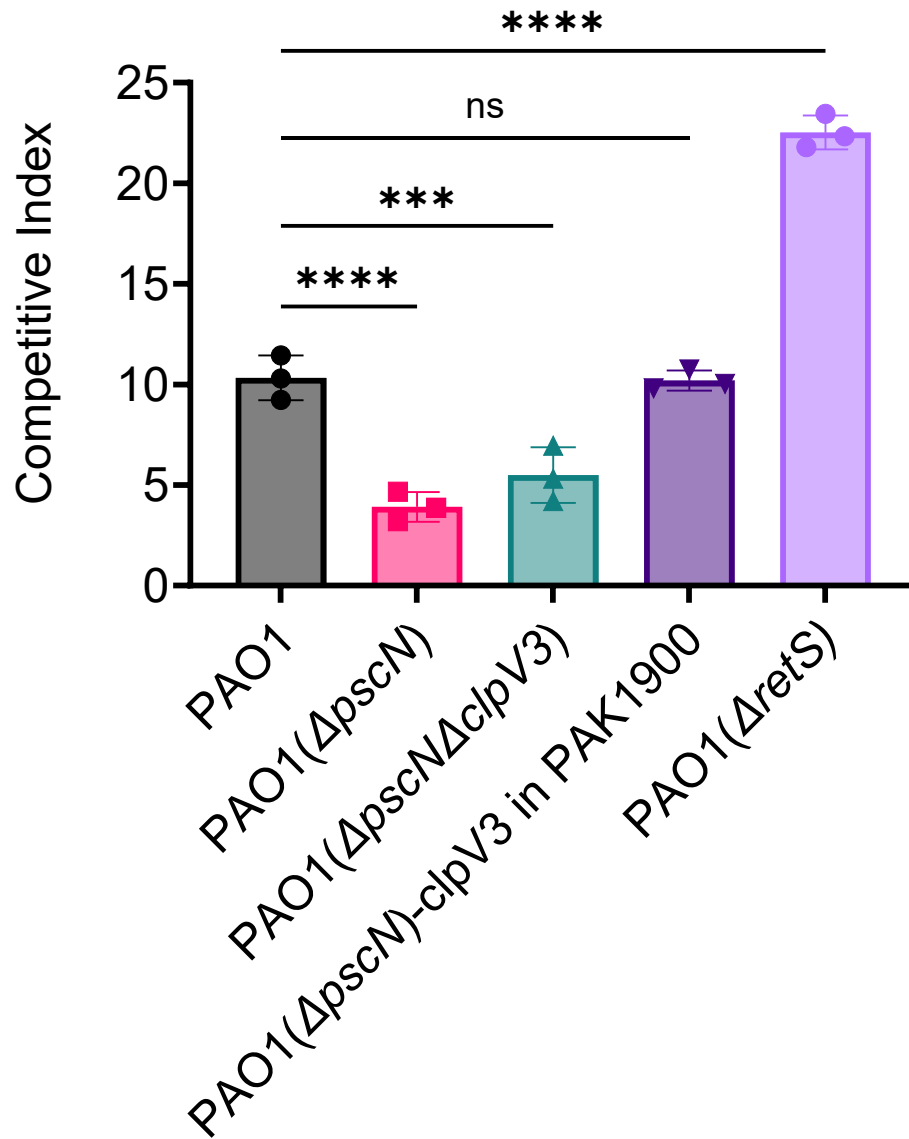


Figure 4.7 Competitive index between *P. aeruginosa* and *E. coli*. The competitive index represents the ratio of CFU of *P. aeruginosa* strains to *E. coli*. PAO1(ΔretS) harbors a constitutively active T6SS (T6SS+) and was used as a positive control. Data was analyzed by one-way analysis of variance (ANOVA). Error bars indicate standard deviations. ns, not significant, and **** $p < 0.0001$.

4.3.5 Transcriptional activity of H3-T6SS is ATPase clpV3-specific

To verify whether the regulatory effect of ATPase (clpV3) on H3-T6SS is specific or if it's a general effect of other ATPases, we first constructed the knockout mutant of PAO1($\Delta PA4595$). PA4595 is an ATPase belonging to the REG subfamily of the ATP-binding cassette (ABC) family and known as artR, anticipated to be a soluble ABC ATPase, showing 74.77% similarity to the REG subfamily protein EttA found in *E. coli* (Boel et al., 2014). Members of this subfamily lack transmembrane domains and are composed of two ABC ATPase domains that are fused in tandem (Kerr, 2004). It is also not part of the ATPases of different protein secretion systems (T1SS-T6SS), and no member of this subfamily has been studied for their involvement in the regulation of T6SS so far. To further study, we constructed a single-copy chromosomally integrated CTX-H3-lux reporter in PAO1 and CTX-H3-lux-PAO1 ($\Delta PA4595$) by inserting the CTX-H3-PAO1 reporter into the chromosomes of PAO1($\Delta PA4595$). We compared the promoter activities by measuring these strains' luminescence (CPS) for 24 hrs. We also checked the OD_{600nm} over time. The results were expressed by CPS/OD_{600 nm} and continuously monitored in an automated plate reader. Analyzing the expression patterns of H3-T6SS between PAO1 and PAO1 ($\Delta PA4595$), we found that the deletion mutant of PA4595 does not have any effect on the expression of H3-T6SS (**Figure 4.8**) compared with the wild-type strain, as both strains showed similar patterns in terms of their promoter activities. The highest level of expression of H3-T6SS reported for the knockout mutant, PAO1 ($\Delta PA4595$), was around 10.5 hrs, which was close to that of the wild-type PAO1 in terms of time. Thus, based on these findings, it is interesting to note that the impact of transcriptional activity of H3-T6SS is only on the ATPase clpV3-specific rather than the effect on the general ATPase present in *P. aeruginosa*.

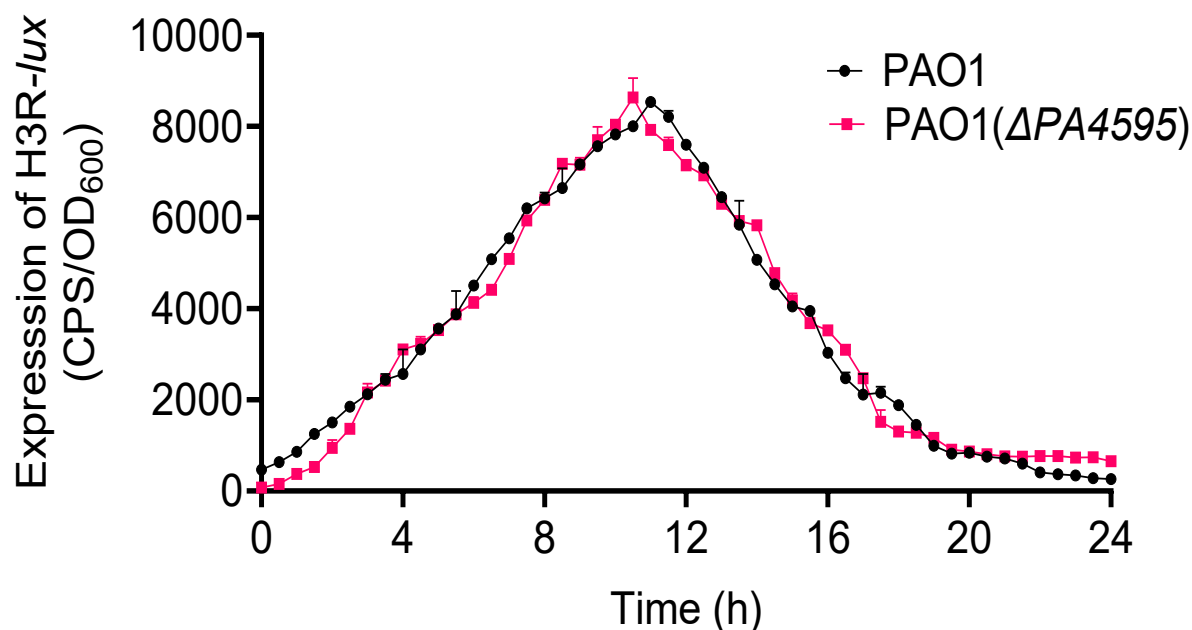


Figure 4.8 The deletion of PA4595 shows no effect on the expression of H3-T6SS. The CTX-H3-T6SS reporter fusion was inserted into the chromosome to measure the promoter activity of the H3-T6SS gene in PAO1(ΔPA4595). The error bars show standard deviations, and the findings are the average of three independent experiments. The results showed that the deletion mutant PA4595 does not have any effect on the expression of H3-T6SS.

4.3.6 Multiple phenotypes of *P. aeruginosa* depend on the ATPase, pscN, and clpV3

Among the gram-negative bacteria, *P. aeruginosa* is the most studied and referred to as a model microorganism for investigating the phenotypes associated with virulence factors (Liao, Huang, Wang, Yao, & Lu, 2022). ATPases play a crucial role in virulence by facilitating the recognition, unfolding, and secretion of virulence factors of *P. aeruginosa* (Laventie et al., 2019). Given that the roles of ATPase, clpV3, and pscN in *P. aeruginosa* virulence remain incompletely understood, we further investigated their potential roles in the bacterium's pathogenicity,

considering prior research linking T6SS and T3SS to these virulence factors. We assessed various phenotypes of PAO1($\Delta clpV3$) and PAO1($\Delta pscN$), such as pyocyanin production, biofilm formation, and motility, and compared the results with those of PAO1 and the complementation strains.

Among the virulence factors, phenazine pyocyanin is a blue-green metabolite that increases *P. aeruginosa*'s capacity to induce chronic lung infection (Lew, Chong, & Lau, 2025). To investigate the roles of both ATPases, *pscN* and *clpV3*, in phenotypes, we first tested pyocyanin production in the knockout mutants of PAO1 ($\Delta clpV3$) and PAO1 ($\Delta pscN$), and in the double-deletion mutant PAO1 ($\Delta clpV3\Delta pscN$). As shown in **Figure 4.9**, the first evident distinction was that the production of pyocyanin in PAO1($\Delta pscN$) was significantly higher when compared to the wild-type PAO1. In comparison to that, PAO1($\Delta clpV3$) showed lower pyocyanin production (4.3-fold lower) than the wild-type strain. Furthermore, compared to the wild-type PAO1, the double deletion mutant PAO1($\Delta clpV3\Delta pscN$) showed lower pyocyanin. We conducted further investigation into the impact of the *clpV3* and *pscN* on *P. aeruginosa* virulence factors by overexpressing *clpV3* and *pscN*. The full-length *clpV3* and *pscN* genes were cloned into shuttle vector pAK1900 and transferred into PAO1 by electroporation to construct the overexpression strains of PAO1($\Delta clpV3$)-*clpV3* in pAK1900 and PAO1($\Delta pscN$)-*pscN* in pAK1900. The results of complementation strains showed similar trends of pyocyanin production as reported by the wild-type, PAO1.

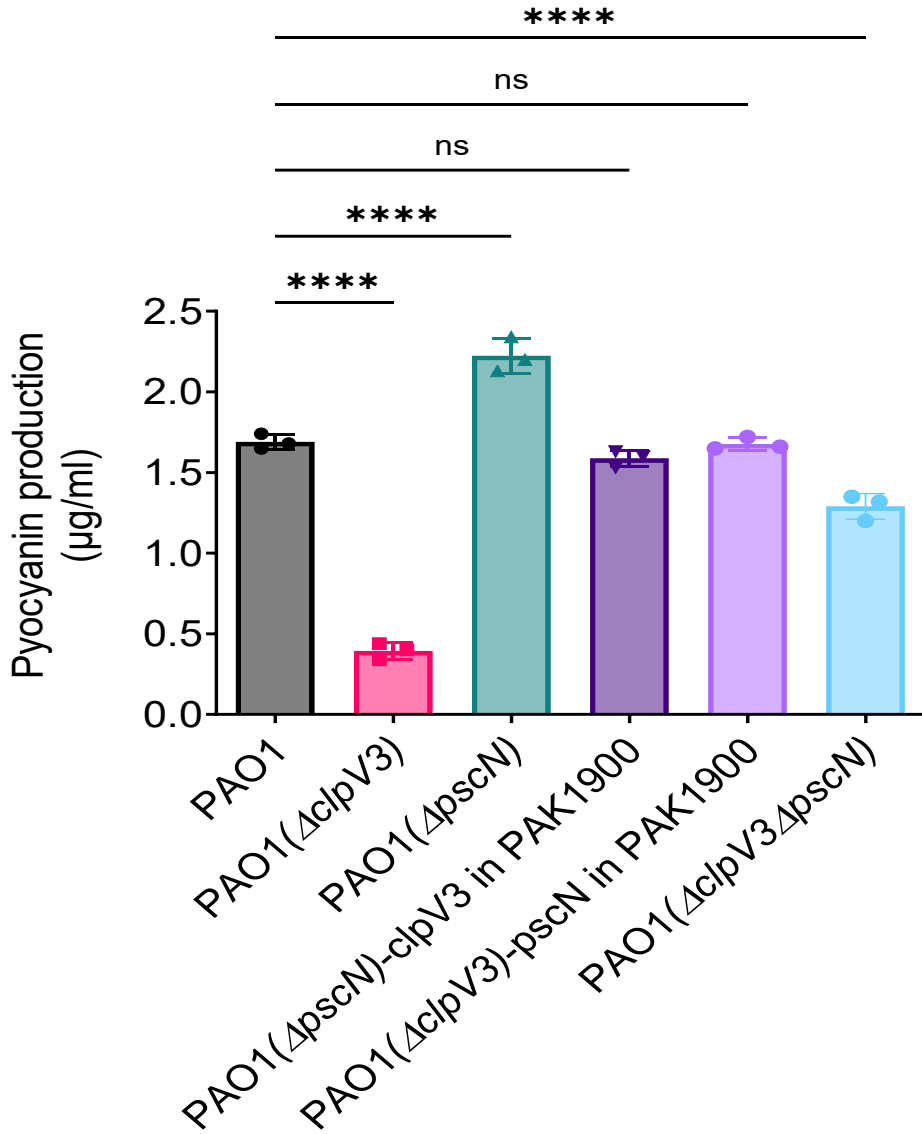


Figure 4.9 Deletion of *pscN* and *clpV3* affects pyocyanin production. Pyocyanin was measured in PAO1(Δ *pscN*) and PAO1(Δ *clpV3*) with PAO1 or the complementation strains. PAO1(Δ *pscN*) showed higher pyocyanin production, whereas PAO1(Δ *clpV3*) exhibited significantly lower pyocyanin production compared to PAO1. Data were analyzed using one-way ANOVA with a t-test and Tukey's multiple comparisons test. Results representative of three separate experiments with comparable outcomes, carried out in triplicate. Error bars indicate standard deviations. ns, not significant, and **** $p < 0.0001$.

