Structural study of ExsA, the regulator of Type III Secretion System of *Pseudomonas aeruginosa*

Yi Xiao

Master of Science
in
Biological Sciences

Florian D. Schubot
Ann M. Stevens
David R. Bevan
Bingyu Zhao

April 25, 2013
Blacksburg, Virginia, USA

Keywords: *Pseudomonas aeruginosa*, Type III secretion system, ExsA, structure, crystallization, ExsD, ExsACDE pathway

Structural study of ExsA, the regulator of Type III Secretion System of *Pseudomonas aeruginosa*

Yi Xiao

Abstract

The Type III secretion system (T3SS) of *Pseudomonas aeruginosa* uses a needle-like protein apparatus to detect eukaryotic host cells and translocate effectors directly into the host cell. The effectors are also known as cytotoxins, which cause disruption of a series of signaling events in the host cell, facilitating the infection by *P. aeruginosa*. As the T3SS is antigenic and the expression of T3SS is energy-consuming, it is highly regulated where several regulatory proteins interact with each other and control the expression of T3SS genes. Among these proteins, ExsA, the master regulator of T3SS in *P. aeruginosa*, is of great importance as it is a transcriptional activator that activates the expression of all T3SS genes. Also, as ExsA belongs to the AraC protein family which only exists in bacteria and fungi, it makes an excellent potential target for drugs against *P. aeruginosa* related infections. With a combination of molecular biology tools and structural biology methods, we solved the N-terminal domain structure of the ExsA protein in *P. aeruginosa.* The model of the ExsA N-terminal domain has enriched our knowledge about ExsA dimerization and can serve as the base for mapping the interaction interfaces on ExsA and ExsD. Further, we have found two homologues of ExsA by structural alignment, which share a lot of similarities and have conserved amino acid residues that are important for ligand binding. The fact that both of these two proteins are regulated by small ligands rather than proteins also raises the possibility that ExsA may have a second regulatory mechanism under which ExsA is regulated by a small ligand, which so far has not been observed or reported by researchers. In order to map the binding site of ExsA on its anti-activator ExsD, we removed the coiled-coil region (amino acid residue 138-202, the potential binding site) of ExsD, based on the

structure of ExsD. We surprisingly found that the ExsD variant without the coiled-coil region readily inhibits ExsA-dependent *in vitro* transcription. This result rules out other possibilities and makes us focus on the N-terminus and adjacent regions of ExsD for the interface with ExsA. Moreover, in order to gain a comprehensive understanding of the dynamics of the regulation of T3SS in *P. aeruginosa*, we have begun to build a mathematical model of the T3SS regulatory pathways. We are measuring the cellular concentrations of T3SS regulatory proteins with quantitative molecular biology methods such as quantitative western blot, quantitative PCR and quantitative mass spectrometry. We have determined the cellular level of ExsA and ExsD proteins under different physiological conditions, and found that some factors such as temperature have a significant impact on the levels of ExsA and ExsD. This study has thus unveiled some unknown features of the T3SS of *P. aeruginosa* and its related infections.

Acknowledgements

I am particularly grateful to Dr. Florian Schubot, my advisor, for his encouragements and guidance. Dr. Schubot expressed a genuine dedication to his students; and that was the key to my achievements at Virginia Tech.

I would like to thank my committee members, Dr. Ann Stevens, Dr. David Bevan and Dr. Bingyu Zhao, for their support, encouragements and advices.

I would like to thank Dr. Howard Robinson, for his help with X-ray diffraction assays and data collection.

I would like to recognize the assistance provided by Xing Jing and Cory Bernhards on this project, and the other members of the lab for the friendly and professional atmosphere.

I have a special thought for my wife and my parents. They had waited to see this day. I am sincerely grateful to my family for their support through every choice I have made in my life.

Table of Contents

List of Figures

List of Tables

Chapter One: Literature Review

1.1 Pseudomonas aeruginosa and related infections

The opportunistic pathogen *P. aeruginosa* is a Gram-negative bacterium which can cause acute and chronic infections in wide range of animal and plant hosts [1-4]. As a ubiquitous bacterium, *P. aeruginosa* exists in water, soil and throughout all environments. *P. aeruginosa* has emerged as an important pathogen during the past two decades, as in most hospitals it causes 10%-20% of infections in patients, including bloodstream, surgical site, and soft tissue infections. *P. aeruginosa* is also a frequent cause of pneumonia and urinary tract infections in hospital patients and accounts for an estimated 13% of all nosocomial intensive care unit infections in the United States [5]. *P. aeruginosa* ranks among the five most common bacterial pathogens, and patients who are hospitalized for extended periods are frequently colonized by *P. aeruginosa* and are at increased risk of developing infection [6]. Due to the prevalence of multi-drug resistant strains, current antibiotic therapies are frequently ineffective and *P. aeruginosa* infections are associated with unacceptably high morbidity and mortality rates. For example the mortality rate for *P. aeruginosa*-associated pneumonia is 50% [7]. Under non-favorable conditions *P. aeruginosa* can form a biofilm which makes it more resistant to antibiotics and other adverse factors. *P. aeruginosa* biofilm is also the cause for chronic infections. *P. aeruginosa*–associated chronic lung infections are the leading cause of mortality in Cystic Fibrosis patients. Failure of current treatments to clear persistent *P. aeruginosa* infections results in the gradual deterioration of lung function [8, 9].

1.2 Type III Secretion System

Many Gram-negative bacteria employ the type III secretion system (T3SS) to detect the presence of eukaryotic cells and translocate proteins that facilitate virulence [10]. Those bacteria include well-known mammalian pathogens such as *Yersinia pestis*, *Salmonella enterica*, *Chlamydia species*, *Vibrio species*, and *P. aeruginosa* [11]. This system is important for pathogenesis, and contributes greatly to disease severity and

persistence of acute infections, as relative risk of mortality is associated with expression of T3SS secretory proteins. Disruption of the T3SS leads to a dramatic attenuation as a *P. aeruginosa* T3SS knockout strain shows reduced virulence [12-14].

The T3SS of *P. aeruginosa* consists of 43 coordinately regulated genes that encode components of the needle-like secretion apparatus, a translocon, and factors that regulate secretion [15, 16]. T3SS secretion apparatus is sometimes known as the "molecular syringe", whose secretion apparatus exports toxins across the bacterial cell envelope. The translocon of the T3SS is responsible for injecting these toxins into the host cell [10]. The T3SS can be divided into several parts: I. Proteins that structurally build the T3SS molecular syringe. II. Proteins (cytotoxins) that are translocated into host cells. III. Chaperones that help the secretion of their partners. IV. Proteins that regulate the T3SS expression and the secretion process [17]. The T3SS apparatus is structurally complex. More than twenty distinct proteins assemble into a type of molecular syringe: two rings (also known as the bases) embedded into inner membrane and outer membrane, respectively; a connector component to link the two rings and a needle component outside of the membrane to inject potent cytotoxins (effectors) directly into host cells. (Figure 1.1) Thirty-six genes that are involved in the synthesis and regulation of the T3SS are encoded from five operons clustered together in *P. aeruginosa* chromosome. The structural components of the T3SS apparatus are broadly conserved among the different pathogens [11].

The nature and function of translocated cytotoxins vary so as to fit the need of various organisms. The T3SS of *P. aeruginosa* translocates four known cytotoxins (ExoS, ExoT, ExoU, and ExoY) into host cells [18]. ExoS has been found to be a bifunctional protein have two functional domains: the amino-terminal domain, when expressed in host cells, results in the disruption of actin filaments in a dose-dependent manner, while expression of the carboxyl-terminal domain of ExoS in eukaryotic cells leads to the ADP-ribosylation of Ras and uncoupling the Ras-mediated signal transduction [19, 20].

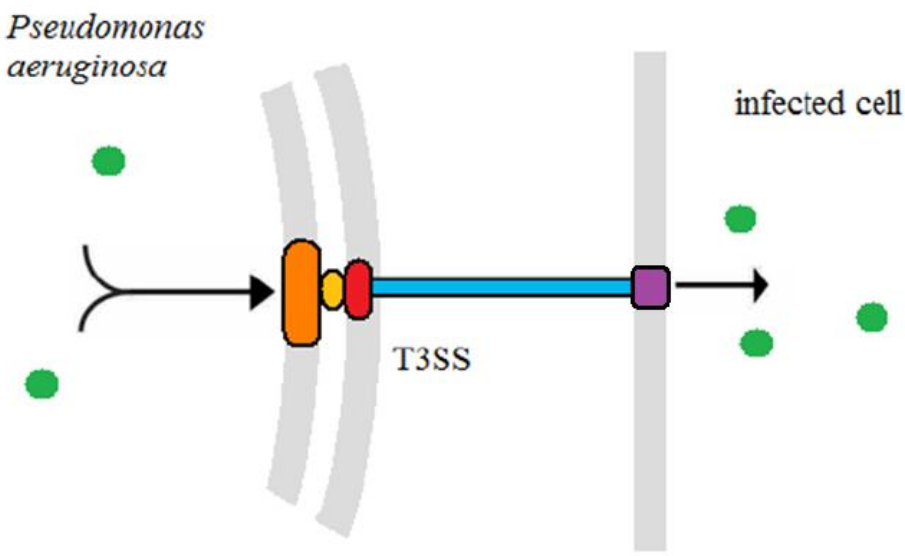

Figure 1.1. The T3SS apparatus. When attached to a eukaryotic host cell, the T3SS apparatus translocates cytotoxins directly into the host cell, which cause disruption of a range of signaling events in the host cell.

The GAP domain of ExoS targets Rho, Rac, which are small GTPases that function to maintain the cytoskeleton and cell shape. By trapping the Rho and Rac GTPases in the inactive GDP-bound form, the host cell actin cytoskeleton is disrupted [21]. The ADPRT domain of ExoS targets host cell factors that are involved in cell cycle progression, protein trafficking and apoptosis [19]. Following the infection of *P.aeruginosa*, ExoS has several adverse effects on the host cell, including cell death, cytoskeleton disruption, and reduction of cell-cell adherence, which are involved in the capacity of *P. aeruginosa* to interfere with epithelial wound healing [22]. ExoT has a 76% primary amino acid identity with ExoS, but is encoded by a different gene in the same operon. Transient intracellular expression of ExoT in eukaryotic cells causes rounding and actin reorganization, and it has also been shown that ExoT is a GTPase-activating protein for RhoA, Rac1, and Cdc42, which are factors involved in actin organization, exocytosis, cell cycle regulation and phagocytosis [23]. ExoU is found to be a phospholipase, when transiently expressed in eukaryotic cells, it correlates with a loss in eukaryotic cell viability by causing a disruption of lipolytic activity, which is indispensable to cellular metabolism as it affects biogenesis of membranes [24]. Injection of ExoU in yeast causes visible

membrane damage on different organelles and fragmentation of vacuoles, and injection of ExoU in mammalian cells results in irreversible cellular membrane damage and rapid cell death [25]. ExoU is likely to be involved in acute infection of *P. aeruginosa*, as it is found that an ExoU knockout strain no longer causes acute cytotoxicity [26]. ExsY has been found to be an adenylate cyclase which requires activation by calmodulin in the eukaryotic cells. Infection of CHO cells with ExoY-producing strains of *P. aeruginosa* resulted in the intracellular accumulation of cAMP [27]. In sum, the cytotoxins delivered into host cells by *P. aeruginosa* through T3SS can trigger a range of events including a rearrangement of the actin cytoskeleton, disruption of cell signaling and apoptosis, causing epithelial damage and bacterial dissemination, which are thought to facilitate the infection.