Biofilms, composed of extracellular polymeric substances (EPS), are accountable for a variety of infections and play a key role in the pathogenesis of *P. aeruginosa*, and are closely linked with the protein secretion system (H. Y. Liu, Prentice, & Webber, 2024). In this study, we examined the formation of biofilm in the constructed deletion mutant to find out the functional role of clpV3 and pscN. Our results (**Figure 4.10**) demonstrated that both clpv3 (2.7-fold) and pscN (1.21-fold) showed significantly lower biofilm compared to the wild-type strain, PAO1, and the complementation strains. In addition, in contrast to the PAO1, the double knock-out also showed a lower biofilm formation trend.

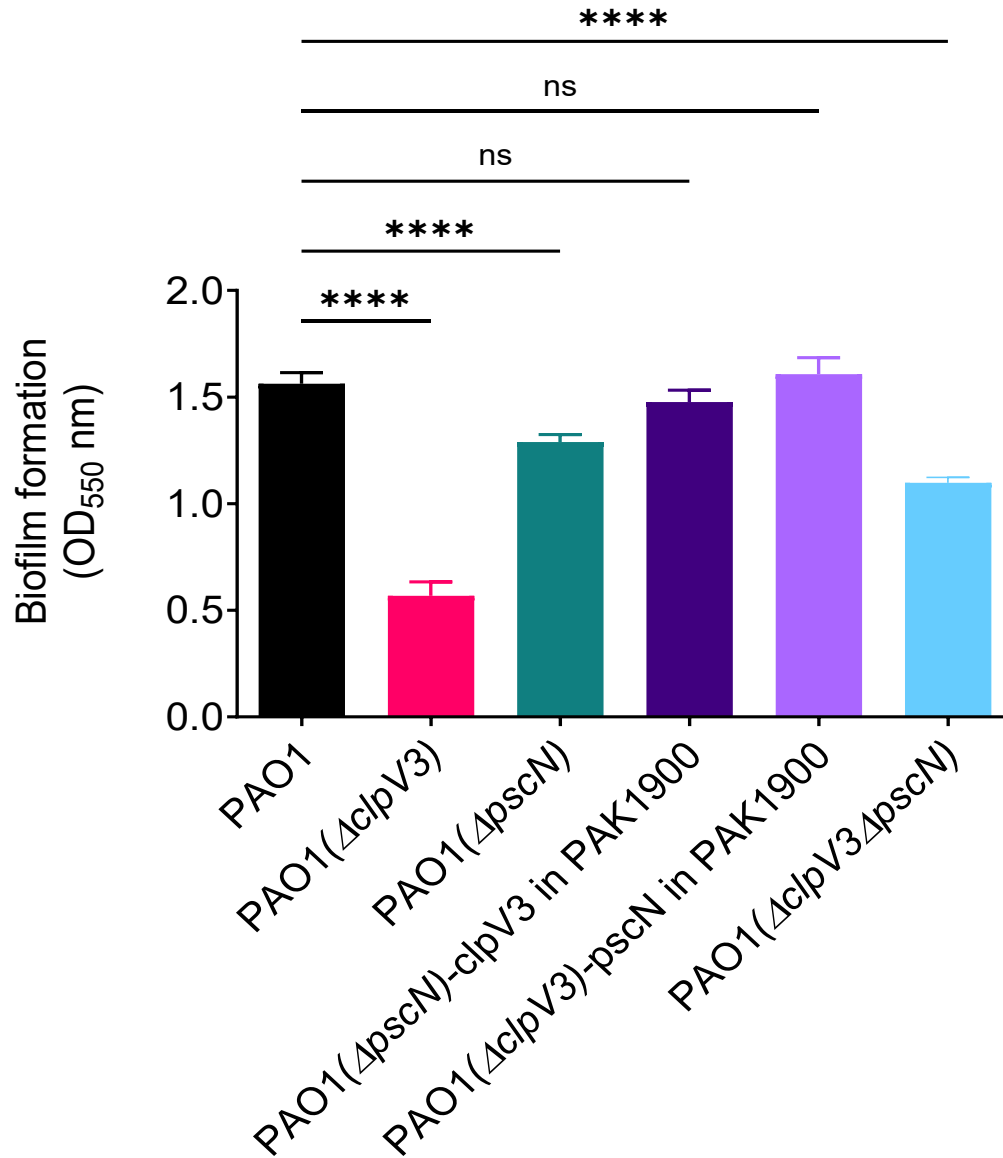


Figure 4.10 Biofilm was reduced in PAO1(Δ pscN) and PAO1(Δ clpV3). Biofilm formation was measured in PAO1(Δ pscN) and PAO1(Δ clpV3) with PAO1 or the complementation strains in a 96-well plate. Data were analyzed using one-way ANOVA with a t-test and Tukey's multiple comparisons test. Results representative of three separate experiments with comparable outcomes, carried out in triplicate. Error bars indicate standard deviations. ns, not significant, and **** $p < 0.0001$.

We further elucidated the effect of both ATPases by examining bacterial motilities, as studies have found that T6SS and T3SS can regulate bacterial interaction and colonization by controlling the swimming and swarming motility (Qin et al., 2022). As shown in **Figure 4.11**, upon the deletion of *pscN*, swarming motility is significantly reduced compared to PAO1, whereas the swimming motility is unaffected. In contrast, swarming motility is significantly reduced in the *clpV3* knockout mutant. Overall, these results indicate that some virulence factors in *P. aeruginosa* are influenced by the ATPase of T6SS and T3SS, *clpV3*, and *pscN*, respectively.

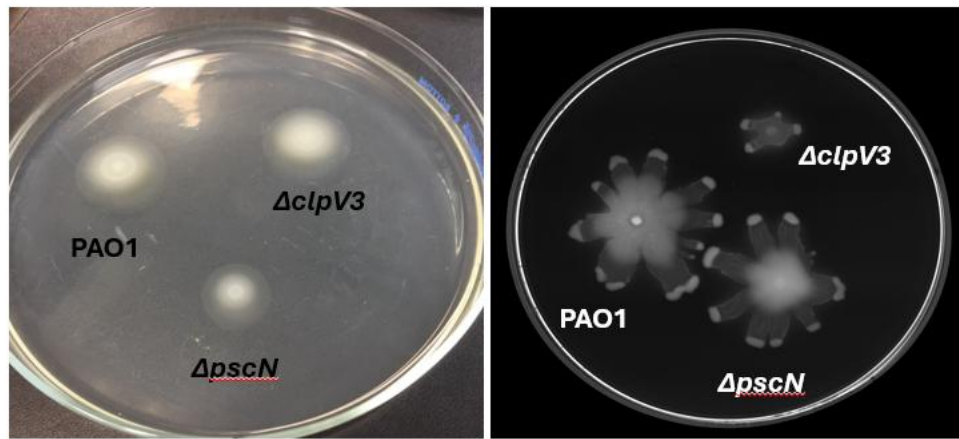


Figure 4.11 Deletion of *pscN* and *clpV3* affects swimming (left panel) and swarming motility (right panel). Inactivation of *pscN* results in reduced swimming motility in PAO1(*ΔpscN*) compared to wild-type PAO1, whereas swarming motility is not affected. The deletion of *clpV3* significantly reduced the swarming motility compared to PAO1.

4.4 Discussion

P. aeruginosa utilizes several protein secretion systems, among which the T3SS and T6SS play key roles in virulence and interbacterial interactions. They deliver effector proteins directly into eukaryotic cells through an extracellular protrusion, or needle, and control the regulation and functional characteristics of genes associated with the virulence of *P. aeruginosa*. The structural assembly and functions of T3SS and T6SS depend on their ATPases. Understanding the molecular mechanisms of the ATPase of T3SS and T6SS is therefore crucial for identifying novel therapeutic targets to combat *P. aeruginosa* infections.

In this study, we characterized two ATPases of the T3SS and T6SS in *P. aeruginosa*, PscN and ClpV3, respectively, and examined their roles in the functional activity of these protein secretion systems. We explored the potential role of ATPases by measuring cellular ATP levels in *P. aeruginosa* and found that ATP levels were significantly increased in the knockout mutants of *pscN*, *clpV3*, and the double mutant *pscNclpV3*. These results suggest that deleting the two major ATPases reduces overall ATP consumption, including protein translocation and cycling, leading to a reduction in overall cellular ATP utilization. PscN, an integral part of T3SS containing Walker A and Walker B motifs, generates energy by hydrolyzing ATP and translocates effector proteins through the extracellular needle into the host cell (Halder et al., 2019). Meanwhile, T6SS effector proteins are delivered via ClpV, which is a cytoplasmic ATPase protein. This system also utilizes energy from ATP hydrolysis, generating the force needed for effector protein secretion (Lennings, Mayer, Makhoul, Brotz-Oesterhelt, & Schwarz, 2019). Since T3SS and T6SS functions are energy-intensive and depend on ATPase proteins, deactivation of *pscN* and *clpV3* is thought to disrupt the cell's ATP pool, causing an overall energy imbalance within the protein secretion system. Therefore, our findings are consistent with a concept in which the

inactivation of secretion ATPases lowers the amount of ATP consumed by these energy-intensive processes, leading to a net increase in the cellular ATP pool and a change in the energetic landscape of *P. aeruginosa*.

PscN (also known as SctN) shares 40-50% sequence homology with other Gram-negative pathogenic bacteria; nonetheless, its heterologous expression in *E. coli* remains challenging (Zarivach, Vuckovic, Deng, Finlay, & Strynadka, 2007). Additionally, it was reported that refolding of PscN is necessary after solubilization of inclusion bodies. Our study revealed that the deletion mutant of *pscN* suppresses the expression of T3SS's effector *exoS* at the transcriptional level. The PAO1($\Delta pscN$) mutant's lower transcriptional level of the T3SS effector *exoS* indicates that PscN activity influences T3SS gene regulation. In *P. aeruginosa*, T3SS is regulated both transcriptionally and post-transcriptionally, and it is closely associated with secretion activation. Our results support this coupling: the system seems to downregulate effector gene expression when the ATPase at the injectisome's base is absent, maybe to prevent synthesis of additional substrates that cannot be effectively exported. This is consistent with theories where signals originating from the injectisome's functional status impact transcription via upstream signaling pathways and global regulators like ExsA. More interestingly, we also found that the transcriptional activities of the effector protein *exoS* were significantly reduced in the deletion mutant PAO1($\Delta clpV3$). The T3SS ATPase, *pscN*, can support or offset the T6SS ATPase, *clpV3*. The possible mechanism could be interaction with the shared chaperone or regulatory pathways that control the gene expression of T3SS effectors, *exoS*. These results offer further proof of T3SS and T6SS crosstalk (**Figure 4.2 and Figure 4.4**) at the ATPase level, an association that points to functional compensation or common regulatory mechanisms within the secretion network.

We also found that when ATPase pscN is knocked out, H3-T6SS loses its ability to function and becomes less competitive. This result was confirmed by a competition assay performed between *P. aeruginosa* and *E. coli*. The result from transcriptional expression levels of H3-T6SS showed that deletion mutants of clpV3 and pscN significantly reduced the expression of H3-T6SS. We found that the complementation of pscN can successfully restore the gene expression of H3-T6SS, which indicates that pscN might play a role in the absence of clpV3 and contribute as an ATPase system for T6SS. The potential cross-talk between pscN and clpV3 is supported by several observations that suggest the following possibilities. First, PscN and ClpV3 are both AAA+ ATPases and may share overlapping accessory proteins or cofactors that modulate energy delivery to secretion systems. Second, both ATPases might be under common global regulatory pathways such as the Gac/Rsm and Vfr pathways, which altogether coordinately regulate protein secretion systems, quorum-sensing, and virulence traits. Third, pscN can activate the expression of H3-T6SS by rebalancing the intracellular ATP level. Our findings suggested that pscN complementation restores H3-T6SS expression in a clpV3-deletion background (**Figure 4.4**), showing that *P. aeruginosa*'s coupling of ATP hydrolysis to secretion system regulation is flexible and that the existence of an active ATPase of the appropriate class is more important than its precise identification.

Our findings indicate that the H3-T6SS transcription is not exclusively dependent on the ClpV3 ATPase. Although deletion of PA4595 (ArtR) did not affect H3-T6SS expression, our data suggest that ATPase-mediated processes potentially involving additional ATPases, such as PscN, may also contribute to the observed regulatory effects. Furthermore, this regulation does not appear to extend broadly to all ATPases in *P. aeruginosa*, indicating a more specific and context-dependent role. Studies showed that ArtR can suppress the transcription of T3SS genes and the

secretion of effectors (Kong et al., 2019). This ATPase class belongs to the REG subfamily, which contains only one gene in its operon and lacks any gene encoding membrane proteins. Interestingly, the regulation of T3SS by ArtR does not depend on its transport activity nor involves Gac-Rsm or Vfr pathways. ATPase activity plays a role in ArtR's regulatory function, relying on two conserved aspartate residues in Walker B motifs. The exact mechanism of ArtR at the gene expression level and the role of ATP hydrolysis are still unclear. It is hypothesized that ArtR influences certain regulatory proteins in the T3SS, leading to the disruption of T3SS gene transcription. Since T3SS activity is energy-demanding, the overall energy status of the cell might be related to it, which needs further confirmation.

The pathogenicity and other characteristics associated with *P. aeruginosa*'s virulence appear to be influenced by the ATPases pscN and clpV3. We have reported the changes in virulence-associated characteristics, including pyocyanin, biofilm, and motility, in the pscN and clpV3 knockout mutant. PCN's functional activity aids in interbacterial competition and stimulates inflammatory responses. Compared to the wild-type PAO1, PAO1($\Delta pscN$) produced a considerably larger amount of pyocyanin. By contrast, PAO1($\Delta clpV3$) produced less pyocyanin than the wild-type strain. These results suggested that ATPase pscN and clpV3 are part of a larger cross-linked regulatory network that affects the virulence of *P. aeruginosa*. Another major virulence factor is biofilm, which is made of extracellular polymeric substance (EPS) and aids in the organism's survival tactics by generating a persistent infection (Di Martino, 2018). Our findings showed that, in comparison to the wild-type strain, PAO1, and the complementation strains, both clpV3 and pscN exhibited noticeably less biofilm. These results indicate that the impact on biofilm development by H3-T6SS and T3SS in *P. aeruginosa* has clinical significance. Bacterial motility is essential to pathogenicity because it colonizes the microbial

surface and aids in the development of biofilm (Liao et al., 2022). When pscN is deleted, swimming motility remains unchanged, whereas swarming motility is drastically decreased in comparison to PAO1. In contrast, the clpV3 deletion mutant exhibits a marked reduction in swarming motility. Overall, these findings suggest that the ATPase of T6SS and T3SS, clpV3 and pscN, respectively, affect virulence factors in *P. aeruginosa*. These findings imply that surface-related group motility behaviors associated with biofilm formation and pathogenicity are preferentially influenced by secretion ATPases, possibly through common regulation via second messengers such as c-di-GMP and cAMP.