1.3 The master regulator of Type III Secretion System - ExsA

The AraC-type transcription factor ExsA is the master regulator that controls the expression of all T3SS-related genes [28, 29]. AraC-family transcriptional regulators control a range of cellular processes, including the regulation of T3SS expression in a number of other pathogens [30, 31]. These proteins are characterized by their conserved AraC domains that interact with specific DNA sequences near the -35 promoter region [32] and facilitate the recruitment of RNA polymerase to the transcription initiation site [33-35]. The regulation of AraC-type transcription factors varies greatly. Most of proteins in the AraC family are regulated by small molecule ligands that bind to the amino terminal domain of the protein. For instance, AraC is a transcription regulator that controls the expression of genes required to uptake and catabolize arabinose. The structure of AraC N-terminal domain has been solved so it is possible to explain the behavior of AraC protein [36]. The AraC protein consists of an N-terminal regulatory domain and a C-terminal DNA binding domain. The two domains are functionally independent as they retain their functions when fused to other proteins. The N-terminal domain of AraC contains an anti-parallel eight-stranded β barrel which is linked to two anti-parallel α helices, each about 20 amino acid residues in length. Arabinose is the

ligand for AraC that controls its DNA binding behavior [37]. The arabinose binding site is located in the pocket formed by the β barrel. AraC functions as a dimer. The dimerization site of AraC protein lies in the α helices [38]. AraC has alternative ways to bind DNA, depending on whether it is arabinose-bound or not. With arabinose bound to the N-terminal regulatory domain of AraC, two AraC molecules form a dimer and bind to the adjacent I1 and I2 sites on the promoter, allowing for the transcription from the *pBAD* and *pC* operons [38]. However, in the absence of arabinose, the AraC dimer adopts a different conformation and binds to I1 and O2 sites on the DNA, which are widely separated. As a result, the DNA forms a loop and the transcription from *pBAD* and *pC* operons is inhibited. (Figure 1.2)

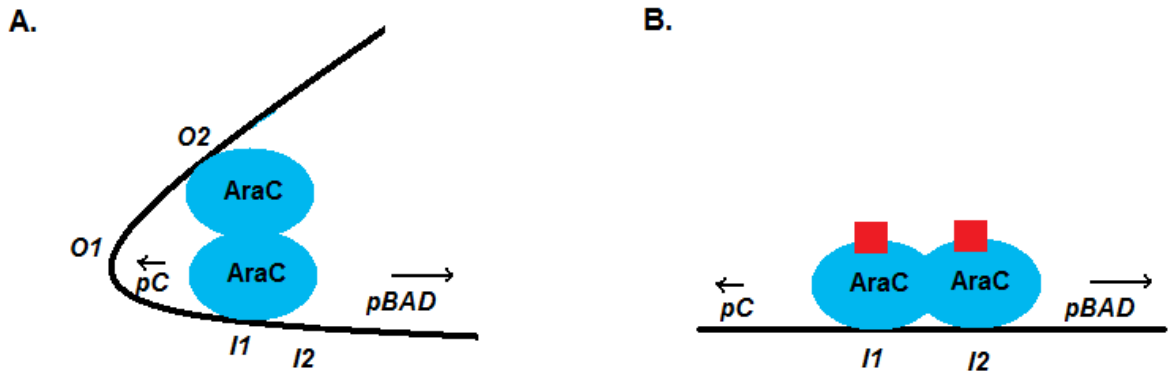

Figure 1.2. A. in the absence of arabinose, AraC dimer represses transcription by forming a DNA loop. B. in the presence of arabinose, the AraC dimer activates transcription.

Interestingly, ExsA and several other regulators of T3SSs constitute a distinct sub-family that is regulated through protein-protein interactions. Other examples include the functional ortholog of ExsA in *V. parahaemolyticus*, InvF in *S. enterica*, and MixeE in *S. flexineri* [39, 40]. However, this does not exclude the possibility that proteins in this sub-family can also associate with carbohydrates like AraC.

ExsA constitutes a particularly attractive target for the development of novel therapeutic options because it belongs to the AraC family of proteins, which is only represented in bacteria and fungi but not in higher eurkaryotes. Drugs designed to target

ExsA are supposed to repress the virulence of *P. aeruginosa,* and may have no side effects on normal functions of other factors in human cell. Thus, a detailed understanding of ExsA's structure, function and regulation will form the foundation for future drug development efforts.

ExsA protein consists of an ~100 amino acid carboxy-terminal domain (CTD) and a ~200 amino acid amino-terminal domain (NTD). The two domains are connected by a flexible linker. The CTD of ExsA contains two helix-turn-helix DNA binding motifs and has been shown to bind a range of T3SS gene promoters and recruit the RNA polymerase component σ^{70} to initiate transcription [41]. The NTD of ExsA is known to have the functions of oligomerization and ligand binding, which is similar to the functions of AraC N-terminal domain. ExsA controls T3SS genes by directly binding to the promoters and activating the transcription of all T3SS genes. All ExsA-dependent promoters contain hexamers that match the consensus -35 and -10 region of σ^{70}-dependent promoters. Compared to the normal 17 bp spacing between the -35 and -10 regions, it is about 21~22 bp for ExsA-dependent promoters, and the spacing is critical for ExsA-dependent transcription. Two ExsA binding sites are upstream of the T3SS genes. An alignment of ExsA-dependent promoters revealed that the ExsA consensus binding site is located around the conserved guanine and cytosine at -47 and -45 positions [42]. Further determinants for ExsA binding include a conserved adenine-rich region centered at the -51 position and several highly conserved nucleotides around the -35 region. The data suggest that there are two distinct ExsA binding sites, one overlapping the -35 region and the other binding site at the adenine-rich position. This finding is confirmed by the nucleotide substitution assay which shows a significant decrease of ExsA-dependent transcription when the nucleotides in the -35 region and -55 region are replaced [40].

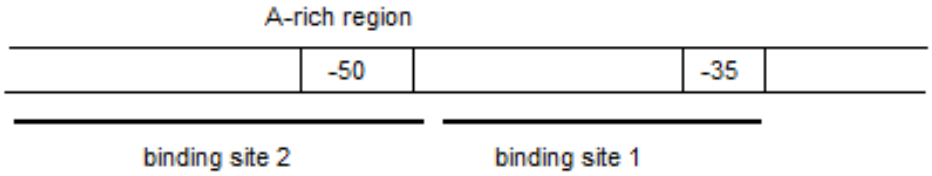

Figure 1.3. The ExsA-DNA binding sites on ExsA-dependent promoters. The binding site 1 overlaps with the -35 consensus and the binding site 2 overlaps with the adenine-rich region upstream of binding site 1.

The ExsA CTD alone can bind ExsA-dependent promoters, shown in the electrophoretic mobility shift assay (EMSA), but the affinity is about five-fold lower than ExsA total protein. In another nucleotide substitution assay, ExsA was shown to bind preferentially to the -35 region, as the substitution assay shows that nucleotide substitutions in the -55 region do not affect the ExsA-DNA binding while substitutions in -35 region almost completely eliminate the binding [42]. By further quantification of the shifts in EMSA, it has been found that ExsA preferentially binds to the first binding site (-35 region), then it recruits another ExsA to the the second binding site (-55 region). Although ExsA CTD can bind to the the first binding site, it fails to recruit another ExsA, as it does not have the NTD for ExsA-ExsA interaction. In the T3SS gene expression assay, both ExsA and ExsA CTD can activate the transcription of the reporter gene, while ExsA NTD cannot as it lacks the DNA binding domain [43]. Thus, the current model of ExsA-dependent transcription activation is: when T3SS is induced, one ExsA binds to the first binding site (-35 region) of the promoter, dimerizes with another ExsA and recruits it to the second binding site (-55 adenine rich region) of the promoter. The ExsA dimer then recruits RNA polymerase to the promoter to initialize transcription. ExsA dimerization leads to a higher affinity for σ^{70} but is not required, since an ExsA monomer alone can also activate transcription of ExsA-dependent genes albeit at a lower efficiency [43].

1.4 Regulation of Type III Secretion System

The expression of the T3SS is under organized regulation as it is highly energy-consuming. The transcriptional activator ExsA is regulated through protein-protein interactions. The most studied signal cascade is the ExsA-ExsC-ExsD-ExsE (ExsACDE) feedback loop that associates the activation of ExsA directly with the activation of the T3SS apparatus. The genes that encode the four regulatory proteins ExsA, ExsD, ExsC and ExsE are distributed over two operons. *exsA*, *exsC*, and *exsE* are all found in one operon, while *exsD* is co-expressed with eleven structural components of the T3SS [44].

ExsD is a 31.4 kD protein that acts as an anti-activator in T3SS regulation. ExsD can bind and inhibit ExsA [45]. Instead of blocking the DNA binding sites on ExsA-dependent promoters, the binding of ExsD results in the reduced DNA binding activity of ExsA, as EMSA results show a significantly reduced DNA binding activity of ExsA when it is bound to ExsD. Also, in an *in vitro* transcription assay, adding ExsD results in direct inhibition of ExsA-dependent transcription. ExsA and ExsD, when co-purifed from *E.coli,* are co-eluted from a gel filtration column [46]. Analytical ultracentrifugation data suggest ExsD forms a 1:1 complex with ExsA to inhibit the transcriptional activity of ExsA [46]. ExsD can also oligomerize in the cells. The ExsD trimer was found to be less efficient in inhibitory functions than a monomer [46]. Thus, there exists equilibrium between ExsD monomer and trimer, which is thought to be another regulatory strategy. The structure of ExsD was recently determined by our lab. Surprisingly, ExsD is found to resemble the structure of KorB, a DNA binding protein that acts as a transcription repressor. This suggests that ExsD may have functions other than binding to ExsA and inhibiting its transcription activity. However, it remains unknown what the function of ExsD is as a DNA binding protein [45, 47].

ExsC is a 16 kD protein that acts as an anti-anti-activator in the T3SS regulation. ExsC can bind ExsD and form a 2:2 complex [48]. Also, ExsC functions as a T3SS chaperone by forming a 2:1 complex with ExsE, a secreted protein that prevents ExsC

from binding to ExsD, when the T3SS is "off" [48]. No other functions have been found for ExsE.

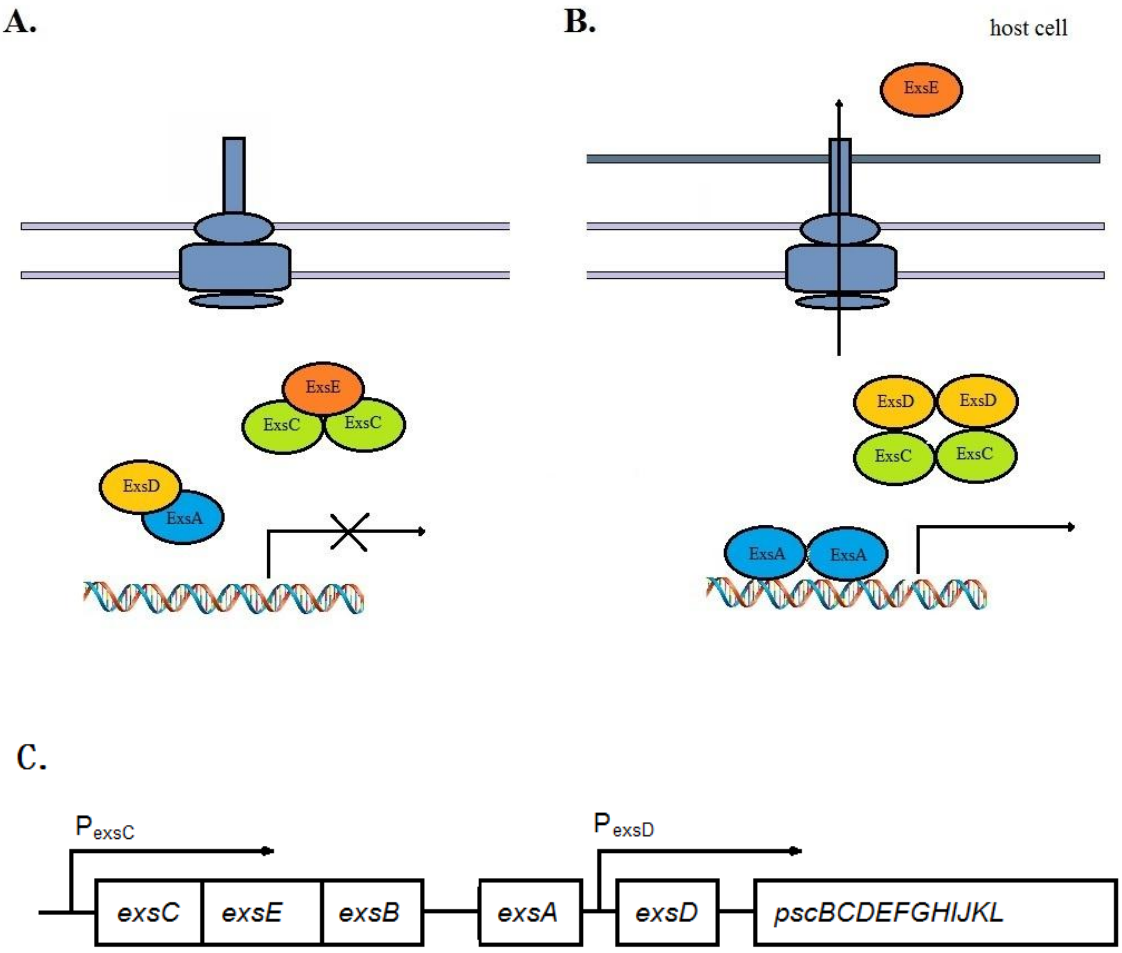

Figure 1.4. A, B. ExsA, ExsD, ExsC, ExsE's interactions when host cell is/is not attached. C. Depiction of the gene arrangement of *exsA*, *exsC*, *exsD*, and *exsE* in the *P. aeruginosa* genome. *exsC*, *exsE*, and *exsA* are co-transcribed, while *exsD* is the first gene in an operon that also encodes eleven structural components of the T3SS.