In summary, our findings showed the connection between ATPases, pscN, and clpV3 and the protein secretion system. The ATPase activities ultimately contribute to the pathogenesis and survival advantage of *P. aeruginosa*. This interconnection suggests that ATPase function directly impacts the virulence and functional physiology of T3SS and T6SS in *P. aeruginosa*.

Determining the structural interfaces between PscN, ClpV3, and their regulatory partners, clarifying the signaling pathways linking ATPase activity to global transcriptional networks, and evaluating ATPase inhibitors in infection models should be the main goal of future investigation. Such studies will be essential to translate the mechanistic insights presented here into novel therapeutic approaches against *P. aeruginosa*.

Chapter 5

Characterize the operon that is regulated by the Fem system

5.1 Introduction

Iron availability is an environmental factor that impacts the composition and function of many microbial communities (Stacy, Abraham, Jorth, & Whiteley, 2016). Iron is essential for bacterial survival, and iron availability is also linked to virulence regulation in *P. aeruginosa* (Frawley & Fang, 2014). *P. aeruginosa* uses iron for its metabolic pathway's regulation, which can be acquired from various sources using siderophores such as pyoverdine and pyochelin (Ganne et al., 2017; Youard, Wenner, & Reimann, 2011). Numerous strategies have been developed by bacteria to actively acquire iron in the host surroundings. One such mechanism is the release of siderophores, which are small molecules that can solubilize iron for bacterial usage (Ellermann & Arthur, 2017). At the host-microbial interface, siderophores can directly affect host functions by altering cellular iron homeostasis. This provides another way for bacteria to shape their immediate surroundings (Wilson, Bogdan, Miyazawa, Hashimoto, & Tsuji, 2016). Iron influences a variety of bacterial interactions with host cells, including susceptibility to phagocytosis, epithelial adhesion, and the translocation of bacterial effectors into host cells (Alves et al., 2010; Ellermann et al., 2015). Both quorum sensing and biofilm formation are affected by iron availability (Banin, Vasil, & Greenberg, 2005). Iron acquisition and cell-to-cell signaling are major mechanisms of interspecies interactions in bacterial species, but their regulation remains poorly understood.

Recent studies found the Fem system to be a major cell surface signaling molecule used by *P. aeruginosa* for transporting iron and other iron-bound siderophores, such as Mycobactin, secreted by *Mycobacterium* species (Llamas et al., 2008). Studies also revealed that the pyoverdine siderophore biosynthesis genes were upregulated by the fem operon, which is

involved in iron transport and uptake, and showed increased expression *ex vivo* (Kordes et al., 2019). The role of the Fem system in the uptake of iron is a very versatile process. First, the uptake of iron is governed by the membrane protein FemA, which transmits a signal to FemR. FemR is known as an anti-sigma factor that facilitates the release of FemI. FemI, in turn, binds to the RNA polymerase and can activate the transcription of the *femA* operon. The *femI-femR* and *femA* operons are autoregulated by the sigma factor FemI.

Previous studies from our laboratory demonstrated that the Fem system regulates multiple virulence-associated traits in *P. aeruginosa*, including pyocyanin production, biofilm formation, *in vivo* pathogenicity, and the expression of H2- and H3-T6SS (Y. Li et al., 2025). Notably, these effects were observed under conditions independent of mycobactin-mediated iron transport, indicating that the Fem system functions within a broader regulatory network beyond iron acquisition. Consistent with this, RNA-seq analysis revealed significant differential expressions of genes within the PA2293-PA2299 operon upon disruption of the FemA (**Table 5.1**). These findings provided a strong rationale for investigating this operon in detail, to determine whether it represents a direct or indirect target of Fem-dependent regulation and to elucidate its potential role in linking iron sensing, signal transduction, and virulence control in *P. aeruginosa*.

Table 5.1 RNA sequencing data showed a significant fold change in genes (PA2293-PA2299) in response to *femA* deletion mutant in *P. aeruginosa*.

Gene name	log2 (Fold Change)	Protein description
PA2293	8.13305	Hypothetical protein
PA2294	4.63421	Probable ATP-binding component of ABC transporter
PA2295	-5.7542	Probable permease of the ABC transporter
PA2296	5.56055	Hypothetical protein
PA2297	17.8252	Probable ferredoxin
PA2298	-5.8611	Probable oxidoreductase
PA2299	5.40626	Probable transcriptional regulator

The genomic region containing PA2293, PA2294, PA2295, PA2296, PA2297, PA2298, and PA2299 in *Pseudomonas aeruginosa* appears to represent a putative operon with potential roles in cellular regulation and transport. PA2294 encodes a predicted ATP-binding component of an ABC transporter, suggesting involvement in substrate uptake or export across the membrane. PA2297 is annotated as a probable ferredoxin, an iron-sulfur protein that typically participates in electron transfer reactions, whereas PA2299 is predicted to function as a transcriptional regulator, implying a possible role in controlling gene expression within this locus. The coexistence of transport, redox, and regulatory functions within the same genomic region suggests that these genes may operate as an interconnected unit rather than as independent elements. Such an organization raises the possibility that this operon contributes to broader regulatory networks in *P. aeruginosa*, including pathways associated with the Fem system and type VI secretion system (T6SS), both of which are important for bacterial

adaptation and pathogenicity. The overall connection of the Fem system and this operon (PA2293-PA2299) is illustrated in **Figure 5.1**. This study demonstrates that the transcriptional regulation of PA2296 and PA229 is mediated by the Fem system under both Mycobactin J-supplemented conditions. Because FemA is a membrane-associated protein, it's likely to function as a surface-localized sensor that detects changes in extracellular iron availability and transduces these signals across the membrane to downstream regulatory components. This Fem-dependent signaling cascade, in turn, modulates the transcription of the operon, linking iron sensing to gene expression control. Moreover, our data indicates that the Fem system, together with this operon, contributes to the regulation of *P. aeruginosa* pathogenicity.

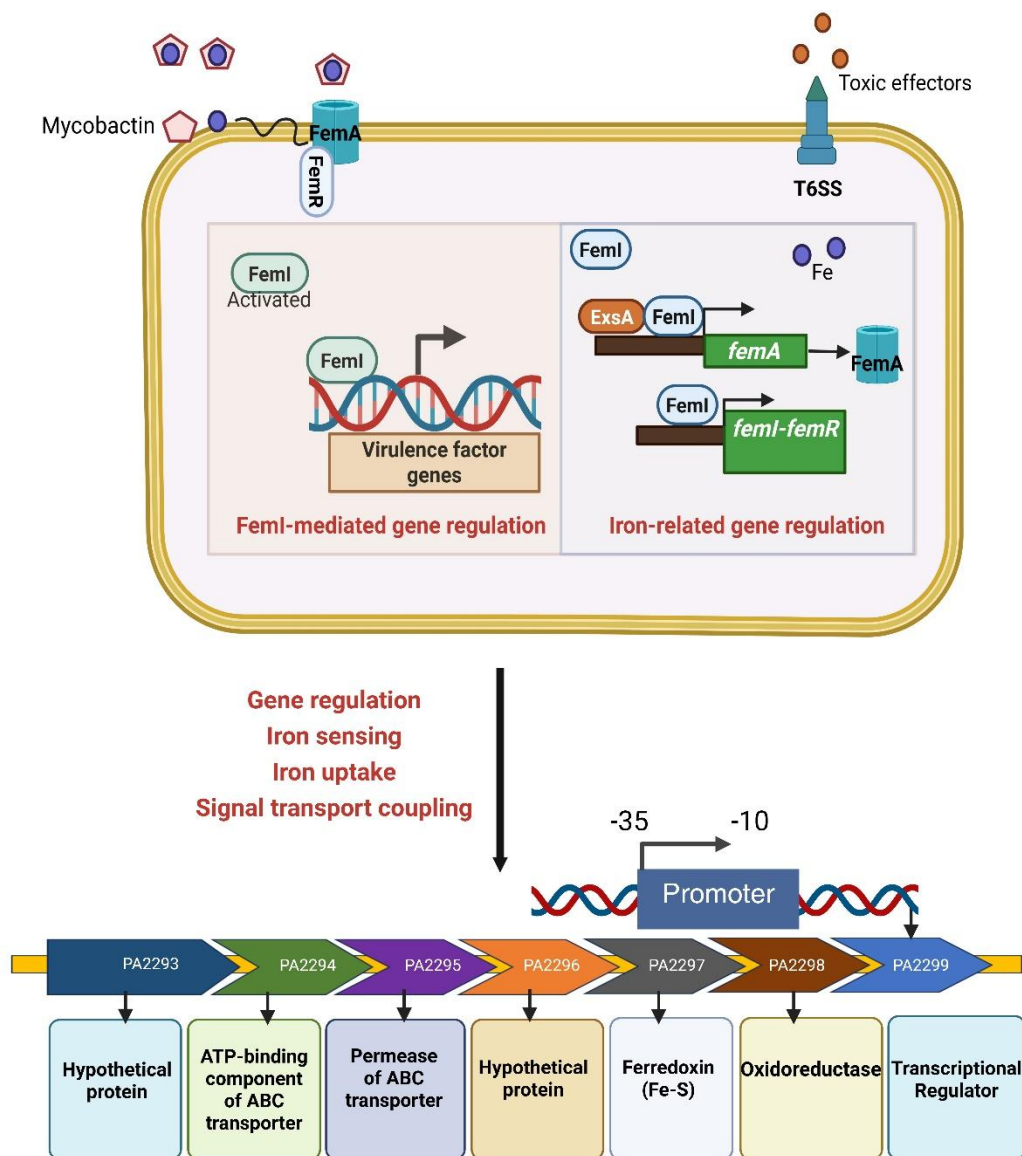


Figure 5.1 Schematic diagram of the potential connection of the Fem system and operon (PA2293-PA2299) in *P. aeruginosa*. The Fem system in *P. aeruginosa* is depicted in this flow diagram, emphasizing its function in iron uptake and regulation. FemA absorbs the siderophore mycobactin in iron-limited environments, which sets off a signal via TonB that activates FemR, which in turn releases FemI to start transcription of the femA operon. The Fem system mediates the transcriptional regulation of the PA2296-PA2299 operon under Mycobactin J-supplemented conditions. The figure was prepared using BioRender.

5.2 Materials and Methods

5.2.1 Bacterial plasmids and strains

For experimental purposes, the bacterial plasmids and strains used in all chapters are listed in **Table 5.2**. *P. aeruginosa* and *E. coli* were cultured on Luria-Bertani (LB) broth and agar at 37°C. *P. aeruginosa* cells were allowed to grow in M9 minimal medium for an iron-limited environment. Mycobactin J was purchased from Allied Monitor (USA). For *P. aeruginosa*, different concentrations of antibiotics were applied, which include trimethoprim (300 µg/ml), tetracycline (70 µg/ml), and carbenicillin (250 µg/ml). For *E. coli*, antibiotic concentrations of 50 µg/ml for kanamycin, 100 µg/ml for ampicillin, and 12.0 µg/ml for tetracycline were used. A *Pseudomonas* isolation agar (PIA) plate was plated with the *P. aeruginosa* mutant PAO1(Δ PA2294), PAO1(Δ PA2295), PAO1(Δ PA2297), PAO1(Δ PA2298), and PAO1(Δ PA2299), which were constructed using tetracycline at a concentration of 300 µg/ml.

Table 5.2 List of bacterial strains and plasmids used.

Bacterial strains and plasmids	Characteristics	Sources/ References
<i>E. coli</i>		
SM10- λ pir	Mobilizing strain, RP4 integrated into the chromosome; Km ^r	Duan Lab
DH5 α	F ⁻ Φ 80lacZ Δ M15 Δ (lacZYA-argF) U169 <i>recA1 endA1 hsdR17</i> (rK ⁻ , mK ⁺) <i>phoA</i> <i>supE44</i> λ - <i>thi-1 gyrA96 relA1</i>	Invitrogen
<i>P. aeruginosa</i> strains		
PAO1	Wild-type <i>P. aeruginosa</i>	Duan Lab
PAO1(Δ PA2294)	PA2294 knockout mutant of PAO1	This study
PAO1(Δ PA2295)	PA2295 knockout mutant of PAO1	This study
PAO1(Δ PA2297)	PA2297 knockout mutant of PAO1	This study
PAO1(Δ PA2298)	PA2298 knockout mutant of PAO1	This study
PAO1(Δ PA2299)	PA2299 knockout mutant of PAO1	This study
Plasmid		
pMS402	Expression reporter plasmid carrying the promoterless <i>luxCDABE</i> ; Km ^r Tmp ^r	(Duan et al., 2003)

pEX18Tc	<i>oriT⁺ sacB⁺</i> gene replacement vector with multiple-cloning site from pUC18; Tc ^r	(Hoang et al., 1998)
pAK1900	<i>E. coli-P. aeruginosa</i> shuttle cloning vector, Amp ^r	(Sharp et al., 1996)
pRK2013	Broad-host-range helper vector; Tra ⁺ , Km ^r	(Ditta et al., 1980)
CTX-6.1	Integration plasmid origins of plasmid mini-CTX- lux; Tc ^r	(Kong et al., 2013)
pEX18Tc- <i>PA2294</i> up+dw	pEX18Tc carrying the upstream and downstream fragments of <i>PA2294</i>	This study
pEX18Tc- <i>PA2295</i> up+dw	pEX18Tc carrying the upstream and downstream fragments of <i>PA2295</i>	This study
pEX18Tc- <i>PA2297</i> up+dw	pEX18Tc carrying the upstream and downstream fragments of <i>PA2297</i>	This study
pEX18Tc- <i>PA2298</i> up+dw	pEX18Tc carrying the upstream and downstream fragments of <i>PA2298</i>	This study
pEX18Tc- <i>PA2299</i> up+dw	pEX18Tc carrying the upstream and downstream fragments of <i>PA2299</i>	This study
CTX- <i>PA2296</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>PA2296</i> containing <i>PA2296</i> promoter region and <i>luxCDABE</i> gene; Km ^r , Tmp ^r , Tc ^r	This study
CTX- <i>PA2297</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>PA2297</i> containing <i>PA2297</i> promoter region and <i>luxCDABE</i> gene; Km ^r , Tmp ^r , Tc ^r	This study
CTX- <i>PA2299</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>PA2299</i> containing <i>PA2299</i> promoter region and <i>luxCDABE</i> gene; Km ^r , Tmp ^r , Tc ^r	This study

5.2.2 Construction of deletion mutant strains

For the construction of a gene knockout strain, we followed the manufacturer's instructions and used the Gibson Assembly® Cloning Kit (New England BioLabs Inc.) to produce deletion mutants of PAO1($\Delta PA2294$), PAO1($\Delta PA2295$), PAO1($\Delta PA2297$), PAO1($\Delta PA2298$), and PAO1($\Delta PA2299$). For PAO1($\Delta PA2294$), we used the primers indicated in **Table 5.3**, the upstream and downstream fragments of PA2294, and the vector (pEX-18Tc), which were amplified to create the deletion construct pEX-18Tc-PA2294up+down. After the purified PCR products were combined with the Gibson Assembly Master Mix, the reaction was incubated for 30 minutes at 50 °C on a thermocycler.

The knockout mutant PAO1($\Delta PA2294$) was obtained through tri-parental mating. The helper strain, *E. coli*, which comprises the recipient strain in addition to the donor strains of pEX18Tc-PA2294up+down and the PAO1 (the recipient strain), was employed in tri-parental mating techniques. Initially, 1X PBS buffer was used to wash the cultures of the donor, helper, and recipient strains after they had been cultivated overnight. Bacterial cultures were combined, spotted on LB agar with the ratio of 2:2:1 (helper: donor: recipient), and incubated for 12 hours at 37°C. The cells were scraped, reconstituted in LB broth, and plated on *Pseudomonas isolation agar* with 300 µg/ml of tetracycline in order to select the merodiploid. We call this procedure the first crossover. After the initial crossover, the colony was streaked in LB agar with 10% sucrose and no NaCl in order to choose a second crossover. PAO1($\Delta PA2294$) was finally confirmed by polymerase chain reaction. Additionally, deletion mutants of PAO1($\Delta PA2295$), PAO1($\Delta PA2297$), PAO1($\Delta PA2298$), and PAO1($\Delta PA2299$) were constructed using the previously mentioned methods and verified by PCR.

Table 5.3 List of primers used.

Primer	Sequence (5'→3')
PA2294-UP-S	AGGTCGACTCTAGAGTGGCGGTACTCGGCT
PA2294-UP-AS	GTGGCGTTTGAATTCCCTCCAGGTTTCAGAT
PA2294-DW-S	GAATTCAAACGCCACTGCCTGGAAGTCTGCTC
PA2294-DW-AS	AGCTCGGTACCCGGGCTGGGCCAGGTTGCA
PA2295-UP-S	AGGTCGACTCTAGAGGGCTGAAGATGCAGACCTTG
PA2295-UP-AS	TGCCGACCAGAATTCCCAGGCCAGATTCAGTTGC
PA2295-DW-S	GAATTCTGGTCGGCATGCTGCTCATCGGCCTCTTC
PA2295-DW-AS	AGCTCGGTACCCGGGACTCCGGCGAAGCGTAATGG
PA2297-UP-S	AGGTCGACTCTAGAGAGCTGCTGAAGAAGGCCGAC
PA2297-UP-AS	CCGCGCCGGGAATTCCTGCGGAAGAAGATGTCCTG
PA2297-DW-S	GAATTCGCGCGCGGTGAAGGTCGAGATTCCCTAC
PA2297-DW-AS	AGCTCGGTACCCGGGCGATGGTCTGGATGGTTTC
PA2298-UP-S	AGGTCGACTCTAGAGAACGTCAGCACCCCTG
PA2298-UP-AS	TCTTCAGGTGAATTCCGAGTACGCCGTTCA
PA2298-DW-S	GAATTCACCTGAAGAAGGACGAGCAGGGAC
PA2298-DW-AS	AGCTCGGTACCCGGGGCGCACGCTTGAGCT
PA2299-UP-S	AGGTCGACTCTAGAGACTATGCGGTGAAGA
PA2299-UP-AS	ACGCATGATGAATTCTGAGCTGGCTGTACA
PA2299-UP-S	GAATTCATCATGCGTATCGAGCGCCTGACC
PA2299-DW-AS	AGCTCGGTACCCGGGGCACCAGGCTGGTCT

P-PA2296-F	TCTTCACCTCGAGGGTACATGGCCTACGAC
P-PA2296-R	AACTAGAGGATCCCCGCAGGTATTTCTCCA
P-PA2297-F	TCTTCACCTCGAGGGAGTTCCACCAGGAAC
P-PA2297-R	AACTAGAGGATCCCCGGCCAGCAGGTCCAT
P-PA2299-F	TCTTCACCTCGAGGGATGAAAGAAGACTTC
P-PA2299-R	AACTAGAGGATCCCCTGATGCGGCTGACGT

5.3 Results

5.3.1 FemA-dependent signaling influences PA2296-PA299 operon expression

The FemA-FemR- FemI cell-surface signaling system detects extracellular cues, including siderophores such as mycobactin J, and relays these signals to influence downstream transcriptional responses. Therefore, comparing promoter activity in the presence and absence of FemA provides insight into Fem-dependent signal transduction. To confirm the regulation of the operon by the Fem system and characterization of the gene products of this operon, we have checked the promoter activities of PA2296, PA2297, and PA2299 in the PAO1 and the deletion mutant of *femA*. In PAO1 and PAO1(Δ *FemA*), we created a single-copy chromosomally integrated CTX-*femA*-lux reporter, and we measured the luminescence in both strains to compare the transcriptional activity. The growth was also monitored continuously for 24 hrs. The expression was normalized to CPS/OD. Transcriptional activity was checked in the M9 minimal medium with and without 1 μ M mycobactin J.

The result showed that the maximal promoter activity of PA2296 and PA2299 was 10-fold (**Figure 5.2**) and 5-fold lower (**Figure 5.4**), respectively, in PAO1(Δ *FemA*) compared with that in the wild-type PAO1. In the presence of mycobactin J, PAO1 exhibited 11-fold higher promoter activity of PA2296 in contrast to the deletion mutant, PAO1(Δ *FemA*). Upon the addition of mycobactin J, PAO1 showed an approximately 4.32-fold higher PA2299 promoter activity relative to the PAO1 (Δ *FemA*) deletion mutant. These findings demonstrate a functional link between the Fem system and the PA2296 and PA2299 operon in *P. aeruginosa*. The reduced promoter activity of PA2296 and PA2299 in the PAO1 (Δ *FemA*) mutant suggests that FemA is required for proper activation of these genes. Given that FemA is an outer membrane TonB-dependent transducer, it likely does not directly regulate transcription but instead participates in

sensing extracellular signals, such as mycobactin J, and transmitting this signal through the FemA-FemR-FemI signaling cascade to modulate gene expression. Our results also indicated that the FemA does not affect the promoter PA2297 in the presence or absence of Mycobactin J (**Figure 5.3**).

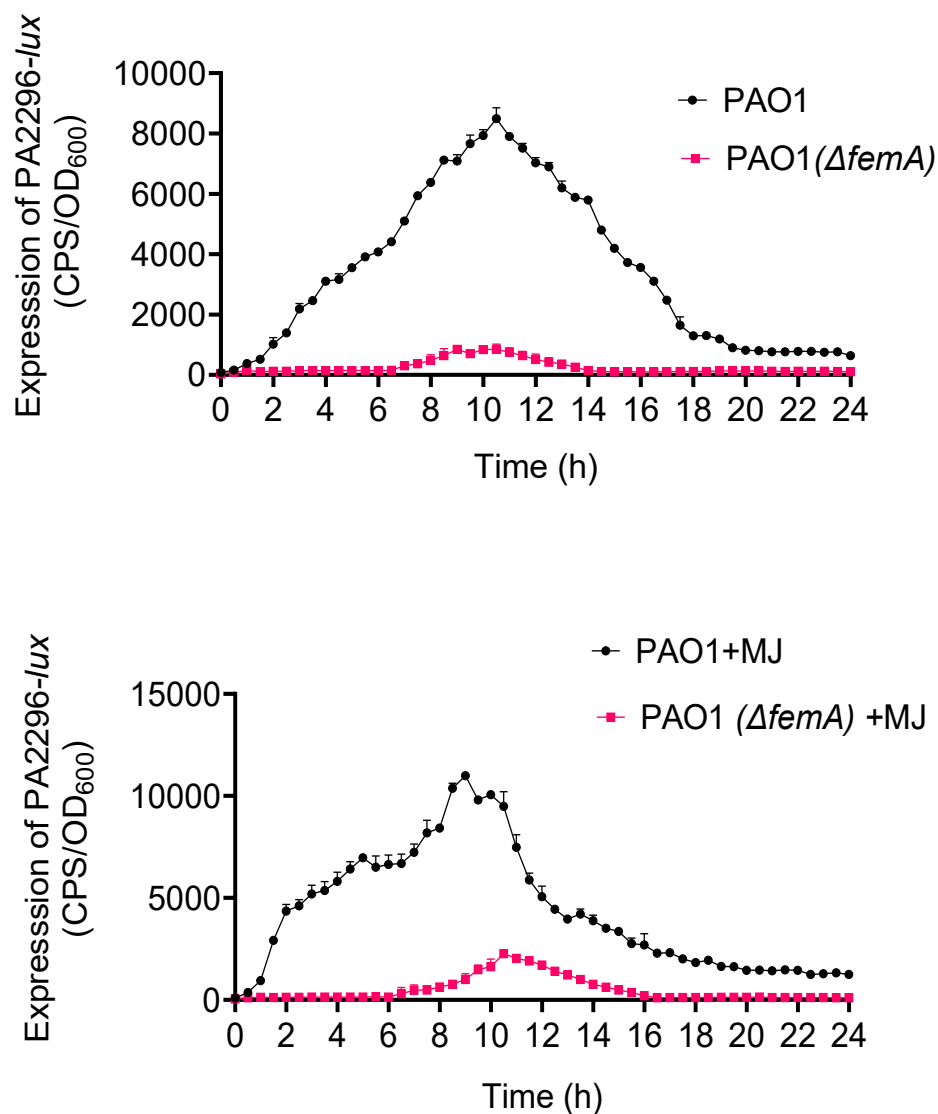


Figure 5.2 Promoter activity of PA2296 decreased in PAO1($\Delta FemA$). The CTX-PA2296 reporter fusion integrated on the chromosome was used to measure the promoter activity of PA2296 in PAO1($\Delta FemA$) in the presence and absence of Mycobactin J. Compared to that in the wild-type PAO1, the promoter activity of PA2296 in PAO1($\Delta FemA$) was significantly lower. The experiments were performed under iron-limited M9 minimal medium. The values represent the average of the triplicate experiments, and the error bars indicate standard deviations.

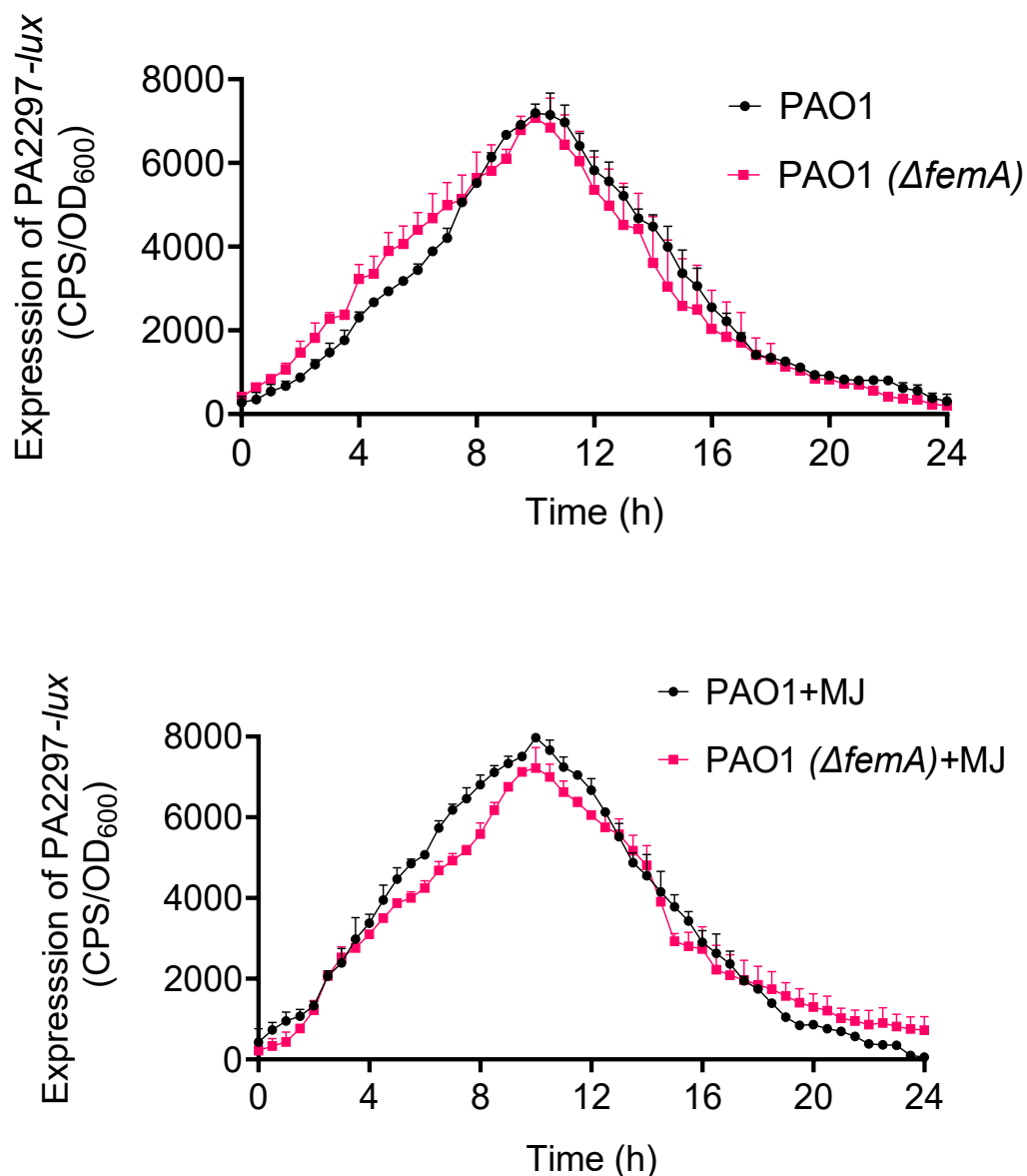


Figure 5.3 FemA showed no effect on operon PA2297. The CTX-PA2297 reporter fusion integrated on the chromosome was used to measure the promoter activity of the PA2297 in PAO1($\Delta FemA$) in the presence and absence of Mycobactin J. The experiments were performed under iron-limited M9 minimal medium. The promoter PA2297 is unaffected by FemA, both with and without Mycobactin J. The values represent the average of the triplicate experiments, and the error bars indicate standard deviations.