T3SS is normally suppressed under non-permissive conditions, such as high Ca^{2+} or not being attached to eukaryotic cells [49]. At this stage, ExsC preferentially binds ExsE, and ExsD binds ExsA and inhibits ExsA-dependent transcription, thus the expression of T3SS is "off". Under inducing conditions (such as low Ca^{2+} or contact of *P. aeruginosa* with eukaryotic cells), the activation of T3SS apparatus results in the translocation of

ExsE from *P. aeruginosa* to host cells, and ExsC is released by ExsE. ExsC then binds and sequesters ExsD to liberate ExsA, which in turn activates the expression of T3SS genes [44]. As ExsA also activates the expression of *exsC, exsE and exsD*, the products will change the cellular concentration of ExsC, ExsE, and ExsD, which regulate the ExsA-dependent transcription. In sum, ExsA regulates the T3SS via a feedback loop together with three other regulatory proteins.

Although the ExsACDE model is widely accepted, there are many details that are not included into the model. For example, the ExsD trimer is thought not to be able to bind ExsA so there may be distinct stable steady states between ExsD monomer and ExsD trimer when *P. aeruginosa* is induced or not. (Figure 1.5)

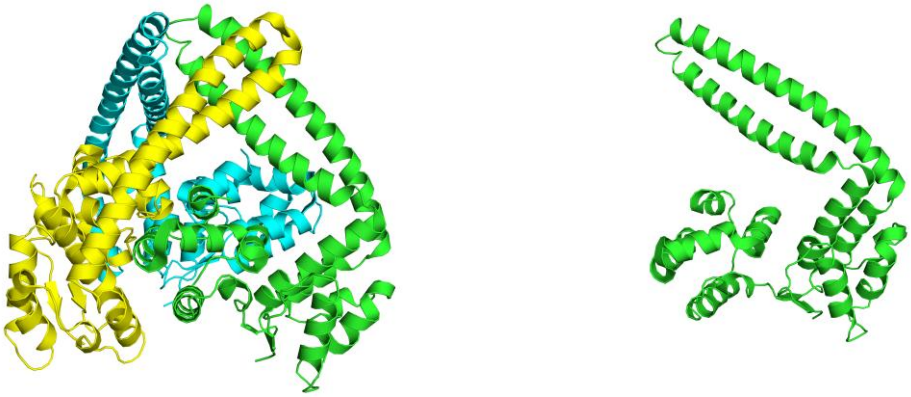

Figure 1.5. ExsD trimer (left) and monomer (right).

Another problem with the current ExsACDE model is the lack of quantitative data. The concentrations of ExsA, ExsD, ExsC, and ExsE in the cell have not been measured thus there is no mathematical model available for the ExsACDE feedback loop to describe the existing equilibrium states under inducing or non-inducing conditions. An experiment has shown that ExsD has a higher affinity for ExsA when both of them are co-expressed in the cell. When they are purified separately and mixed, the affinity between ExsA and ExsD is much lower [44]. This result suggests that ExsD may bind to ExsA during translation. Furthermore, we would also like to know the concentration of ExsA and ExsD in a cell. As we do not know the turnover rate of ExsA or ExsD for now,

we cannot answer the question whether ExsD can facilitate the degradation of ExsA in cell, in which case ExsD should exist in low concentrations in the cell.

The current ExsACDE model does not consider the effects of other important parameters which may affect the regulation T3SS. Most *in vitro* experiments on T3SS regulation were performed under 30^{o}C. However, based on the result from the *in vitro* ExsA-dependent transcription assays, the transcription activity of ExsA is different at 30^{o}C and 37^{o}C. As *P. aeruginosa* targets warm-blooded hosts, it is of great importance to study the regulation of T3SS under 37^{o}C, which is the body temperature for humans. It is possible that the turnover rate of ExsA may increase when the environmental temperature goes from 30^{o}C to 37^{o}C, which may affect the overall landscape of T3SS expression.

1.5 Purpose

The specific aims of this project were to gain a deeper understanding of T3SS regulation, within which the T3SS master regulator ExsA was the focus of the study. ExsA N-terminal domain protein was purified and crystallized and its structure was solved in order to study the function and protein-protein interactions of ExsA. With the structure of the ExsA N-terminal domain as the basis, we found the dimerization interface of ExsA. Based on the structure alignments, we found homologous proteins, which provide information about how ExsA could be regulated (Chapter 2). Also, we constructed several ExsD variants based on the structure of ExsD and tested them in the ExsA-dependent *in vitro* transcription assay, in order to find the ExsA-ExsD interaction interface (Chapter 3). Finally, the cellular concentrations of ExsA and ExsD were measured in order to obtain parameters that would help build the mathematical model of ExsACDE regulatory pathway (Chapter 4).

Chapter Two: Structure of the ExsA N-terminal domain

2.1 Introduction

The experimental (structural and molecular analysis) part of this project was driven by three specific aims. First, ExsA N-terminal domain (ExsA NTD) protein was purified and crystallized for X-ray diffraction analysis, upon which the structural model of ExsA NTD was solved. As the locations of the ExsA-ExsA and ExsA-ExsD interfaces are not known, understanding the molecular basis for these interactions will be invaluable for the development of small molecules that mimic the behavior of ExsD to suppress the virulence of *P.aeruginosa*. The AraC-type transcription factors regulate virtually all aspects of bacterial gene expression, but these proteins are notoriously difficult to crystallize, as a result of their low solubility and temperature sensitivity. While the overall structure of the DNA-binding domain of ExsA can be easily predicted, no structural information exists for the regulatory domain. Therefore, critical questions such as the location of the dimer interface and the identity of residues involved in regulator binding have been difficult to answer. Further, it is also unclear how ExsA recruits the RNA polymerase to the promoters. The structure model of ExsA NTD will help reveal the answers for some of these questions.

2.2 Material and Methods

2.2.1 ExsA N-terminal domain preparation

The gene encoding ExsA with a TEV protease cleavage site upstream of the 5' end was amplified from *P. aeruginosa* genomic DNA and introduced into the pDEST-His-MBP expression plasmid via Gateway cloning technology (Invitrogen). The recombinant plasmid expressed residues 2–278 of the wild-type ExsA protein sequence without modifications. *Escherichia coli* BL21 (DE3) cells were transformed with the plasmid and induced with 1mM IPTG at an OD600 of 0.6. The cells were incubated for 12 h at 18^{o}C,

harvested by centrifugation and the pellet was stored at -80 ^{o}C. Thawed cells were lysed by sonication in binding buffer (500 mM NaCl, 25 mM imidazole pH7.4, 50 mM Tris-HCl pH7.4, 2 mM DTT), containing 1 mM of PMSF. The cell lysate was clarified by centrifugation at 15000g for 1 hour at 4^{o}C and applied onto a 30 ml HisTrap Ni-NTA column (GE Healthcare) (wash buffer: 150 mM NaCl, 25 mM imidazole pH7.4, 50 mM Tris-HCl pH 7.4, 2 mM DTT. elution buffer: 150 mM NaCl, 250 mM imidazole pH 7.4, 50 mM Tris-HCl pH 7.4, 2 mM DTT). The eluate was then applied to an anion exchange column (wash buffer: 50 mM NaCl, 25 mM Tris-HCl pH 7.4, 2 mM DTT elution buffer: 1 M NaCl, 25 mM Tris-HCl pH 7.4). TEV protease was added to cleave the His_6 Maltose binding protein tag. The product ExsA is further purified using a HiTrap Ni-NTA column (wash buffer: 500 mM NaCl, 25 mM imidazole pH 7.4, 50 mM Tris-HCl pH 7.4, 2 mM DTT. elution buffer: 500 mM NaCl, 250 mM imidazole pH 7.4, 50 mM Tris-HCl pH 7.4, 2 mM DTT). ExsA full-length protein was digested by Thermolysin (20 μg ml^{-1}) at 30^{o}C for 1 hour, leaving only the N-terminal domain (verified by mass spectrometry). The reaction was then quenched by adding 2mM $CaCl_2$. The product was applied onto a Superdex260 gel-filtration column (GE Healthcare) (buffer: 500 mM NaCl, 25 mM Tris-HCl pH 7.4, 2mM TCEP). The purified ExsA N-terminal domain protein was concentrated to 1.5 mg ml^{-1}. The selenomethionine-substituted ExsA (SeMet ExsA) was produced using the saturation of the methionine biosynthetic pathway protocol [50] and subsequently treated in the same manner as the original ExsA to yield ExsA NTD. To determine whether the purified ExsA NTD was properly folded, it was applied to the ExsA-dependent *in vitro* transcription assay as an inhibitor. The ExsA-dependent *in vitro* transcription assay is described in Cory Bernhard's work [46].

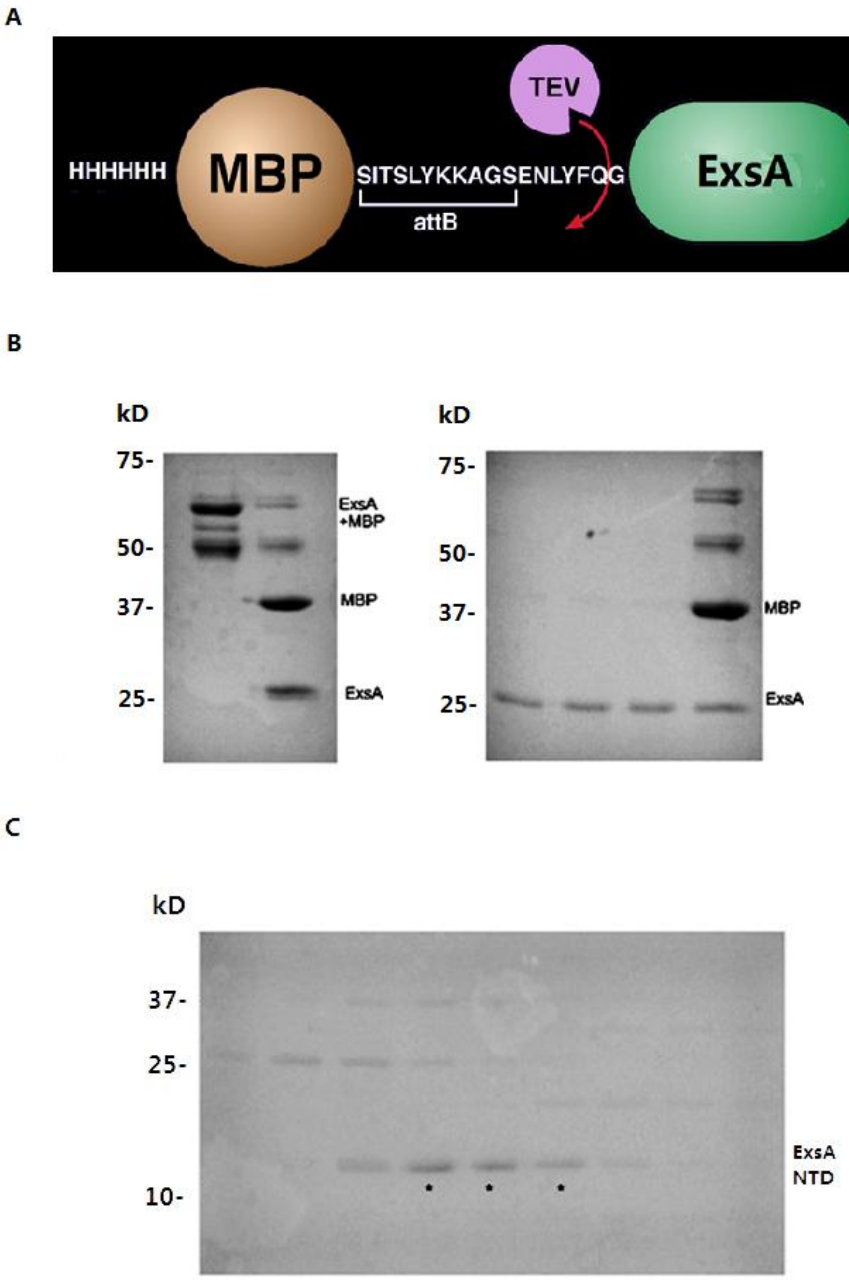

Figure 2.1. A. ExsA-MBP-His_6 recombinant protein and TEV cleavage site. B. ExsA-MBP-His_6 recombinant protein and ExsA & MBP-His_6 after TEV cleavage (left gel). ExsA and MBP-His_6 were separated by passing through a Ni^{2+} column (right gel). The flow through contained ExsA and the eluate contained His_6-MBP. C. After limited proteolysis, ExsA N-terminal domain (ExsA NTD) was generated and further purified by passing through a gel filtration column.

As only properly folded protein can yield crystals, we wanted to show the purified ExsA NTD protein is properly folded and functional. A titration of ExsA NTD protein in the ExsA-dependent *in vitro* transcription assay is done to show that the ExsA NTD protein can inhibit ExsA dependent transcription in a dose-dependent manner, which indicates that the purified ExsA NTD protein competes for the dimerization site of full-length ExsA, resulting in a reduced transcription activity as the DNA binding motif is located in the C-terminal domain of ExsA. The result indicates the ExsA NTD protein is properly folded and functional. (Figure 2.2)

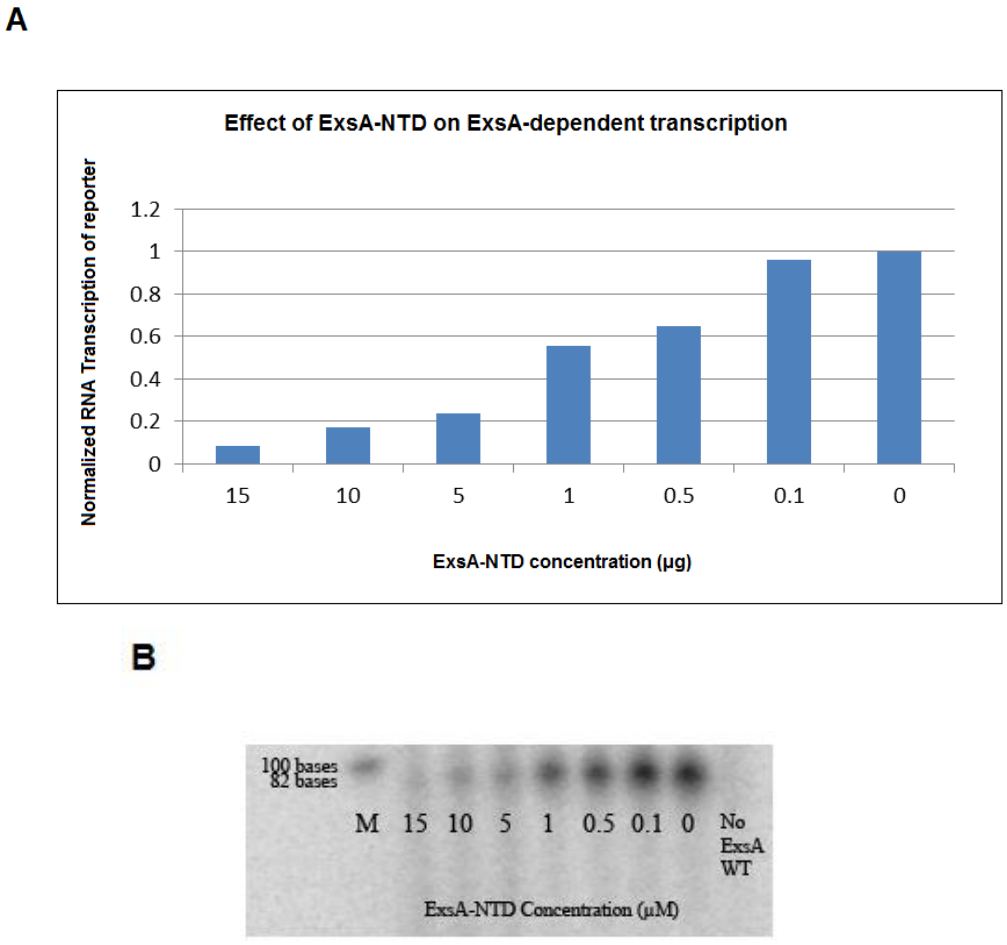

Figure 2.2. A. Titration of ExsA N-ternimal domain in ExsA-dependent *in vitro* transcription. The reporter values were based on the density of radio-labeled mRNA transcript bands. B. The radio-labeled mRNA transcripts.

2.2.2 Crystallization and data collection

Crystallization of the ExsA NTD was performed using the hanging-drop vapor-diffusion method at 25^{o}C. (Figure 2.3) Crystals of ExsA NTD were obtained using a reservoir solution containing 1.6 M $MgSO_4$, 0.1 M MES pH 6.5 and 0.1 M EGTA. The crystallization droplet consisted of 3ul protein solution (1.5 mg ml^{-1} ExsA NTD protein, 500 mM NaCl, 25 mM Tris-HCl pH 7.4, 2 mM TCEP) and 1 µl reservoir solution. Rod-shaped crystals of ExsA NTD appeared after several days. SeMet ExsA NTD was crystallized under identical conditions. The crystals were flash-cooled in liquid nitrogen after soaking in a cryoprotection solution containing 90% reservoir solution and 10% (v/v) glycerol. X-ray data were collected at beamline X29A (National Synchrotron Light Source, Brookhaven National Laboratory) using an ADSC Q315 CCD detector.

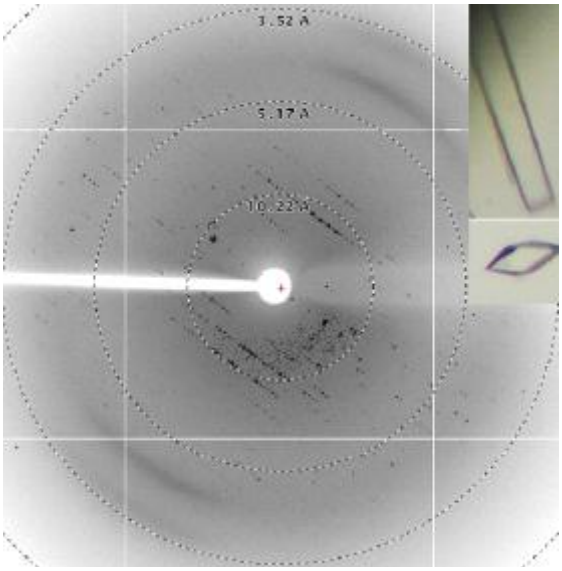

Figure 2.3. X-ray diffraction image and diffraction analysis of ExsA-NTD crystals.

2.2.3 Structure determination and refinement

The X-ray diffraction data were processed using the XDS program package. Initial phases for SeMet ExsA N-terminal domain were obtained by Phaser using the multiple

anomalous dispersion (MAD) [51]. Model building was performed using COOT [52], and Phenix program suite was used for structure solution and refinement [53].

Data-collection statistics	
Space group	$P4_32_12$
No. of moledules in asymmetric unit	2
Uni-cell parameters (Å)	a=69.820 b=69.820 c=191.580 α=90.00 β=90.00 γ=90.00
Mosaicity	0.4
Resolution range (Å)	65.82-2.9
Observed reflections	209145
Unique reflections	11232
Average multiplicity	18.6
Completeness(%)	100.00%
Rmerge	0.105
$< I/\sigma(I)>$	19.5
Refinement statistics	
Resolution range (Å)	65.599-2.9
No. of reflections	11125
R-work	0.2367
R-free	0.2785
R.m.s. deviations	
Bond lengths(Å)	0.004
Bond angles(^{o})	0.822
Ramachandran plot(%)	
Favored	90.27
Allowed	9.4
Outliers	0.34

Table 2.1. Data collection and refinement statistics for ExsA N-terminal domain.

2.3 Results and Discussion

2.3.1 Characterizing of the structure of ExsA N-terminal domain

We have solved the 2.9 Å resolution crystal structure of ExsA N-terminal domain. The crystal contains two ExsA dimers per asymmetric unit. The ExsA N-terminal domain

contains an eight-stranded antiparallel β barrel (β1- β8). The length of the β strands varies but together they form the barrel with an open end. The β barrel is followed by a linker that has a long α helix (α1) followed by another β strand (β9). β9 is then followed by 3 α helices (α2 – α4), α1 and α2 are approximately 15 amino acid residues in length, while α4 is shorter and the amino acid residues following α4 are disordered. The three α helices are packed against the surface of the β barrel. (Figure 2.4)

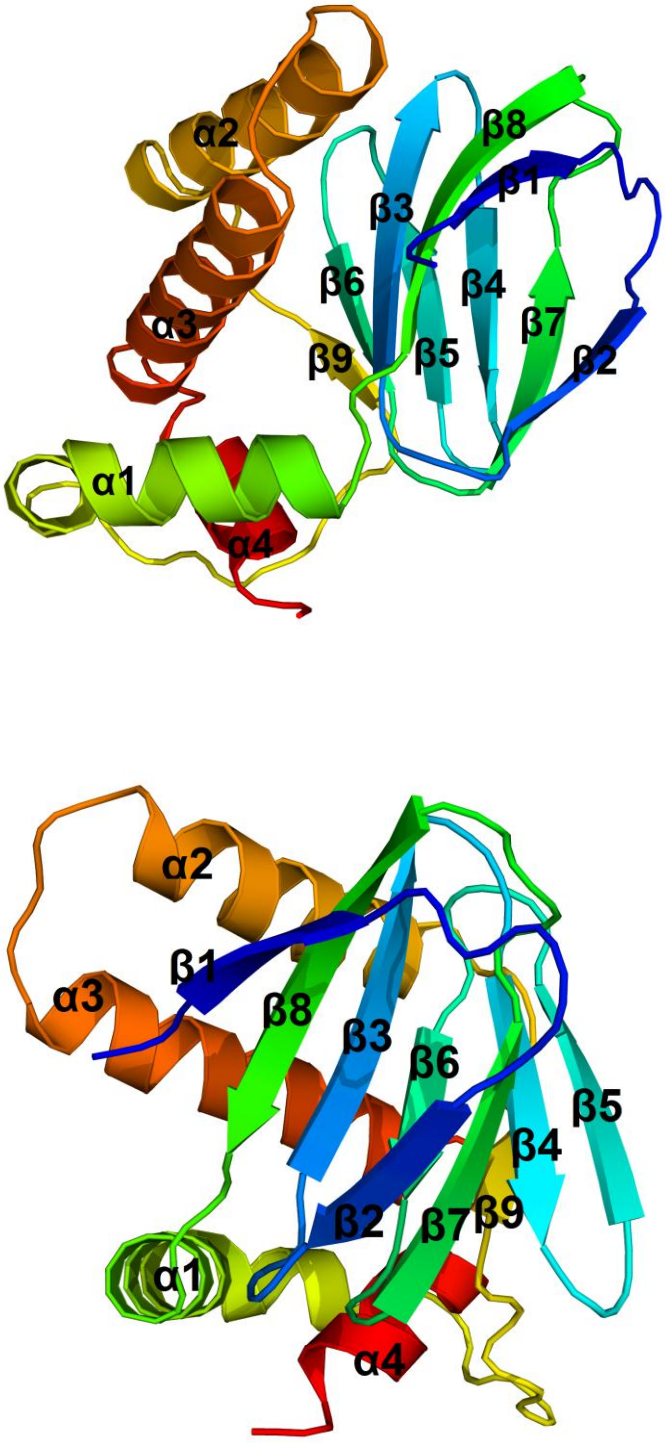

Figure 2.4. Structure model of ExsA NTD amino acids 10-165. The bottom one is the top model rotated left by 90°. The models are in rainbow colors, where purpose stands for the N-terminus and red stands for the C-terminus.