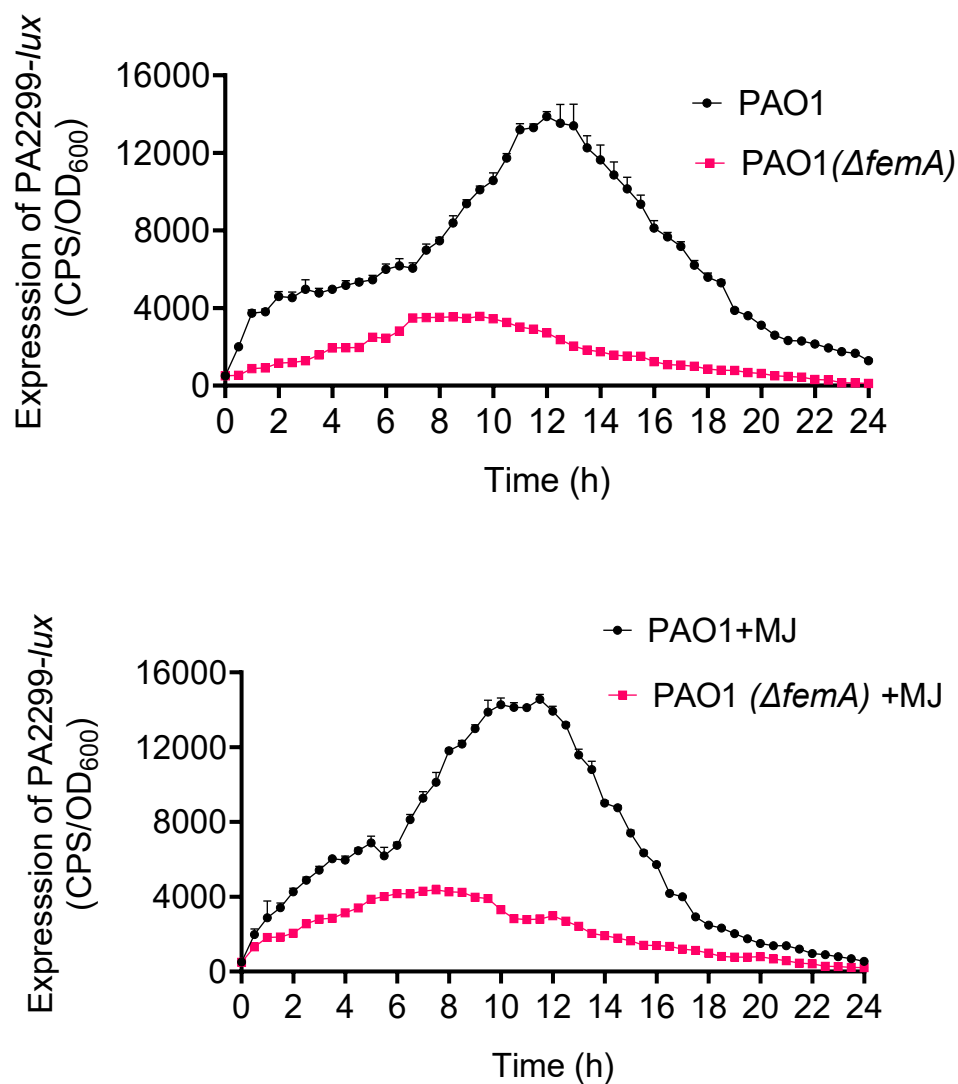


Figure 5.4 Promoter PA2299 activity decreased in PAO1($\Delta FemA$). A chromosomally integrated CTX-PA2299 reporter fusion was used to assess PA2299 promoter activity in the PAO1 ($\Delta FemA$) strain under conditions with and without Mycobactin J. The promoter activity of PA2299 in PAO1($\Delta FemA$) was significantly reduced compared to that in the wild-type PAO1. Experiments were conducted in iron-limited M9 minimal medium. The error bars indicate standard deviations.

5.3.2 Virulence of *P. aeruginosa* is affected by ($\Delta PA2294$) and PAO1 ($\Delta PA2298$)

The virulence factors of *P. aeruginosa* are very versatile, and iron availability plays a critical role in the survival and maintenance of key virulence traits (Sanchez-Jimenez, Marcos-Torres, & Llamas, 2023). To investigate whether a possible regulatory influence exists on phenotypes, we have constructed the deletion mutants PAO1 ($\Delta PA2294$) and PAO1 ($\Delta PA2298$) and further checked the effect of these knockout mutants on the phenotypes of *P. aeruginosa* (e.g., biofilm and pyocyanin) in the presence and absence of Mycobactin J.

Our results showed that (**Figure 5.5**), both deletion mutants, PAO1 ($\Delta PA2294$) and PAO1 ($\Delta PA2298$), showed lower biofilm compared with wild-type PAO1. In the pyocyanin assay, in the presence of Mycobactin J, the knock-out mutant PAO1 ($\Delta PA2298$) showed significantly reduced pyocyanin production compared to the wild-type, PAO1. The knockout mutant PAO1 ($\Delta PA2294$) showed higher pyocyanin production compared to PAO1 (**Figure 5.6**). This is the first report where we have shown the overall impact on biofilm and pyocyanin production upon the knockout of PAO1 ($\Delta PA2294$) and PAO1 ($\Delta PA2298$). This result indicates that PA2296 and PA2298 can influence the virulence of *P. aeruginosa* activities through mycobactin-mediated iron uptake.

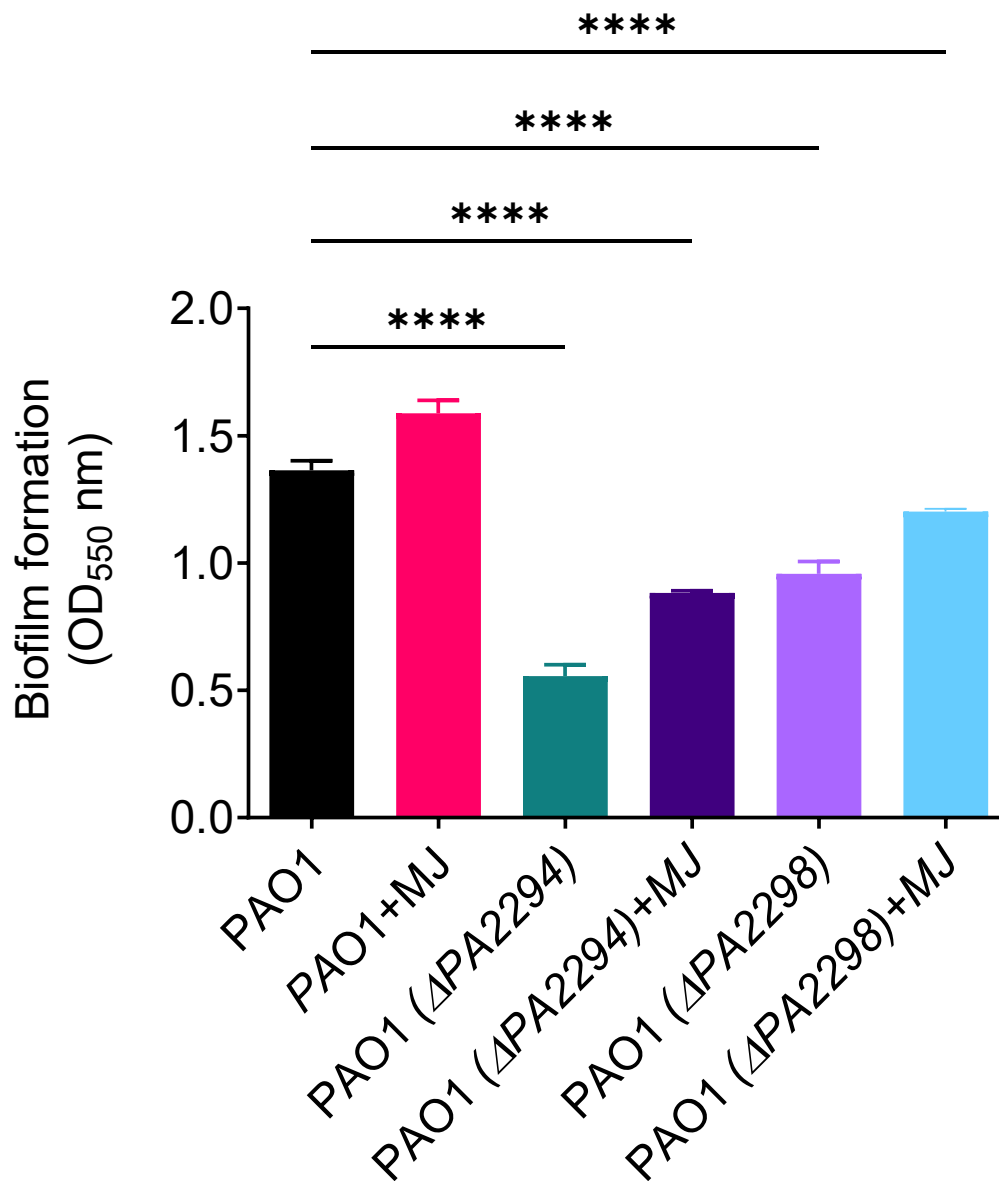


Figure 5.5 Deletion of PA2294 and PA2298 reduced the biofilm. Biofilm formation was measured in PAO1(Δ PA2294) and PAO1(Δ PA2298) with PAO1 in a 96-well plate with or without Mycobactin J. Data were analyzed using one-way ANOVA with a t-test and Tukey's multiple comparisons test. Results representative of three separate experiments with comparable outcomes, carried out in triplicate. Error bars indicate standard deviations. **** $p < 0.0001$.

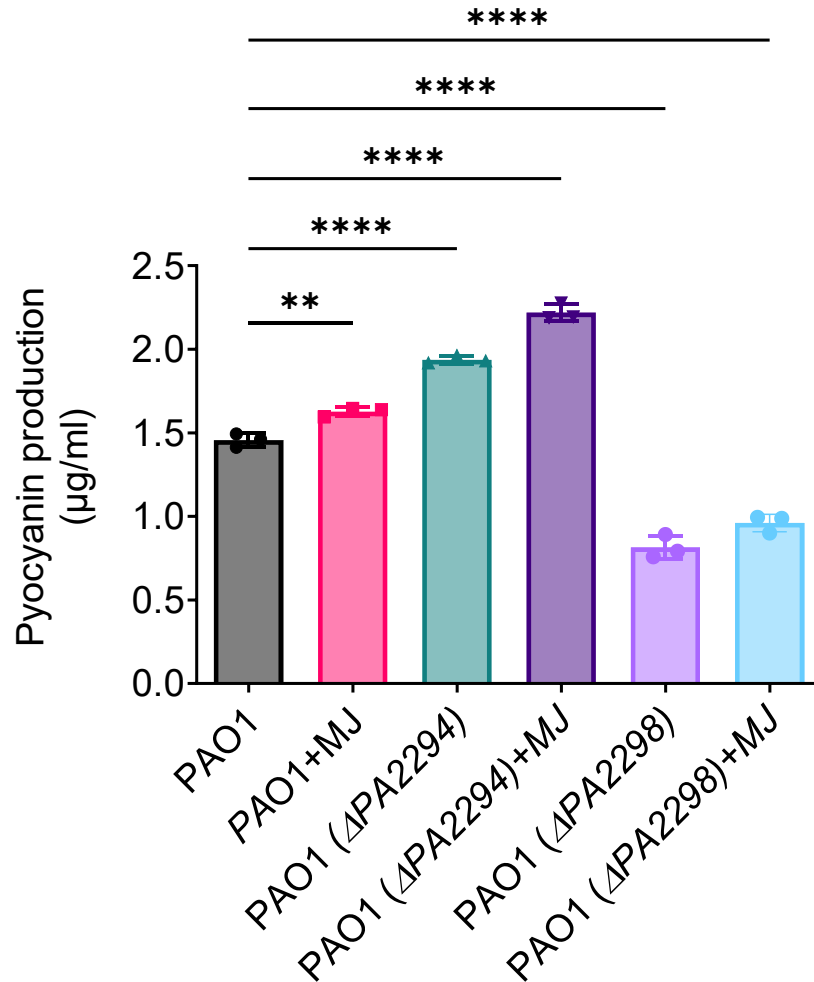


Figure 5.6 Deletion of PA2296 and PA2297 affects pyocyanin production. Pyocyanin was measured in PAO1(Δ PA2296) and PAO1(Δ PA2297) with PAO1 in the presence or absence of Mycobactin J. The knock-out mutant PAO1 (Δ PA2298) produced considerably less pyocyanin than the wild-type PAO1 in the presence of Mycobactin J. In comparison to PAO1, the knockout mutant PAO1 (Δ PA2294) produced more pyocyanin. Data were analyzed using one-way ANOVA with a t-test and Tukey's multiple comparisons test. Results are representative of three separate experiments with comparable outcomes, carried out in triplicate. Error bars indicate standard deviations. ** $p < 0.01$ and **** $p < 0.0001$.

5.4 Discussion

Significant fold changes in the genes (PA2293-PA2299) of this operon in response to *femA* deletion were found by recent RNA sequencing data initiating us to pursue further study to find out the potential connection between this operon and the Fem system (Li, 2020). The findings indicate that, in the presence of the siderophore mycobactin J, deletion of *femA* leads to a decrease in transcription of the PA2296 and PA2299 operon. The virulence traits were also affected by the deletion mutant of PA2294 and PA2298.

We demonstrate that the Fem system is functionally linked to the PA2296-PA2299 operon and regulates its transcription in response to iron-bound mycobactin J, likely integrating membrane-associated sensing by FemA with iron uptake. FemA is necessary for complete transcriptional activation, as seen by the decrease in PA2296 promoter activity in the *femA* deletion mutant in both M9 minimum medium and in the presence of Mycobactin J. A model in which FemA contributes to the activation of the PA2296 and PA2299 putative promoters is supported by the observed changes in transcriptional activity. In minimal medium, promoter activity decreased by approximately 10-fold and 5-fold, respectively, in the PAO1($\Delta femA$) mutant compared to wild-type PAO1. In the presence of Mycobactin J, promoter activity of PA2296 and PA2299 was around 11-fold and 4.32-fold higher in the wild type than in the $\Delta femA$ mutant. Because FemA is a membrane-associated protein, it likely regulates promoter activity through a signaling pathway rather than directly activating transcription. Under these conditions, the PA2296 and PA2299 operons contribute to adaptation to iron limitation, and the Fem system possibly plays a role in the bacterial response to iron stress.

The regulatory model is refined, and an essential internal control is provided by the lack of a discernible FemA action on the putative PA2297 promoter. The promoter activity of PA2297 is

unaffected by Mycobactin J. Instead of acting broadly across several promoters in the region, this data implies that FemA primarily regulates the PA2296 promoter. On the other hand, PA2297 might have a separate promoter that is constitutive or responsive to other regulatory inputs, but not to FemA or to the signal mediated by Mycobactin J employed in this research.

Within this operon, PA2294 encodes an ATP-binding component of the ABC transporter, which may facilitate iron uptake or import of siderophore-bound iron complexes. ABC transporters typically mediate energy-dependent translocation of substrates across cellular membranes through ATP hydrolysis (Rees, Johnson, & Lewinson, 2009). In this context, PA2294 could serve as the inner membrane component that imports iron released from siderophores or other iron-containing complexes into the periplasm or cytoplasm. This transport process would provide the bacterium with a direct mechanism to internalize iron once extracellular sensing by FemA detects iron limitation or siderophore presence.