2.3.2 Characterizing the ExsA dimer

In the crystal there are two ExsA N-terminal domain molecules. Using the 2-fold symmetry of the crystal the two independent molecules may be used to generate two slightly distinct dimers that each might represent the biological dimer of ExsA. In both dimers two α helices (α2 and α3) of one molecule interact with same helices from the other molecule in reversed orientation, forming a four-helical bundle at the interface. While the sequence similarity between the regulatory domains of ExsA and AraC is very low the overall folds are quite similar as discussed below. Interestingly, the arrangement of the two ExsA NTD dimers closely resembles that observed for a dimer of the regulatory domain of AraC (PDB code: 2ARC), with the two pairs of α helices acting as the dimerization interface. (Figure 2.5)

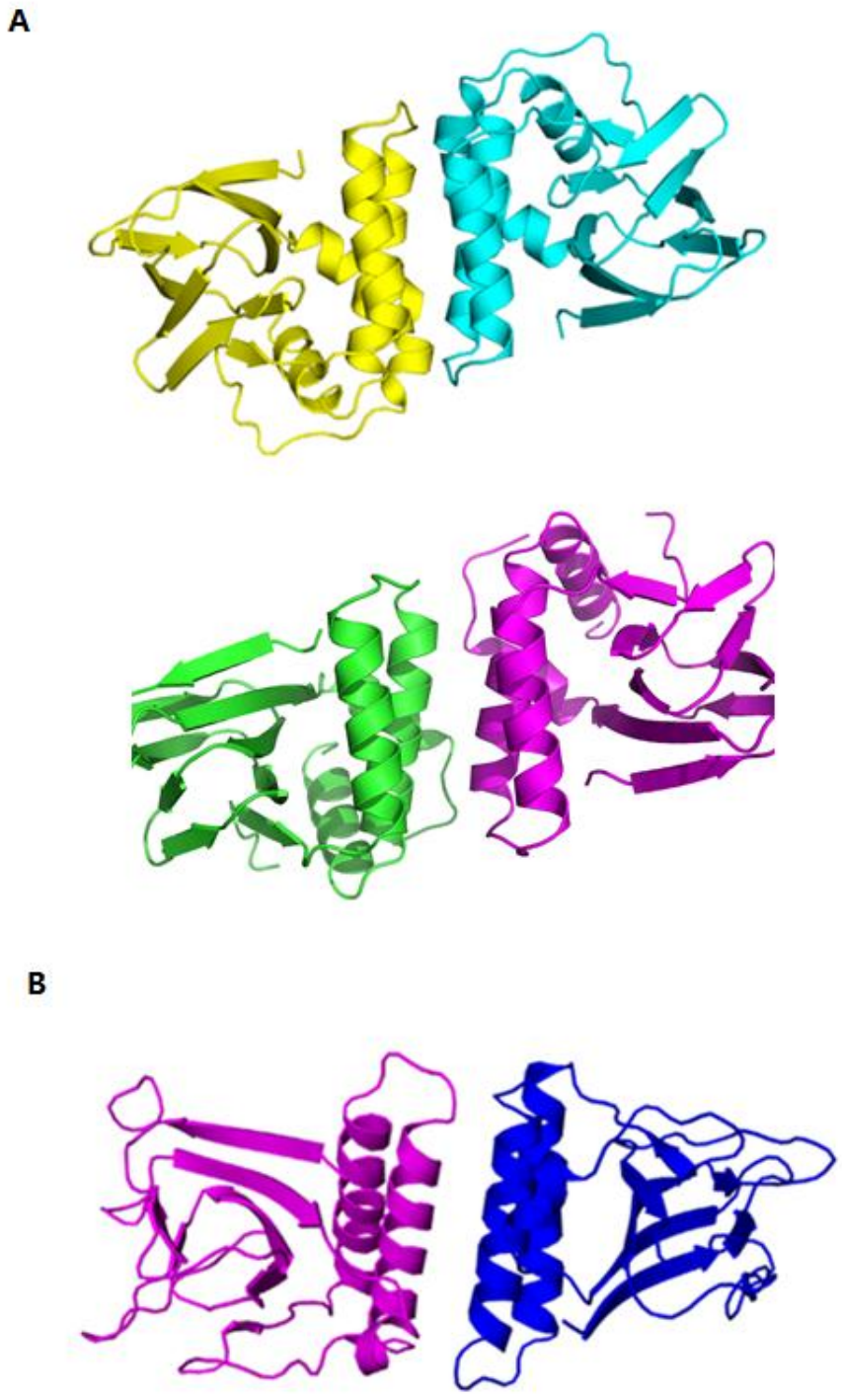

Figure 2.5. A. The two ExsA N-terminal domain dimer found in the crystal. B. AraC N-terminal domain dimer.

One of the most important goals is to find the amino acid residues that are involved in dimerization of ExsA. Although the two ExsA dimers found in the crystals resemble each other, there are still differences. In order to find the region of amino acid residues that is important in the dimerization, we superimposed two chains (A and C) from the different dimers found in the crystal, and examined the spatial position of the other chains (B and D). Although we can see a shift between chain B and D, the region of chain B and D that does not shift much is amino acid residues 134-144 (red region). (Figure 2.6)

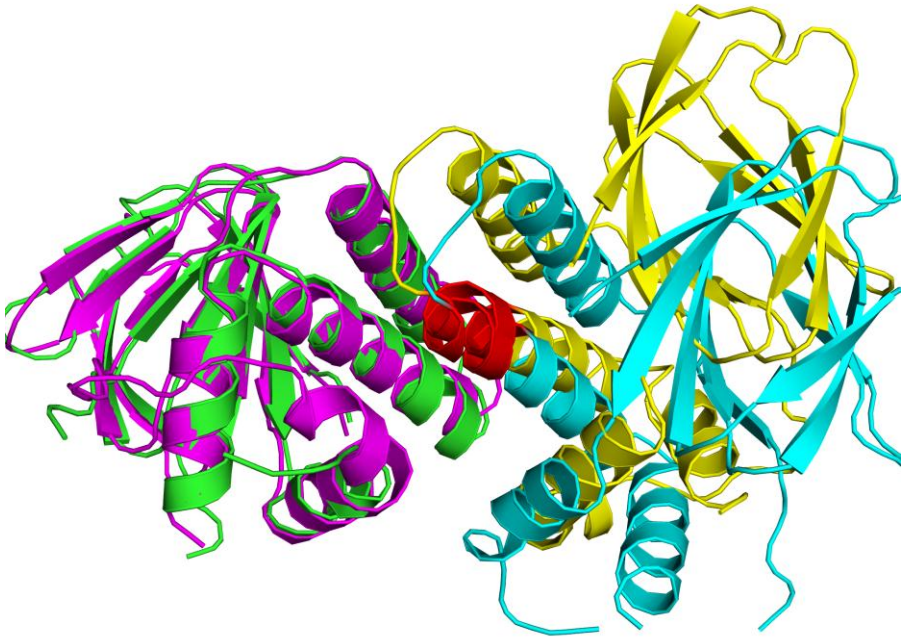

Figure 2.6. Superposition of ExsA N-terminal domain dimers in the same asymmetric unit. Green and purple chains are A and B, Yellow and cyan chains are C and D. The red part specifies the most stable region where the key amino acid residues for dimerization are located (amino acid residues 134-144).

Based on the dimer interface in ExsA N-terminal domain structure, we have found the amino acid residues that interact with nearby residues. For the two dimers we found in the crystal, the interacting amino acid residues are mainly located in the range of

residues 135-151. The results obtained from two dimers overlap but one dimer has more amino acid residues involved in dimerization than the other, suggesting the dimerization of ExsA is dynamic. One interesting finding we have is the R91 on the α1 helix is involved in the dimerization of ExsA. I.t interacts with the R91 and E143 on the other molecule of ExsA dimer (Figure 2.7). Our collaborator Timothy Yahr (University of Iowa) has corroborated this interface demonstrating that several residues at this interface are indeed critical for ExsA dimerization (personal communication).

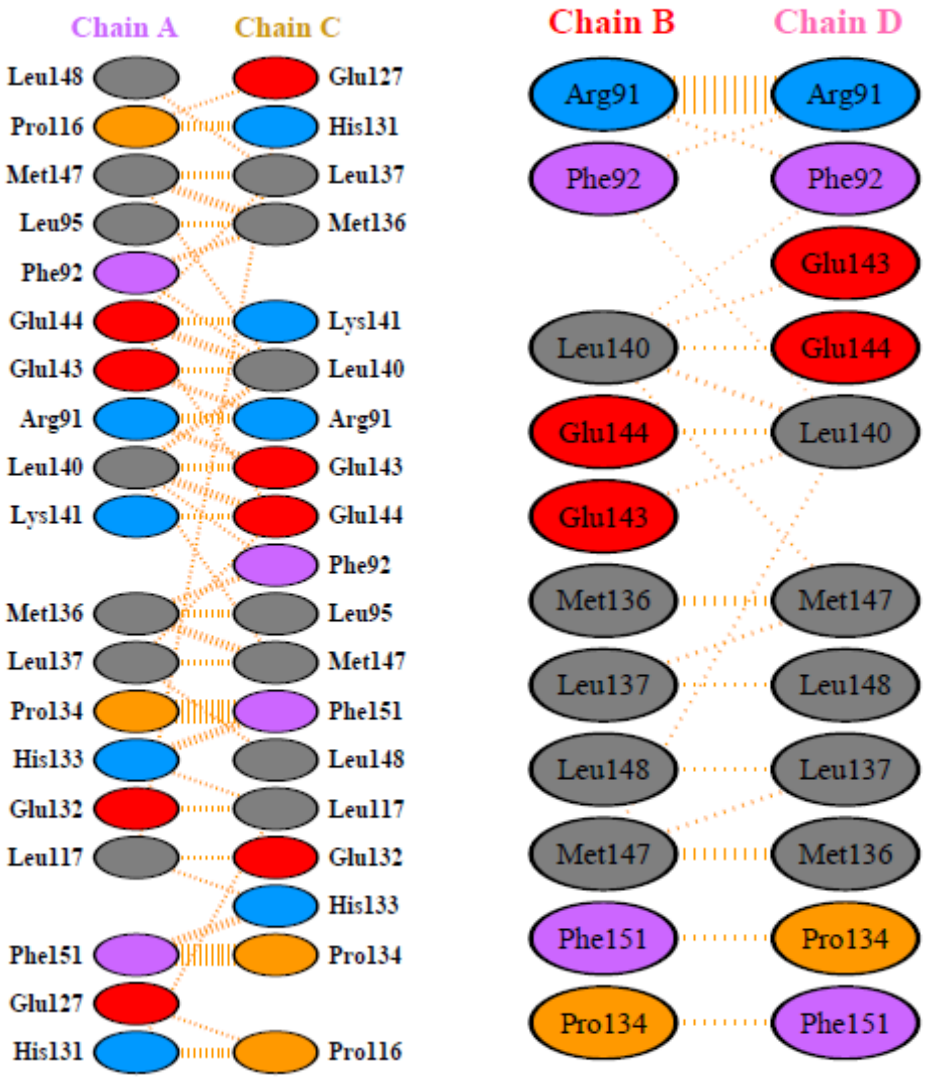

Figure 2.7. Amino acid residues involved in ExsA dimerization. A and C are two molecules of one of the dimers found in the crystal. B and D are two molecule of the other dimer.

2.3.3 Characterizing ExsA N-terminal domain homologues

Based on the structure of ExsA NTD, we would also like to know the interface of the ExsA and ExsD interaction. We expected that the homologues of ExsA might share some similarities not only in structure by also in function. In order to find structural homologues of ExsA NTD, we applied structure alignment to the ExsA NTD structure against structures in the online protein databank [54]. The structure alignment attempts to find similarities between two structures, based not on the sequence alignment but on the tertiary structure of the molecules, thus it can establish homology of proteins with low sequence similarity, where evolutionary relationships between proteins can be hardly detected by standard sequence alignment techniques. Structural alignment can therefore be used to imply evolutionary relationships between proteins that share very little common sequence [55]. The method we used for evaluating the similarity between proteins is Distance Aligned matrix method (DALI). DALI is a common and popular structure alignment method that breaks the input structures into hexapeptide fragments and calculates a distance matrix by evaluating the contact patterns between successive fragments [56]. Sequence alignment on ExsA does not yield any proteins with high similarity, but structure alignment on ExsA NTD structure on DALI server finds two similar structures, ToxT (PDB code: 3GBG, r.m.s.d 3.0) and AraC NTD (PDB code: 2ARC, r.m.s.d 3.3). Both ToxT and AraC belong to the AraC family. ToxT is a protein found in *V. cholera*, which is a Gram-negative bacterium that can cause an acute intestinal infection known as cholera. In addition, ToxT regulates two primary virulence factors of *V. cholera* [57]. AraC is the regulator of arabinose catabolism in *E. coli* [58]. (Figure 2.8)

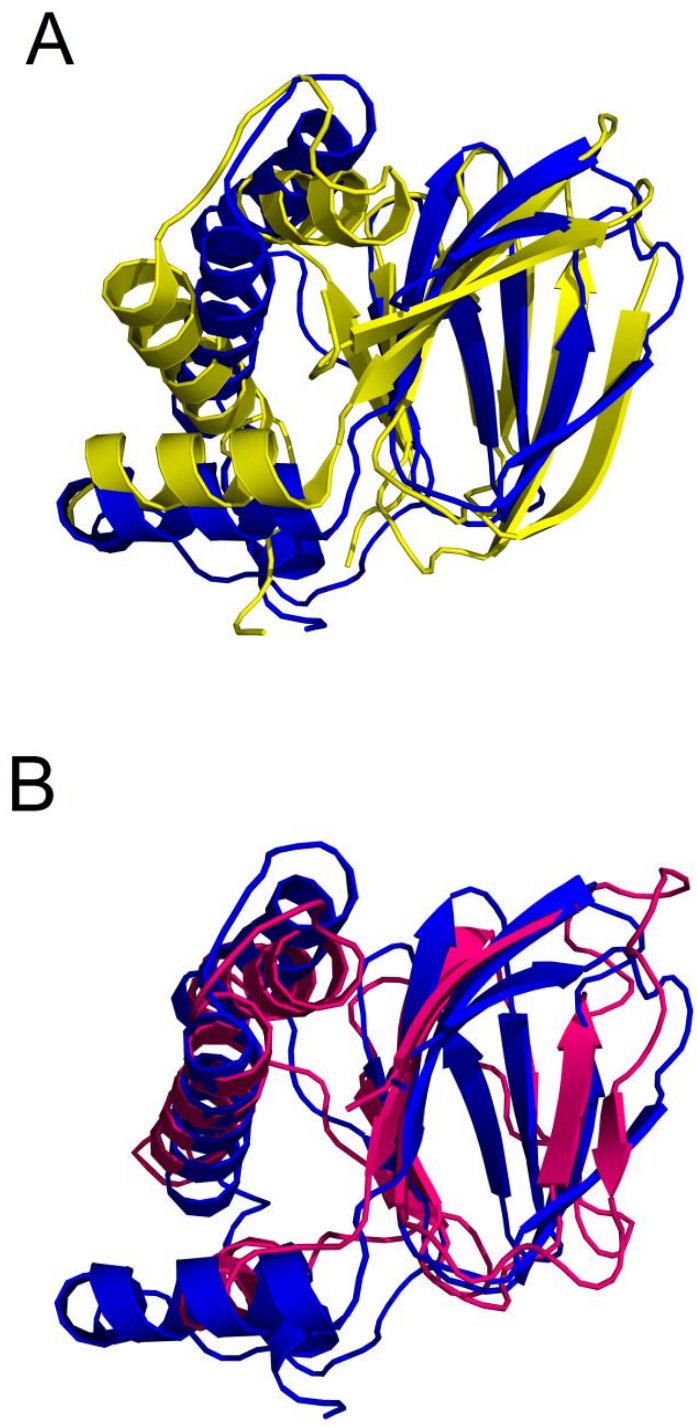

Figure 2.8. A. Superposition of ExsA N-terminal domain (blue) and ToxT N-terminal domain (yellow). B. Superposition of ExsA N-terminal domain (blue) and ToxT N-terminal domain (magenta).

From the structures we find that the N-terminal domain of ExsA resembles that of ToxT, and they are both similar to the structure of N-terminal domain of AraC. All three of the proteins contain the anti-parallel β barrel, and the shape and volume of the barrel are similar to one another. In AraC, the β barrel contains the binding site for arabinose, and there is an N-terminal arm (amino acid residues 1-10) to "cover" the "opening" of the barrel which is supposed to stabilize the binding of arabinose via hydrogen bonds. The N-terminal arm is not found in either ExsA or ToxT. The ToxT structure is found to be bound to a sixteen-carbon fatty acid cis-palmitoleate, which is found to be a repressor for ToxT. Y20, F22 and F33 on the N-terminal domain, as well as V261, M259, M269 and Y266 of the C-terminal domain of ToxT help stabilize the ligand in the pocket [59]. So far there is no evidence to show that ExsA can bind any small ligands, however, a close comparison between the binding pocket between AraC and ExsA reveals some conserved features. The R38, H80, Y82, H93 and W95 in AraC are the residues that form hydrogen bonds with arabinose directly and indirectly [38], while in ExsA, R25, N27, Y33, W17 and W77 are corresponding residues that are at the similar position in the space, which may also form hydrogen bonds with a ligand when it is bound (Figure 2.9). As for ToxT, K28 in ExsA is close to K31 in ToxT, which forms salt bridges with the negatively charged carboxylate head group of the fatty acid as well as K230 from the C-terminal domain of ToxT. However, the binding pocket of ExsA is occupied by several bulky side chains. Thus, it is less likely for ExsA to accommodate a large ligand such as a fatty acid. Another possibility is that the β barrel pocket is where the interaction between ExsA and its regulator ExsD occurs. ExsD may bind to the pocket and change the conformation of ExsA which in turn prevents it from activating transcription. Other than the β barrel, all three proteins contain two helices that are aligned anti-parallel. These two α helices are the interface for dimerization of ExsA and AraC with arabinose bound to it, while ToxT was found to be monomer in the crystal.

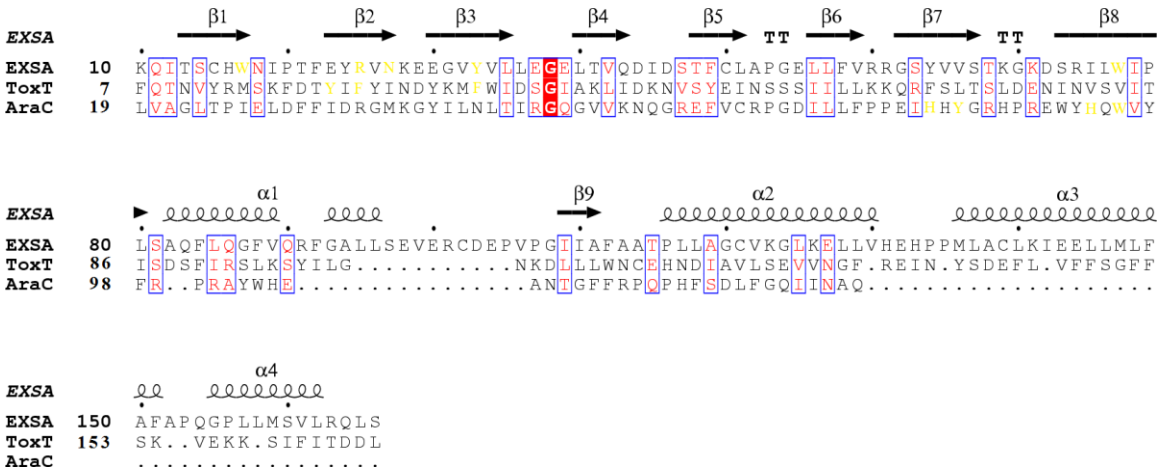

Figure 2.9. Structural-based sequence alignment of ExsA N-terminal domain with ToxT and AraC. Amino acid residues marked yellow are the ones involved in ligand binding (potential ligand binding residues for ExsA).

Chapter Three: Mapping the interface of the ExsA and ExsD interaction

3.1 Introduction

After we solved the structure of the ExsA NTD, we next asked where the interface the ExsA and ExsD interaction is. There are two potential binding sites on ExsD, one is the amino terminus of ExsD because we believe it to be overlapping or in the vicinity of the ExsC binding site. The other is the coiled-coil region of ExsD (amino acid residues 138-202), because such regions are frequently involved in protein-protein interactions. Having developed an ExsA-specific *in vitro* transcription assay, we tested a series of truncated ExsD variants for their ability to interfere with ExsA function. Using the ExsD structure as a guide, we generated two recombinant ExsD variant proteins, one of which is the two α helices (amino acid residues 138-202), and the other part is the rest of the ExsD with the gap of amino acid residues 138-202 bridged by four glycines.

3.2 Material and Methods

3.2.1 Preparation of ExsD coiled-coil region deleted variant

The ExsD coiled-coil deleted region (ExsD Δ138-202aa) gene is constructed via two rounds of PCRs. In the first PCR reaction, ExsD 1-137aa was amplified with primers forward: 5’-GTGGAGAACCTGTACTTCCAGGGTATGGAGCAGGAAGAC-3’, backward: 5’-C TTCGCCAGTGCCGATCCTCCTCCTCCCTGGTCGAGCAGGCT-3’, ExsD 203-276aa was amplified with primers forward: 5’-CGGGTCAACCTCGGAGGA GGAGGATCGGCACTGGCG-3’, backward: 5’- GGGGACAACTTTGTACAAGAAAG TTGCTCATA CTGGCAGAGCTGA-3’ from *P. aeruginosa* genomic DNA. At the end of ExsD 1-137aa and the beginning of the ExsD 203-276aa there was a short DNA sequence encoding 4 continuous glycines. The two products obtained from the first PCR were then used in the second PCR as templates to amplify the ExsD Δ138-202 with primers forward: 5’-GT GGAGAACCTGTACTTCCAGGGTATGGAGCAGGAAGAC-3’, backward: 5’-

GGGGACAACTTTGTACAAGAAAGTTGCTCATACTGGCAGAGCTGA-3'. The ExsD Δ138-202aa gene was then introduced into pDONR201 plasmid and later introduced into the pDEST-His-MBP expression plasmid via Gateway cloning technique. *E. coli* BL21 (DE3) cells were transformed with the plasmid and induced with 1mM IPTG at an OD600 of 0.6. The cells were incubated for 12 h at 18^{o}C, harvested by centrifugation and the pellet was stored at -80 ^{o}C. Thawed cells were lysed by sonication in binding buffer (500 mM NaCl, 25 mM imidazole pH7.4, 50 mM Tris-HCl pH 7.4, 2 mM DTT), containing 50 mM of PMSF. The cell lysate was clarified by centrifugation at 15000g for 1 hour at 4^{o}C and applied onto a HisTrap Ni-NTA column (GE Healthcare) (wash buffer: 150 mM NaCl, 25 mM imidazole pH 7.4, 50 mM Tris-HCl pH 7.4, 2 mM DTT. Elution buffer: 150 mM NaCl, 250 mM imidazole pH 7.4, 50 mM Tris-HCl pH 7.4, 2 mM DTT). The coiled coil region of ExsD was found to be unstable and most of it degraded during purification. However, the coiled-coil deleted region of ExsD was stable. TEV protease was added into the eluate to cleave the MBP-His_6 tag, and the product ExsD coiled coil deleted protein was further purified using a HiTrap Ni-NTA column (wash buffer: 150 mM NaCl, 25 mM imidazole pH 7.4, 50 mM Tris-HCl pH 7.4, 2 mM DTT. elution buffer: 150 mM NaCl, 250 mM imidazole pH 7.4, 50 mM Tris-HCl pH 7.4, 2 mM DTT). (Figure 3.1) The ExsA-dependent *in vitro* transcription assay is described in Cory Bernhard's work [46].

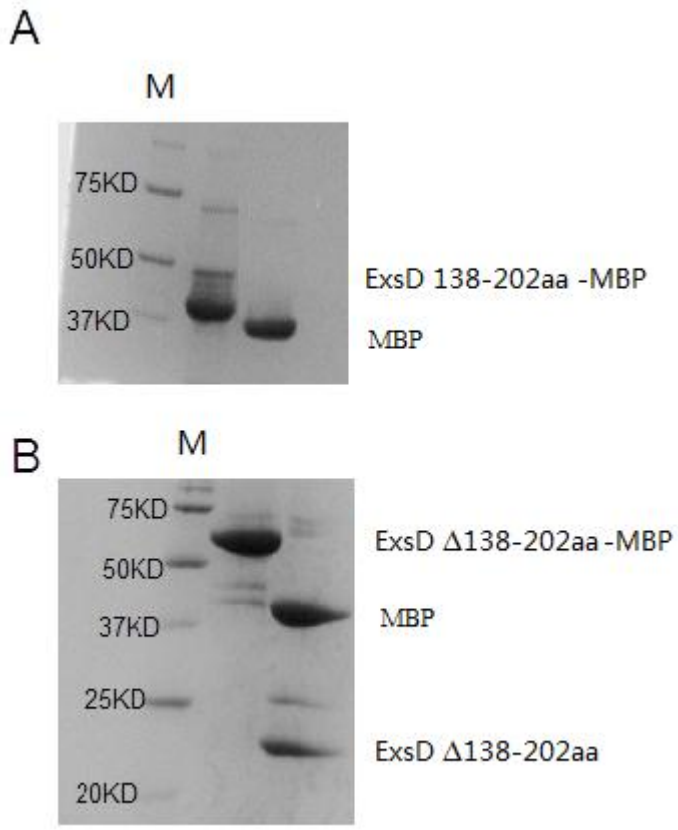

Figure 3.1. A. Purification of ExsD 138-202aa. B. Purification of ExsD Δ138-202aa.

3.3 Results and Discussion

3.3.1 Characterizing the inhibitory effect of ExsD Δ138-202aa in ExsA-dependent *in vitro* transcription assay

The ExsD Δ138-202aa deleted protein was applied to ExsA-dependent *in vitro* transcription assay at 37^{o}C, with full-length ExsD protein as control. The result shows that ExsD Δ138-202aa inhibits ExsA-dependent transcription in a dose dependent manner. Surprisingly, ExsD Δ138-202aa has a higher inhibitory effect on ExsA-dependent transcription than ExsD full-length protein. (Figure 3.2) As indicated by the result, 5 μM of ExsD Δ138-202aa has a similar inhibitory effect of 50 μM ExsD full-length protein, suggesting that the coiled coil region of ExsD may not have any effect on ExsD-ExsA interaction.