The downstream gene PA2299 encodes a predicted transcriptional regulator, which may serve as a local effector connecting Fem-dependent signal transduction to operon-specific gene expression. Upon activation of the Fem cascade, initiated by FemA's extracellular sensing and transmitted through FemR and FemI (Llamas et al., 2008), PA2299 could be either directly activated or post-translationally modified to fine-tune the transcription of transport and virulence-related genes, including the PA2296-PA2299 operon itself. This regulatory feedback would ensure a coordinated response coupling iron acquisition with metabolic and pathogenic pathways.

A plausible model emerges where FemA detects extracellular siderophores (e.g., mycobactin J), FemR-FemI transduce this signal across the membrane, and PA2299 acts as a transcriptional

regulator that modulates the expression of PA2296, PA2299, and related downstream genes to optimize iron uptake. This concerted regulation may enhance *P. aeruginosa* fitness under iron-limited conditions, common in host environments, thereby promoting virulence. The functional link among the Fem system, the ABC transporter PA2294, and the transcriptional regulator PA2299 underscores an integrated signaling and transport mechanism that coordinates iron sensing, uptake, and gene regulation in response to environmental iron cues.

Our study also demonstrated that the PAO1 (Δ PA2294) and PAO1 (Δ PA2298) deletion mutants have an iron-dependent role in virulence-associated characteristics. Compared to wild-type PAO1, both mutants produce less biofilm, suggesting that the PA2294-PA2298 region promotes biofilm formation. PAO1 (Δ PA2298) exhibits decreased pyocyanin under Mycobactin J-mediated iron limitation, indicating that PA2298 has a positive effect on pyocyanin production, while PAO1 (Δ PA2294) exhibits increased pyocyanin, suggesting that PA2294 may function as a negative regulator. Together, these findings reveal PA2294 and PA2298 as novel participants that connect biofilm formation and pyocyanin production in *P. aeruginosa* to mycobactin-mediated iron uptake.

Altogether, these results have several implications. Firstly, they create a direct regulatory link between the PA2296 and PA2299 operon and the Fem system, offering a mechanistic foundation for additional functional research on the role of this gene in fitness and possible virulence. Secondly, they suggest that Fem-dependent regulation of PA2296-PA2299 could improve survival in host conditions with limited iron, which is a crucial element of pathogenic success. Lastly, this study draws attention to the need for more research, including locating the FemA binding location within the PA2296 and PA2299 putative promoter, figuring out whether iron or other ligands affect FemA activity.

Chapter 6

Examine the involvement of PA1611 in the mucin response of *P. aeruginosa*

A manuscript reporting this section is under preparation, and a draft has been written. The manuscript will be authored by **Haque, M. M.**, Bhagirath, A, and Duan, K. (2026), and the tentative title is “PA1611 expression in *Pseudomonas aeruginosa* isolates and its environmental cues”.

Author contributions: MMH, AB, and KD directed and designed the research. MMH and AB conducted the experiments. MMH and AB analyzed the data. Article writing was performed by MMH, AB, and KD.

6.1 Introduction

The prominent ability of *P. aeruginosa* to form biofilms in different environments is facilitated by various factors, which include nutritional adaptability and gene regulation mechanisms such as two-component regulatory systems (TCSs), quorum sensing (QS), and bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) signaling systems. In *P. aeruginosa* PAO1, the RetS-GacS/GacA-RsmA system, which involves hybrid histidine kinases and traditional two-component systems (TCSs), is essential for biofilm development and virulence regulation (Bhagirath et al., 2017; Kong et al., 2013). In this system, GacS/A acts as a traditional TCS, whereas RetS, LadS, and PA1611 are hybrid histidine kinases (HHKs). The exact environmental cues that activate these TCSs are not yet fully understood. LadS has been shown to respond to environmental calcium to mediate acute-chronic disease transition (Broder, Jaeger, & Jenal, 2016). It has been demonstrated that RetS suppresses *pel* genes that encode the biofilm's polysaccharide components (Vanessa I. Francis et al., 2018; Moscoso et al., 2011). When GacS is activated, GacA is phosphorylated, which triggers the transcription of regulatory short RNAs such as RsmY and RsmZ. Following their binding to and sequestration of the posttranscriptional activator RsmA, these short RNAs promote the formation of biofilms and restrict bacterial motility, Type IV pili, and the action of toxin secretion systems, such as T3SS (Burrowes, Baysse, Adams, & O'Gara, 2006; Andrew L. Goodman et al., 2004; Kay et al., 2006). T3SS is a needle-like injectosome that delivers toxic effectors (ExoS, Y, T, U) into host cells, causing cell damage and death. Active T3SS is a hallmark of the acute phase of *P. aeruginosa* infection (Kay et al., 2006).

Prior research in our lab has shown that overexpression of PA1611 in PAO1 promotes biofilm formation while suppressing T3SS expression and swarming motility (Bhagirath et al., 2017;

Kong et al., 2013). Studies also demonstrated that PA1611 interacts directly with RetS, which in turn alters the signaling between GacS and GacA. The downstream effects are mediated by GacA via RsmA-Y/Z (Kong et al., 2013). Even though PA1611 has a major regulatory role in *P. aeruginosa* PAO1 when overexpressed, its comparatively modest expression in typical laboratory settings has raised concerns regarding its significance in regulating virulence factors such as T3SS and biofilm formation. Similarly, it remains an unsettled question regarding how PA1611 expresses and functions in strains other than PAO1.

Recent works showed that mucin promotes the ability of RetS to directly inhibit GacS-GacA activity in the *P. aeruginosa* PA14 strain. The signaling depends on both the signal-binding Dismed2 domain of RetS as well as its cytoplasmic phosphorelay domains (B. X. Wang et al., 2021). The effect of mucin on *exoS* and biofilm formation could potentially be explained by the direct interaction between mucin and RetS. In this study, we report that the *PA1611* knockout mutant of PAK showed significant changes in both T3SS activity and biofilm formation. Our results further indicate the potential connection between mucin and PA1611 and its regulation. Collectively, our findings suggest that PA1611 acts as a hybrid sensor kinase in *P. aeruginosa*, sensing mucin glycan signals and regulating both T3SS and biofilm development.

6.2 Materials and Methods

6.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used are listed in **Table 6.1**. All cloning processes were carried out using *Escherichia coli* strain DH5 α , while conjugative DNA transfer was carried out using *E. coli* strain *Sm10 λ pir*. Tetracycline (Tc) at 70 μ g/ml in LB or 300 μ g/ml in *Pseudomonas* Isolation Agar (PIA), Carbenicillin (Cb) at 250 μ g/ml in LB, and Trimethoprim (Tmp) at 300 μ g/ml in LB were the antibiotics used for *P. aeruginosa*; Kanamycin (Kn) at 50 μ g/ml and Ampicillin (Amp) at 100 μ g/ml in LB were used for *E. coli*.

Table 6.1 List of bacterial strains and plasmids used in this study.

Bacterial strains or plasmids	Characteristics	Source/References
<i>E. coli</i>		
SM10- λ <i>pir</i>	Mobilizing strain, RP4 integrated into the chromosome; Kn ^r	(Simon, Priefer, & Puhler, 1983)
<i>P. aeruginosa</i>		
PAO1	Wild-type <i>P. aeruginosa</i>	This lab
PAK	Environmental strain of <i>P. aeruginosa</i>	This lab
PA(Δ PA1611)	PA1611 Knockout mutant of PAO1	This lab
PAK(Δ PA1611)	PA1611 knockout mutant of PAK	This study
Plasmids		
CTX6.1	Integration plasmid origins of plasmid mini-CTX-lux; Tc ^r	This lab
pAK1900	<i>E. coli</i> - <i>P. aeruginosa</i> shuttle cloning vector carrying <i>Plac</i> upstream of MCS; Ap ^r ; Cb ^r	This lab
pAK1611	pAK1900 with a 2024 bp fragment of <i>PA1611</i> between <i>SalI</i> and <i>HindIII</i> ; Ap ^r , Cb ^r	This study
CTX- <i>exoS</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoS</i> containing <i>exoS</i> promoter region and <i>luxCDABE</i> gene; Kn ^r , Tmp ^r , Tc ^r	This Lab
pMS402	Expression reporter plasmid carrying the promoterless <i>luxCDABE</i> gene; Kn ^r , Tmp ^r	(Duan, Dammel, Stein, Rabin, & Surette, 2003)
pEX18Tc	oriT ⁺ sacB ⁺ gene replacement vector with multiple-cloning site from pUC18; Tc ^r	(Hoang et al., 1998)
CTX- <i>exoS</i>	Integration plasmid, CTX6.1 with a fragment of pKD- <i>exoS</i> containing <i>exoS</i> promoter region and <i>luxCDABE</i> gene; Kn ^r , Tmp ^r , Tc ^r	This Lab

6.2.2 Construction of deletion mutant PAK(Δ PA1611) and complementation strain

To create an unmarked deletion mutant of PA1611, we used the Gibson Assembly® Cloning Kit (New England BioLabs Inc) following the manufacturer's instructions. The deletion construct pEX-18Tc-PA1611 was assembled by amplifying the upstream and downstream fragments of PA1611 and the vector (pEX-18Tc) using the primers listed in **Table 6.2**. The PCR products were purified and mixed with the Gibson Assembly Master Mix. The reaction mixture was incubated in a thermocycler at 50 °C for 15 minutes. The PA1611 knockout mutant, PAK(Δ PA1611), was obtained using tri-parental mating as reported previously (Hmelo et al., 2015). Briefly, overnight cultures of the donor strain *E. coli* containing the plasmid pEX18Tc-PA1611, the helper strain containing pRK2013, and the recipient PAK strain were collected and resuspended in PBS. The bacteria were mixed in a ratio of 2:2:1 and then spotted on an LB agar plate. After culturing at 37 °C overnight, the bacteria were scraped off and re-suspended in 500 µl of LB. The diluted suspensions were spread on PIA plates containing tetracycline at 300 µg/ml. The obtained colony was streaked on No-salt LB supplemented with 10% sucrose to screen for a second recombination event. The resultant PA1611 knockout mutant was verified by PCR with primers C-PA1611-F/C-PA1611-R and designated as PAK(Δ PA1611).

For complementation, we employed the multi-copy-number *E. coli*-*P. aeruginosa* shuttle vector pAK-1900 (Poole, Neshat, Krebs, & Heinrichs, 1993). Full-length PA1611 was PCR-amplified using primers listed in **Table 6.2**. The PCR products were digested with *Sph*I and *Hind*III and then cloned into pAK-1900 under control of a *lacUV5/lacIq*-regulated *T7* promoter. The cloned PA1611 sequence was confirmed by DNA sequencing. The constructed plasmid was then transformed into *P. aeruginosa* by electroporation resulting in the strain Δ PA1611 (Δ PA1611 (pAK-PA1611)).

Table 6.2 Primers used in this study.

Primer	Sequence (5'-3')
PA1611-Forward	GTAGTCGACGAAGGAGAAGAACCATG
PA1611-Reverse	GCTAAGCTTGGCGAACAGATGTTCACT
Gibson-1611-Vector-Forward	GCACGCCGATCTGCAGGTCTGACTCTAGAG
Gibson-1611-Vector-Reverse	CTACCTCGGCGCATGCAAGCTTGGCACT
Gibson-1611-UP-Forward	CGACCTGCAGATCGGCGTGCTCTACGGCA
Gibson-1611-UP-Reverse	GATGTCACGGTGAGCAACAGGGTCAGCAGC
Gibson-1611-DW-Forward	CTGTTGCTCACCGTGACATCGAACGAAACC
Gibson-1611-DW-Reverse	GCTTGCATGCGCCGAGGTAGAAACCGAC
C-PA1611-Forward	CCAGCAGCAGCGGCAACGACGA
C-PA1611-Reverse	TTGCCATACTTGCCGCCGACATCG

6.2.3 Mucin preparation

Mucin was prepared as per protocol described previously (B. X. Wang et al., 2021). 10 g/L mucin (Sigma Type III) was added to M9 salt media with supplement containing glucose, MgSO₄, CaCl₂, and casamino acid. The media was autoclaved at 121°C for 15 min. Cellular debris was removed after centrifugation for 30 minutes at 4°C at 8,000 x g. Amicon ultra centrifugal 100 kDa filters (Sigma) were used to remove the high molecular weight components from the mucus. Mucin was further passed through a 0.45 µm Millipore syringe filter to sterilize and isolate soluble intact glycoproteins. Pseudomonas was cultured in M9 minimal media containing supplements.

6.2.4 Bacterial twitching motility assay

Bacteria were assessed for twitching motility according to the protocol described previously (Rashid & Kornberg, 2000). The medium for the twitching motility assay consisted of 4 g/L tryptone, 2 g/L yeast extract, 2 g/L NaCl, 1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, solidified with 1% agar. Twitching motility was evaluated by seeding stationary-phase cells onto the centers of 100 mm diameter motility plates using a sterile toothpick. For twitching motilities, the incubation was for 24 h at 37°C. The diameters of the migration front were measured, and photographs were taken with a Vilber Lourmat Fusion FX7 imager.

6.3 Results

6.3.1 PA1611 regulates virulence and key phenotypes in the PAK strain

Previous results from our lab established that overexpression of PA1611 enhances biofilm formation and represses T3SS (Kong et al., 2013). To determine whether these effects are consistent, we selected the PAK strain of *P. aeruginosa* as a reference strain due to its well-documented behavior, significant PA1611 expression, and practicality for genetic manipulation compared to clinical isolates. We constructed a PA1611 knockout in the PAK strain (denoted as PAK Δ 1611) and created a mutant complementation strain (denoted as PAK1611) and checked the key phenotypes such as biofilm formation and motility of those strains.

Activation of PA1611 in PAO1 is associated with an increase in biofilm formation, and this result was confirmed in the PAK strain, where the wild-type strain produced significantly more biofilm than the PA1611 deletion mutant PAK(Δ PA1611) (**Figure 6.1**). The complementation strain, PAK1611, showed a similar pattern of biofilm formation to the wild type. The PAK Δ 1611 mutant not only showed reduced biofilm formation but also displayed enhanced swimming, swarming,

and twitching motility (**Figure 6.2**). These alterations were reversed to wild-type phenotypes in the mutant complementation strain PAK-1611. Altogether, these results suggested that PA1611 can regulate multiple virulence traits, such as biofilm formation and motility, and contribute to the pathogenicity of *P. aeruginosa*.