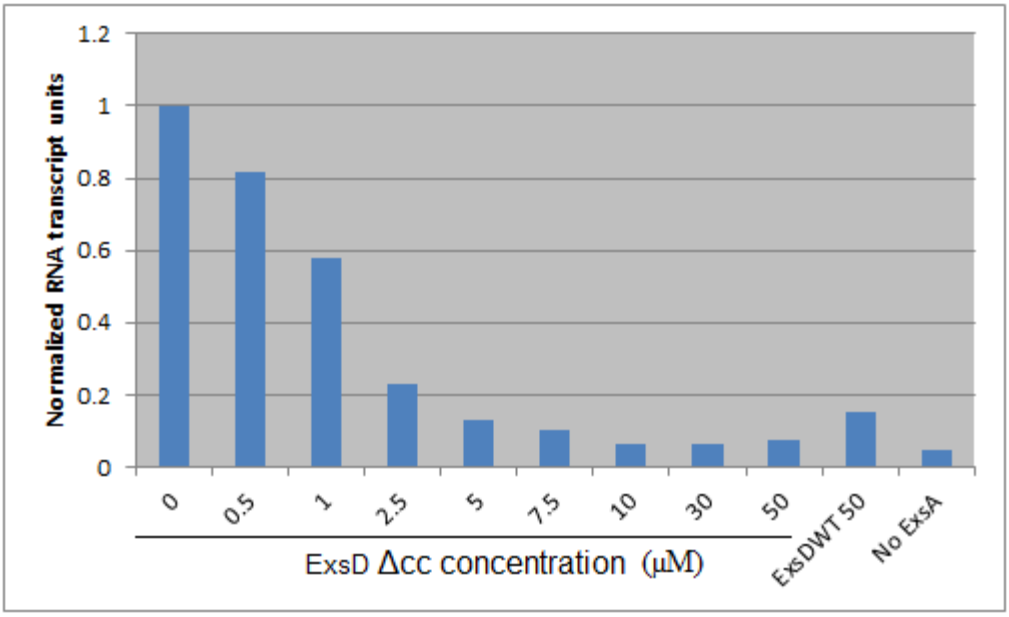

Figure 3.2. Titration of ExsA N-ternimal domain in ExsA dependent transcription

The result is contrary to our expectation, as we thought the coiled-coil region of ExsD may act as the interface for the ExsD-ExsA association, since coiled coil regions are normally involved in protein-protein interaction (for example, ExsA dimerization). However, it indicates the fact that ExsA and ExsC may compete for the binding site on ExsD, as ExsC is found to bind the first 40 amino acid residues of the N-terminus of ExsD. In order to test it, ExsD amino acid residues 1-46 (ExsD 1-46aa, purchased peptide) and ExsD Δ1-20 amino acid residues (ExsD Δ1-20aa, purified ExsD protein digested in limited proteolysis) were also tested in the ExsA-dependent *in vitro* transcription assay by. In Cory Bernhards' work in our laboratory, ExsD 1-46aa and ExsD Δ1-20aa were shown to only inhibit ExsA-dependent transcription at a high concentration (>30 μM), suggesting ExsD 1-46aa and its adjacent region of ExsD are both important for ExsA inhibition. (Figure 3.3)

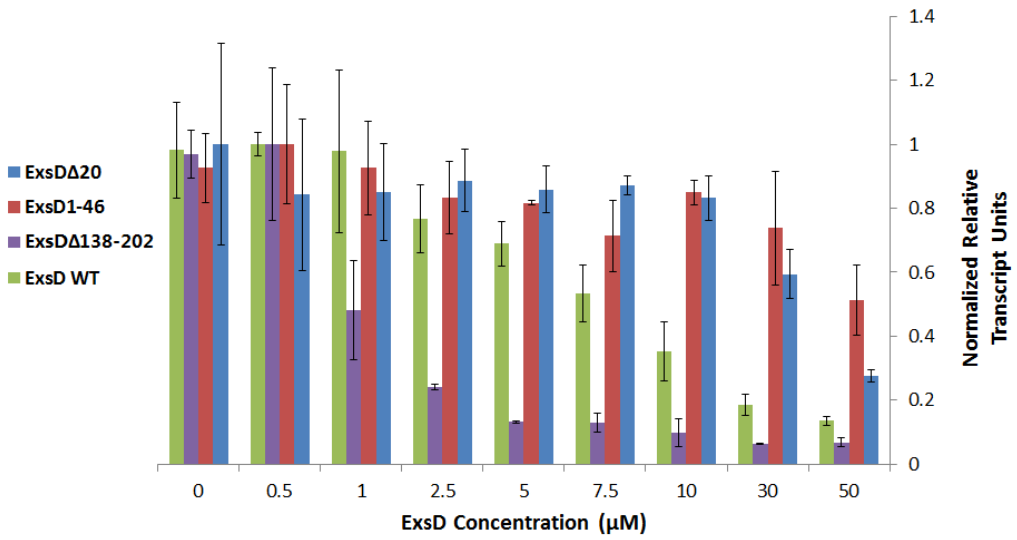

Figure 3.3. Titration of ExsD variants in ExsA-dependent in vitro transcription. ExsD Δ20: ExsD 1-20aa deleted region. ExsD 1-46: purchased ExsD 1-46aa peptide. ExsD Δ138-202: ExsD coiled-coil (amino acid residues 138-202) deleted region. ExsD WT: full-length ExsD protein.

Based on the result of the ExsA-dependent *in vitro* transcription assay, the ExsD coiled-coil deleted protein has an inhibitory effect on ExsA that is similar to that of ExsD M59R, which is a mutant that exists as a monomer (determined by analytical gel filtration assay). We think that the coiled-coil region of ExsD may stabilize the ExsD trimer, as we know that it is the ExsD monomer that inhibits the ExsA-dependent transcription. Also, we found that the yield of ExsD Δ138-202aa was lower than that of ExsD full-length protein, which is probably due to the reduced stability of the variant protein. Now we have shown that the coiled-coil region of ExsD does not inhibit ExsA. It may have the function of stabilizing the ExsD trimer, however, it remains unknown whether the coiled-coil region of ExsD has any other functions. (Figure 3.4)

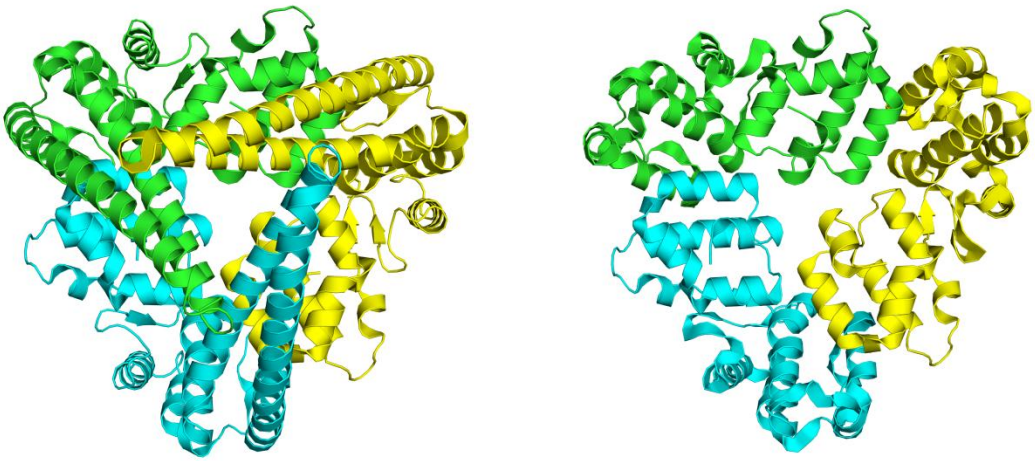

Figure 3.4. ExsD trimer (left) and ExsD coiled coil deleted region trimer (right)

In sum, we have determined that the coiled-coil region of ExsD is not necessary for the inhibition of ExsA-dependent *in vitro* transcription. In Cory Bernhards' work in our lab, the ExsD N-terminal region has also been investigated and the results suggest that the N-terminus of ExsD and its adjacent region are involved in the ExsA-ExsD interactions. Our finding can serve as a guide for more specific mapping of the amino acid residues that are important for ExsD to bind ExsA and inhibit the transcriptional activity of ExsA, and it also provides information for the design of drugs that mimic the function of ExsD.

Chapter Four: Model the ExsACDE feedback loop

4.1 Introduction

Our last aim is to build a mathematical model of the ExsACDE regulatory pathway. It is an energy-consuming process for *P. aeruginosa* to produce the components of T3SS. Thus for the most part of the life of *P. aeruginosa* the T3SS genes are down-regulated unless it has access to host cells. ExsA activates the transcription of T3SS system proteins when *P. aeruginosa* is induced at low Ca^{2+} condition or attached to a eukaryotic cell. The knowledge of the levels of transcription and translation of the T3SS regulatory factors including ExsA, ExsD, ExsC and ExsE under different physiological conditions will yield important information about how T3SS system regulation work, and a mathematical model can be built with ordinary differential equations based on the parameters derived from quantitative molecular biology assays such qPCR and qWestern blot, which measure the relative/absolute level of RNA and protein in cell. With the model, we will be able to predict the behaviors of *P. aeruginosa* virulence under different circumstances.

4.2 Material and Methods

An initial experiment was done to determine the RNA level of *exsA* in *P. aeruginosa* when cultured at either 37^{o}C or 30^{o}C, as the *in vitro* transcription assays done in our lab indicate there is a difference in the inhibitory effect of ExsD on ExsA at different temperatures. We also wanted to know the change of *exsA* RNA level when *P. aeruginosa* is cultured under T3SS inducing conditions. As we want to know the regulation of ExsA on both P_{exsC} and P_{exsD} promoters, we designed primers that amplify *exsC, exsA* and *pscB* genes. *P. aeruginosa* PAO1 and a PAO1 *exsD* knockout strain were cultured in LB overnight. On the second day, 100 µl of overnight culture was added to the TSB containing 100 mM monosodium glutamate and 1% glycerol. EGTA (2mM) is added to the culture to create the low Ca^{2+} condition which stimulates the expression of T3SS genes. Cells are harvested with the OD ~ 0.8 by centrifugation at 4500g for 1min. Total

RNAs are isolated from cells with Trizol reagent followed by a DNase treatment (Promega RQ1 RNase-free DNase), cDNAs are obtained by reverse transcription (High Capacity cDNA Reverse Transcription Kits, Applied Biosystems). Relative mRNA levels of *exsA*, *exsC* and *pscB* are determined by qPCR with *rpoD* as reference gene with BIORAD C1000 Thermal Cycler. Primers: *exsA* forward: 5'-ATGTCGGTCCTGCGGCA ACTGAGC-3', backward: 5'-GCGCGGCGAAACCCCATAGACACT-3'. *exsC* forward: 5'-AGCGGCAGCGTCTGTTGCTGGAG-3', backward: 5'-GGGTCAGTTGCGCTGCG AGAATCT-3'. *pscB* forward: TCGATGCGCAGGTGGTACGAA, backward: TGGATCA TCTGTTGAGCGGATTGG. *rpoD* forward: 5'-GGGCGAAGAAGAAATGGTC-3', backward: 5'-CAGGTGGCGTAGGTGGAGAA-3'.

The protein level of ExsA and ExsD were determined by quantitative western blotting. *P. aeruginosa* PAO1 and PAO1 *exsD* knockout strain were cultured in LB overnight. On the second day, 100 µl of overnight culture was added to the Tryptic Soy Broth containing 100mM monosodium glutamate and 1% glycerol. EGTA (2 mM) is added to the culture to create the low Ca^{2+} condition which stimulates the expression of T3SS genes. Cells are harvested with the OD ~ 0.8 by centrifugation at 4500g for 15min. For each pellet 1 ml of lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM lysozyme) were added and cells were resuspended by pipetting. Cells were incubated on ice for 10 minutes, followed by sonification with a BRANSON sonifier 450 for 30 seconds (duty cycle: constant, output: 3). Precipitations were cleared by centrifugation at 14000 rpm at 4^{o}C for 30 minutes. Total protein concentration was measured with Bradford. For quantitative western blot, cell lysate containing 80 mg total protein was applied to SDS-PAGE. Proteins on the gel were then transferred to an Immuno-Blot PVDF membrane (BIORAD). The membrane was blocked with 5% non-fat milk in TBST (150 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.05% Triton X-100) for 30 minutes. ExsA/ExsD antibody was added to the blocking solution in 1:10000 dilution. After incubating the membrane overnight, the membrane was washed three times with TBST (10 minutes each time). Immun-Star Goat Anti-Rabbit (GAR)-HRP secondary antibody (BIORAD) was added to blocking solution and the membrane was incubated for 2 hours. After washing three times with TBST, SuperSignal West Pico Chemiluminescent

Substrate (Thermo) was added to the membrane and the membrane was developed with film. Band density was measured with ImageQuant (GE Healthcare).