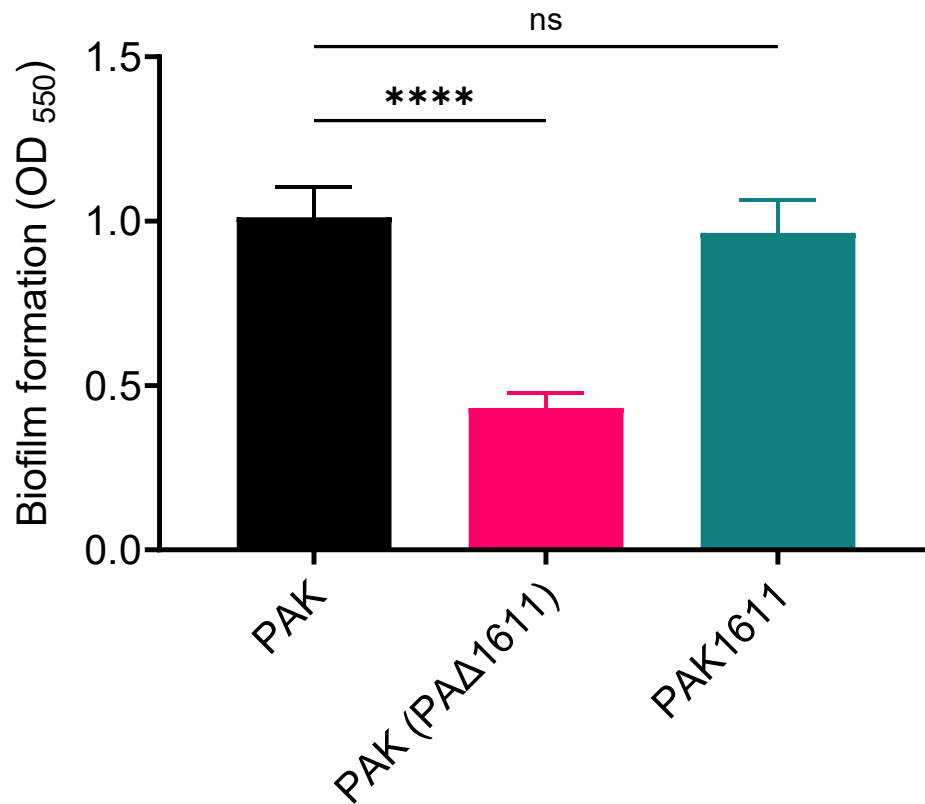


Figure 6.1 Deletion mutant PAK ($\Delta PA1611$) reduced biofilm. Biofilm formation was measured in PAK, PAK ($\Delta PA1611$), and the complementation strain PAK1611 in a 96-well plate. Data were analyzed using one-way ANOVA with a t-test and Tukey's multiple comparisons test. Results representative of three separate experiments with comparable outcomes, carried out in triplicate. Error bars indicate standard deviations. ns, not significant, and **** $p < 0.0001$.

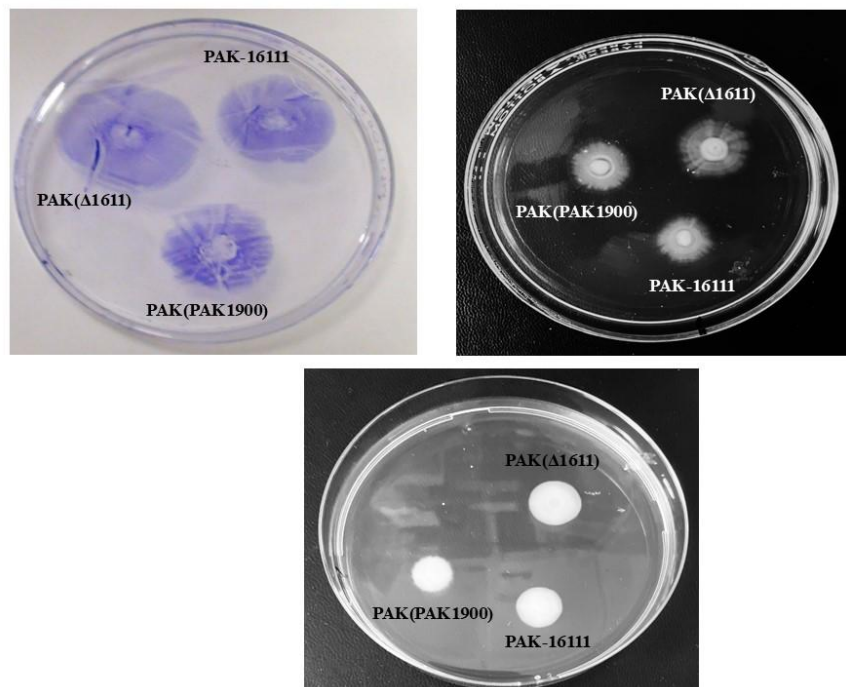


Figure 6.2 Deletion of PAK ($\Delta PA1611$) affects the twitching, swarming, and swimming motility. Inactivation of PA1611 in PAK results in enhanced twitching, swarming, and swimming motility in PAK ($\Delta PA1611$) compared to wild-type PAK.

6.3.2 Mucin affects T3SS in *P. aeruginosa* PAK through PA1611

Given the robust expression of PA1611 in the PAK strain, we sought to examine its response under conditions relevant to CF lung infection. To determine whether T3SS gene expression was affected by PAK through PA1611, we constructed promoter reporter strains of T3SS, CTX-exoS-PAK ($\Delta PA1611$) and a complementation strain, CTX-exoS-PAK1611, that was integrated into the chromosome of the PAK strain. The promoter activity of *exoS* was monitored over an extended period of growth in the presence of various concentrations of mucin. The standardized media with the addition of mucin, designed to mimic CF sputum, can provide valuable insights into *P. aeruginosa's* in vivo behavior.

Subsequently, we supplemented the M9 minimal medium with 0.5 and 1% mucin to gauge its

impact on PA1611 and *exoS* promoter activity while concurrently tracking bacterial growth.

Figure 6.3 illustrates that the presence of 0.5 % and 1% mucin significantly reduced *exoS* promoter activity.

In terms of the mucin regulation, our results suggest that, in addition to the regulation of *retS* by mucin via the Gac-Rsm pathway, mucin can also affect the *retS*-Gac pathway through PA1611, by activation of its expression. However, mucin signaling through PA1611 leads to a regulatory outcome that is opposite to that observed with mucin-mediated activation of RetS, where RetS siphons phosphate from GacS, rendering GacA inactive and RsmA active.

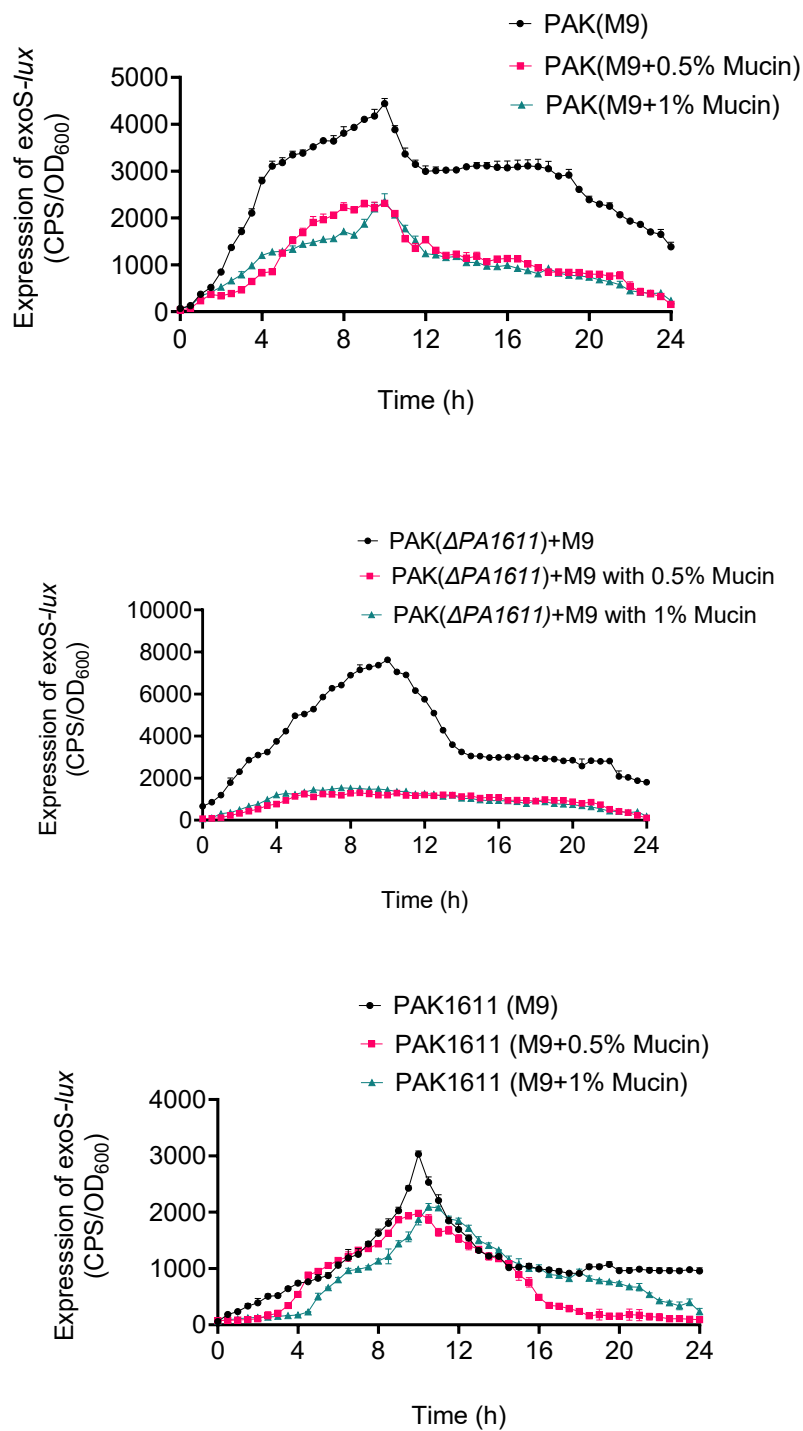


Figure 6.3 Effect of mucin on the expression of T3SS effector, *exoS*. The CTX-*exoS*-T3SS reporter fusion integrated on the chromosome was used to measure the promoter activity of the *exoS* gene in PAK, deletion mutant, PAK (Δ PA1611), and the complementation strain.

Luminescence was measured as relative luminescence units (RLU), calculated by normalizing cps (counts per second) to optical density at 600 nm (OD₆₀₀). The graph presents the average values from three independent experiments.

6.3.3 PAK (Δ PA1611) reduced biofilms in *P. aeruginosa* in the presence of mucin

To further characterize the response to mucin on biofilms through PA1611, we compared the effects of mucin on biofilm formation in the PAK wild-type, PAK (Δ 1611), and complementation strains PAK1611. Our results showed that the presence of the PA1611 mucin deletion mutant resulted in a lower biofilm percentage than in the wild-type PAK (**Figure 6.4**). These results of biofilm formation assay activity in PAK wild type, PAK Δ 1611, and PAK1611(complementation) in the presence (0.5% and 1%) and absence of mucin are critically important and showed a PA1611-dependent mechanism (how PA1611 responds to mucin) in *P. aeruginosa*. The biofilm formation test further demonstrated the significant effect of mucin on the regulation of the protein secretion system.

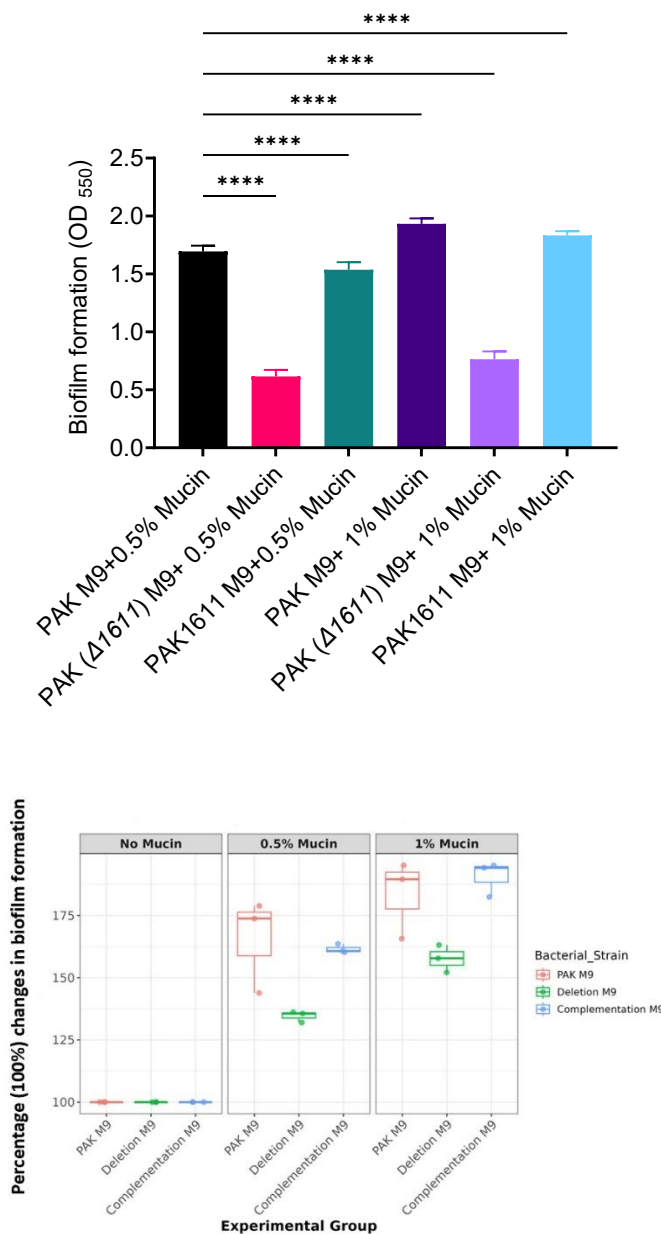


Figure 6.4 Deletion mutant PAK (Δ PA1611) showed reduced biofilm with the addition of mucin. Biofilm formation was evaluated in 96-well microtiter plates, and the result showed the overall biofilm formation in the presence of mucin (0.5% and 1%). The results showed that in the presence of mucin, the PA1611 deletion mutant had a lower biofilm percentage than the wild-type PAK. Data was analyzed using one-way ANOVA with a t-test. Error bars indicate standard deviations. **** $p < 0.0001$.

6.4 Discussion

Previous research has identified PA1611 as a crucial regulatory gene in *P. aeruginosa*, influencing both biofilm formation and the expression of the T3SS (Bhagirath et al., 2017; Kong et al., 2013). Despite this, PA1611 is expressed at very low levels in the *P. aeruginosa* PAO1 strain under standard laboratory conditions. The specific environmental cues that trigger PA1611 and its role in chronic disease progression remain elusive. Considering PA1611's pivotal role in *P. aeruginosa*'s pathogenicity, its significance in environmental and clinical settings prompted further investigation in clinical and environmental strains. Given the substantial genomic diversity among *P. aeruginosa* clinical isolates, our lab previously confirmed the presence of PA1611 via PCR. Subsequently, we also assessed its promoter activity in LB media to evaluate expression levels.

Research has shown that colonizing *Pseudomonas aeruginosa* isolates are genetically distinct variants of their environmental progenitors (Hansen et al., 2012). The unique ecological pressures within the cystic fibrosis (CF) lung environment exert a profound influence on the regulation of gene expression and virulence in *P. aeruginosa*. Thus, studying PA1611 expression in authentic CF lung conditions would involve using patient-derived CF sputum. However, this approach may be fraught with potential experimental inconsistencies, such as variations in the clinical stage of disease at the time of sputum collection or the presence of antibiotics in the samples.

For studying gene expression, the PAK strain was chosen since its genetic sequence is well known (Cain et al., 2019). Notably, the removal of mucin from the medium resulted in the highest levels of *exoS* expression. Consequently, we introduced 1% mucin to the medium and found that *exoS* promoter activity was significantly reduced under these conditions, compared to

the control. Mucin, a major constituent of the airway mucus, is notably affected in CF, with its levels reported to exceed 2% (w/v) in CF patients (A. Boyd & Chakrabarty, 1995). This increased concentration of mucins, which are negatively charged and viscous, is hypothesized to impact the function of the T3SS in *P. aeruginosa*. The physical properties of mucins could potentially interfere with the rotation of the T3SS needle and the subsequent secretion of effectors, thereby affecting the bacterium's virulence mechanisms (Bansil & Turner, 2006).

The pathophysiology of *P. aeruginosa* is known to be significantly influenced by mucin, a high molecular weight glycoprotein with a rich array of carbohydrate side chains. Prior investigations have shown that mucin can stimulate biofilm formation and impede bacterial movement. It also enhances survival and may confer antibiotic resistance through the activation of interactions between mucin and FliD, an adhesin (Landry, An, Hupp, Singh, & Parsek, 2006). Additionally, biofilms formed in the presence of mucin are notably distinct from those developed in standard culture media, exhibiting a more densely packed and flattened morphology (Landry et al., 2006). These findings align with our study's results, further corroborating the influential role of mucin in shaping the biofilm architecture and the overall virulence strategy of *P. aeruginosa*.

According to recent research, mucin enhances RetS's capacity to block GacS-GacA activity in the *P. aeruginosa* PA14 strain directly. Both RetS's cytoplasmic phosphorelay domains and its signal-binding Dismed2 domain are necessary for signaling (B. X. Wang et al., 2021). During *P. aeruginosa*'s planktonic development phase, RetS and GacS form a domain-swapped hetero-oligomer. Unknown cues then drive this hetero-oligomer to dissociate, relieving GacS inhibition and promoting the production of biofilms (Ryan Kaler, Nix, & Schubot, 2021). GacS's hallmark motifs necessary for its atypical autokinase process are revealed by the structure of the D1 domain of GacS and the identification of an unnamed functional ND domain that is necessary for

GacS's complete autokinase activity (Fadel et al., 2022). RetS reduces GacS signaling by removing phosphoryl groups from GacS-P, inhibiting GacS autophosphorylation, and having transmitter phosphatase activity against the GacS-P receiver domain (V. I. Francis et al., 2018). To confirm whether the biofilm regulation in the PAK strain was mediated by the GacS/GacA-RetS-PA1611-RsmA/Y/Z pathway, we examined the expression of its key components in a mutant strain. To confirm a rescue-of-expression, we also examined their levels in a complementation strain. The findings from this study indicate that the biofilm regulatory effects we observed are, at least in part, facilitated by this canonical signaling pathway.

This study underscores the intricate nature of gene regulation crucial for the survival and persistence of *P. aeruginosa*. It further highlights the importance of deciphering the interplay between signaling pathways in bacterial virulence, the GacA/S-PA1611-RetS-RsmA/Y/Z pathway in *P. aeruginosa*. Studies also showed that *P. aeruginosa* Gac/Rsm signaling system homeostasis is contributed to by direct inhibition of RetS synthesis by RsmA (Corley, Intile, & Yahr, 2022). Delving deeper into the complex signaling mechanisms that influence key regulators in *P. aeruginosa* is vital for developing targeted and effective therapeutic strategies.

Chapter 7

Conclusions and future directions

7.1 Conclusion

7.1.1 Virulence and secretion system regulation in *P. aeruginosa*

P. aeruginosa is an opportunistic pathogenic bacterium that can be found in both biotic and abiotic environments. It is the major cause of microbial dysbiosis in a wide range of patients with pulmonary infections, burn wounds, urinary tract infections (UTIs), and other diseases that compromise the immune system. It forms biofilms, develops resistance to antibiotics, and produces an arsenal of virulence factors that make *P. aeruginosa* a major life-threatening pathogen. Bacteria in biofilms communicate with each other using signals known as quorum-sensing. However, the molecular mechanism of bacterial signaling molecules and protein secretion systems, T3SSs and T6SSs in *P. aeruginosa*, the global regulators, is not fully known. T3SSs and T6SSs are the major virulence determinants and help to translocate toxins into target cells. In this study, the molecular basis and regulatory pathways of bacterial cell-to-cell interaction in *P. aeruginosa* in relation to T3SSs and T6SSs were characterized and discovered.

7.1.2 NorCBD-mediated nitric oxide homeostasis

We report the identification of *norCBD* as an operon that modulates the transcriptional activity of H2-T6SS. Both transposon insertion at *norCBD* and the deletion of the *norCBD* genes significantly reduced the CTX-H2-T6SS reporter activity. As the transcriptional regulatory Dnr activates H2-type VI secretion system (H2-T6SS) in response to NO, experiments were carried out to examine whether *norCBD* deletion caused intracellular NO accumulation, which in turn disrupted Dnr-dependent regulation of H2-T6SS and virulence factors. The NO levels and Dnr-regulated gene expression were measured, and several virulence-related phenotypes were examined. The effects of NO donor sodium nitroprusside (SNP) and NO scavenger carboxy-phenyl-tetramethylimidazolineoxyl (CPTIO) were also tested. The data obtained indicate that

deletion of *norCBD* led to intracellular NO accumulation, reduced H2-T6SS expression, and affected motility, pyocyanin production, and biofilm formation. Complementation of *norCBD* on a plasmid in the deletion mutant was able to restore H2-T6SS expression and the examined phenotypes to the wild-type levels. Treatment with CPTIO also restored H2-T6SS expression in the PAO1(Δ *norCBD*). These results indicate that NorCBD plays a critical role in maintaining NO homeostasis that is necessary for effective Dnr-mediated gene regulation and multiple virulence-related traits, highlighting the importance of redox balance in coordinating respiration and pathogenesis in *P. aeruginosa* (Chapter 3).

7.1.3 ATPases and energy-dependent functions of T3SS and T6SS

In this study, we characterized two key ATPases, PscN and ClpV3, associated with T3SS and T6SS, respectively, in *P. aeruginosa*. Our findings demonstrate that disruption of *pscN* and *clpV3* affects the expression of both T3SS and H3-T6SS, influences virulence-associated phenotypes, and alters intracellular ATP levels. Given that T3SS and T6SS are highly energy-demanding systems, fluctuations in the cellular ATP pool may directly affect their function and efficiency. Although the precise mechanisms by which intracellular ATP levels influence secretion system regulation remain to be fully elucidated, our findings suggest that PscN and ClpV3 function as metabolic checkpoints linking cellular energy status to secretion system activity and pathogenicity (Chapter 4).

7.1.4 Fem-associated operon and virulence regulation

Recent RNA sequencing data from our lab showed the significant fold changes in genes (PA2293-PA2299) in *P. aeruginosa* in response to the FemA deletion mutant, a potential cell surface signaling molecule. In our study, we found that this operon relates to the Fem system, as

the PA2294 operon was shown to be regulated by FemA. Our results also showed that the potential virulence factors, such as biofilm and pyocyanin, are highly affected when we knock out the PA2294 and PA2298 genes. These findings greatly increase our comprehension of the regulatory processes and potential connection of these operons and the Fem system and their possibly link with T6SSs (Chapter 5).

7.1.5 Environmental sensing via PA1611

Our study also revealed that PA1611 functioned as a key regulator for biofilm formation and T3SS, and was responsive to the presence of mucin, a key environmental factor within the CF lung environment. These results underscore the importance of PA1611 in the pathogenicity of *P. aeruginosa* and emphasize the need for integrating different strains in research to understand the full spectrum of the pathogen's regulatory pathways (Chapter 6).

7.1.6 Concluding perspective

In summary, the signaling dynamics within *P. aeruginosa* are more complex and require further investigation to be fully elucidated. The unique functioning of these signaling pathways, which contribute to T3SSs and T6SSs in promoting survival and pathogenicity, underscores the need for further studies, particularly those focusing on external factors that shape bacterial virulence. A nuanced understanding of the interactions between the host and the bacterial response is crucial for effective infection control and will potentially lead to the development of novel antibacterial targets for *P. aeruginosa* and better management of its activities and microbial communities, including biofilms and human microbiota. A proposed model is presented in **Figure 7.1** as an overall summary of this thesis.

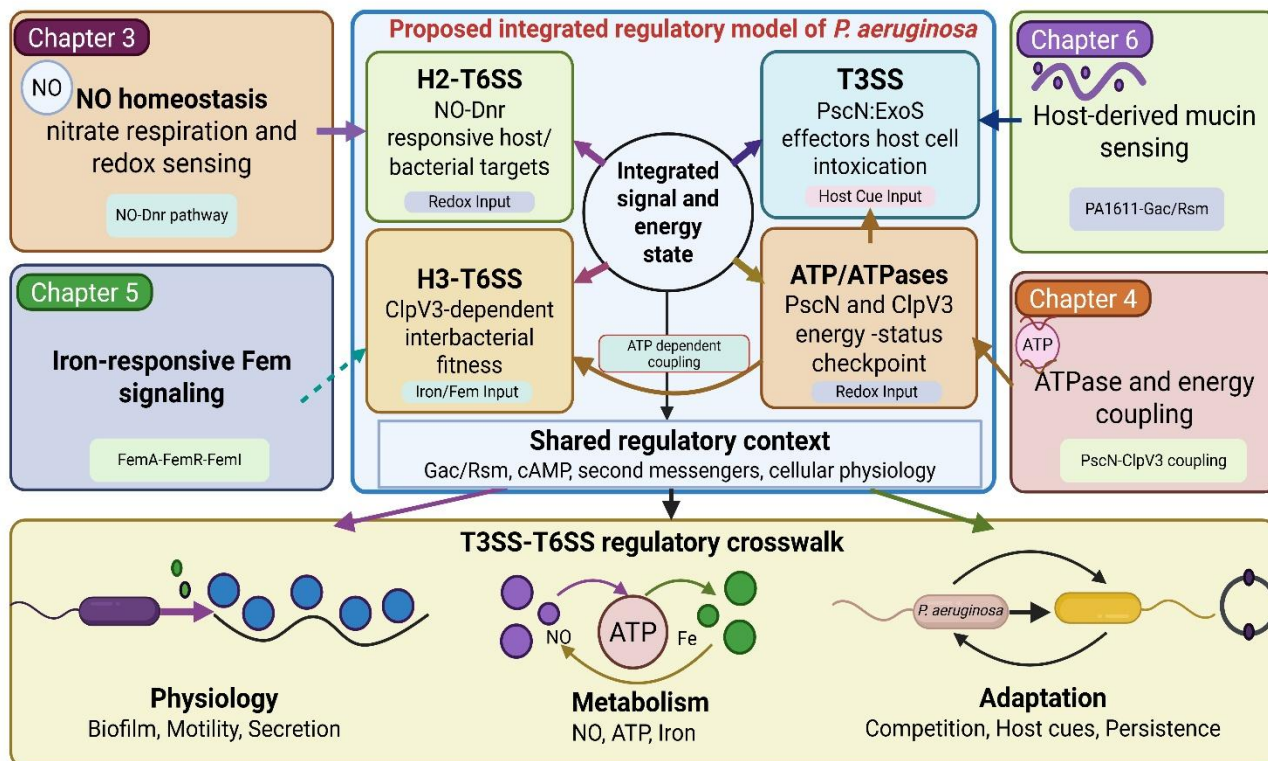


Figure 7.1 Proposed integrated regulatory model of *P. aeruginosa*. This figure depicts the overall summary of this thesis. In *P. aeruginosa*, distinct regulatory pathways connect the T3SS and T6SS, influencing bacterial physiology, metabolism, and environmental adaptation. The figure was prepared using BioRender.

7.2 Future Directions

7.2.1 Genetic interactions among the NO, cAMP-Vfr, and c-di-GMP circuits

Our study provides a brief association between *P. aeruginosa* and redox status, secretion-system control, and virulence characteristics, unified by NO homeostasis. Future studies are required to map the complex genetic connections among the nitric oxide, cAMP-Vfr, and c-di-GMP circuits and to fully understand the underlying mechanisms among them. In addition, transcriptional profiling of the *norCBD* deletion mutant needs to be performed to determine the global effect of *norCBD* inactivation and how the metabolic pathway is affected via *norCBD*. Also, RNAscope could be used to detect bacterial RNA, thereby tracking spatiotemporal RNA expression changes across differentiation stages during bacterial infections with *Pseudomonas aeruginosa*. This will provide stage and niche-specific readouts, supporting lineage conclusions with in situ, quantitative evidence, while targeting anti-virulence therapies.

7.2.2 Characterization of protein-protein interactions of T3SS and T6SS dependent ATPase

For future investigation, identifying protein-protein interactions between clpV3 and popN (known to interact with pscN), pscN and TssA, and TssC (known to interact with clpV3) is necessary. Molecular modeling and ligand binding interaction need to be revealed by AlphaFold, HADDOCK, and ZDOCK. To better define ATPase function, structural and mutational studies, and the effect on virulence should be evaluated using infection models for future investigation. In addition, we may be able to learn more about the detailed functioning of these secretion systems if we discover new T3SS and T6SS-dependent effectors. Thus, proteomics and mass spectrometry could be used to determine which secreted effectors are connected to T3SSs and T6SSs in *P. aeruginosa*.

7.2.3 Characterization of the function of the gene products of this operon

Our studies revealed the potential connection and regulation of operon PA2267-PA2299 by the Fem system. For future studies, firstly, the function of this operon's gene products needs to be examined, which includes substrate transport, product synthesis, and the role in bacterial physiology. Secondly, how the synthesis of ferredoxin is connected and regulated by FemI/A system needs to be investigated to reveal the underlying molecular mechanism. Finally, the regulatory mechanisms (e.g., regulator binding and sites), how this operon is interlinked with the Fem system, need to be further studied.

7.2.4 Using multi-omics approaches to complete pathway mapping

It will be interesting to understand PA1611's global impact under mucin exposure and other environmental clues. For this, proteomic and transcriptomic analyses need to be done to determine the downstream genes impacted by the PA1611 gene. Furthermore, metabolomics studies could be conducted to reveal the molecular mechanisms correlated with signaling pathways. In addition, the structural dynamics of PA1611 could be investigated to identify ligand-binding pockets that could react to mucin and other host signals connected with protein secretion systems of *P. aeruginosa*.

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