4.3 Results and Discussion

As expected, *exsA, exsC* and *pscB* RNA levels increased significantly when cells were cultured under T3SS inducing condition at both 30^{o}C and 37^{o}C for wild type *P. aeruginosa,* compared to cells cultured under non-inducing conditions. In the *exsD* knockout strain, the RNA levels of *exsA, exsC* and *pscB* are also higher than those in the wild type strain, which agree with the fact that the T3SS is deregulated in an *exsD* knockout strain. The RNA levels of *exsA, exsC* and *pscB* do not increase much when the *exsD* knockout strain is cultured under T3SS inducing condition, indicating that the ExsACDE regulation is non-functional without ExsD. When cells are grown at 37^{o}C, *exsA, exsC* and *pscB* RNA levels are significantly higher than those in cells grown at 30^{o}C. Interestingly, the RNA levels of *exsA, exsC* and *pscB* at 37^{o}C without induction are close to the levels of *exsA, exsC* and *pscB* RNA at 30^{o}C with induction. This result may suggest that T3SS expression is activated at 37^{o}C, which is the body temperature of human beings. (Figure 4.1) For most work on T3SS the experiments are done at 30^{o}C. But the recent RNA sequencing study on the transcriptome of *P. aeruginosa* suggests that the T3SS genes are activated at 37^{o}C, which agrees with our findings [60]. In sum, our result suggests a different mechanism that regulates the expression of T3SS at 37^{o}C, when *P. aeruginosa* enters the host's body.

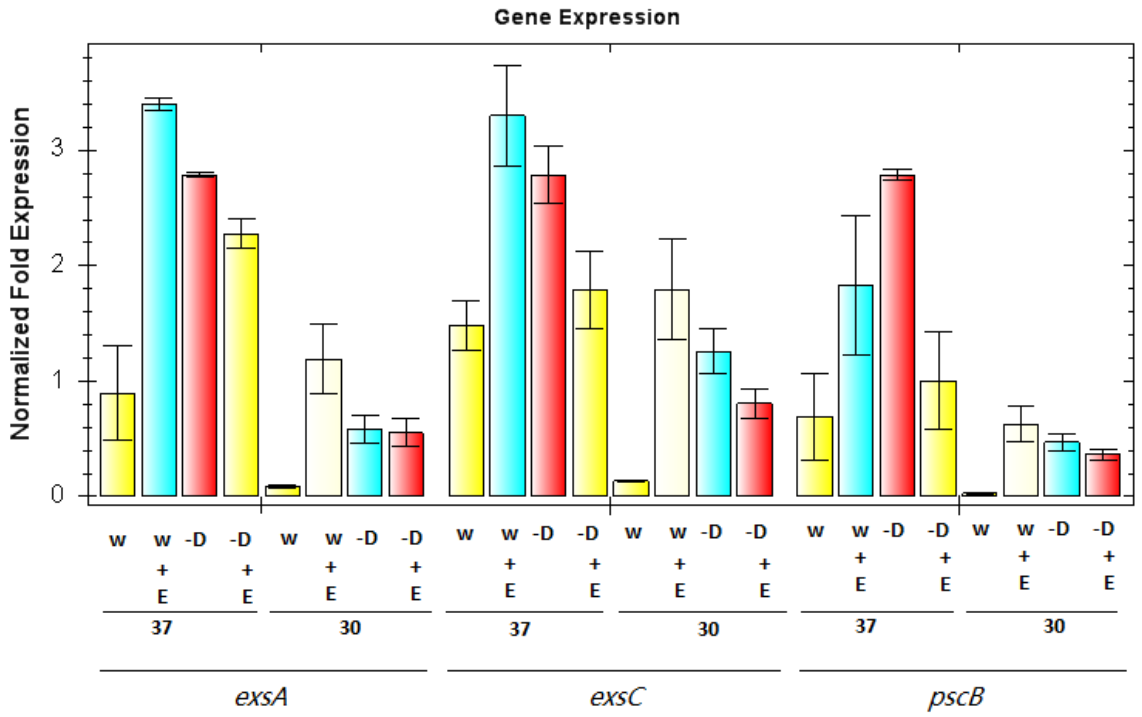

Figure 4.1. The *exsA exsC* and *pscB* RNA levels in both wild type strain (w) and *exsD* knockout strain (-D) grown at both 37 ℃ and 30 ℃, in the presence of EGTA (+E) or not. Amount of mRNAs is determined by quantitative PCR. The experiment is done in triplicates, with values normalized to *rpoD*.

Though mRNA level can represent the transcription rate of genes, it does not necessarily indicate the protein levels in the cell, as translation and protein turnover rates are not taken into consideration. Thus we wanted to determine the cellular concentrations of ExsA and ExsD from cells cultured at either 37 ℃ or 30 ℃, under either T3SS inducing condition or not. Quantitative western blotting method was used for protein measurement. Based on the density of the protein band, the ImageQuant software can determine the relative amount of protein. Thus, before measuring the amount of ExsA/ExsD proteins, a standard curve is created with purified ExsA/ExsD proteins using quantitative western blot, which demonstrate good linearity between band density and amount of protein (Figure 4.2).

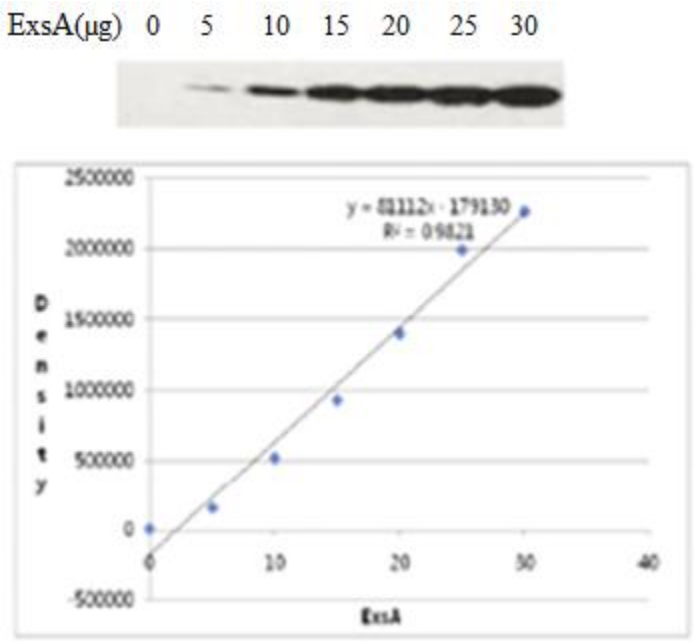

Figure 4.2. Standard curve created by adding 0 µg-30 µg purified ExsA protein.

Next we measured the concentration of ExsA protein in *P. aeruginosa* grown at 37^{o}C and 30^{o}C. At 37^{o}C, the amount of ExsA in the wild type strain is about half of that in the *exsD* knockout strain, which agrees with qPCR results. The estimated cellular concentration of ExsA is 16 nM in the wild type strain and the concentration of ExsA is 31 nM in the *exsD* knockout strain at 37^{o}C. ExsA is not detected in wild type cells when grown at 30^{o}C under non-inducing conditions. In the *exsD* knockout strain of *P. aeruginosa*, the concentration of ExsA protein is 27 nM (Figure 4.3). This is expected result as we think the T3SS expression is shut down when *P. aeruginosa* is in the non-inducing environment. However, ExsA expression is detected at 37^{o}C, suggesting that the T3SS is on at 37^{o}C, even without induction. Our finding is interesting as it implies that the temperature may have an impact on the regulation of the T3SS.

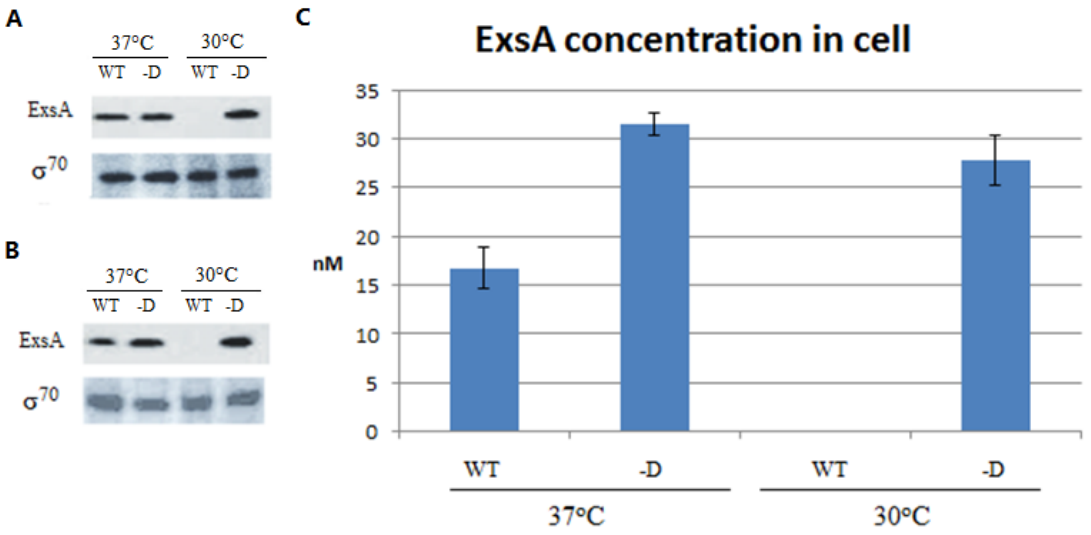

Figure 4.3. The ExsA protein levels in both wild type (WT) and *ΔexsD* mutant (-D) grown at both 37 ℃ and 30 ℃ within 60 μg total proteins. Amount of ExsA protein is determined by quantitative western blot. A and B are duplicate western blot results, each is blotted with a standard curve, respectively. C. Quantified results of A and B, cellular concentration of ExsA.

Further, we would also like to know the concentration of ExsA and ExsD in cell when the T3SS is induced or not. This may answer the question of how temperature affects the regulation of T3SS. To induce T3SS, we create a low Ca^{2+} condition by adding EGTA to the cultures (2 mM EGTA final). The quantitative western blot result show something interesting about ExsD: at 30^{o}C, ExsD could not be detected when *P. aeruginosa* was grown under the non-inducing environment. However, ExsD was 25 nM in cells when T3SS was induced at 30^{o}C. At 37^{o}C, cellular concentration of ExsD was 17 nM when T3SS was not induced, compared to 11 nM when T3SS was induced, which agrees with the qPCR result. (Figure 4.4) The same experiment was done for ExsA, the image was in poor quality due to the poor antibody against ExsA thus the bands could not be quantified. However, the film generally showed almost the same pattern as ExsD quantitative western blot result.

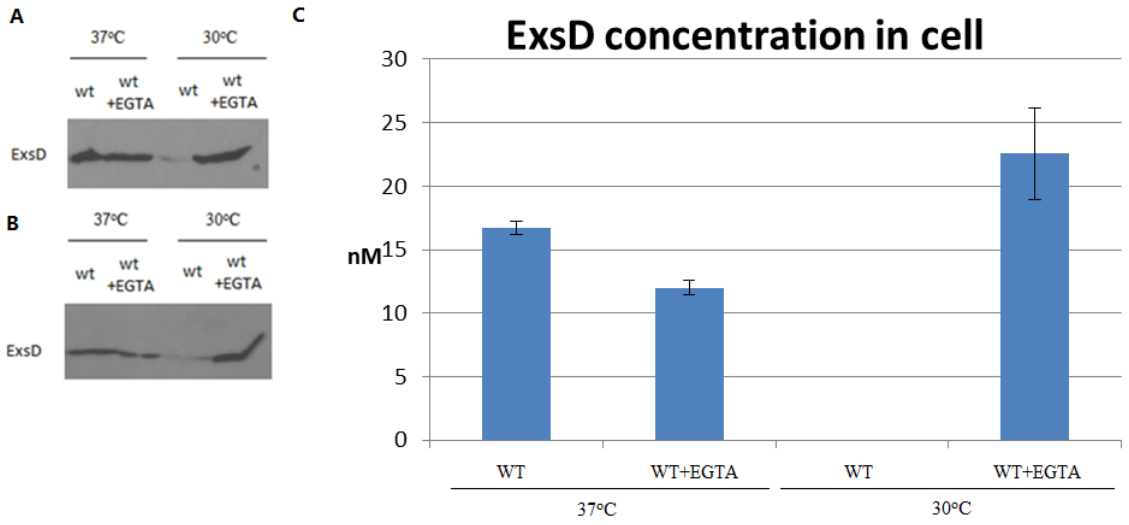

Figure 4.4. Quantitative western blot showing cellular concentration of ExsD in wild type strain grown at both 37 ℃ and 30 ℃. The cell lysate samples loaded contained 80 μg total proteins. A and B are duplicate western blot results, each is blotted with a standard curve, respectively. C. Quantified results of A and B, cellular concentration of ExsD.

Combining the qPCR and quantitative western blot results, we draw the conclusion that the transcription level and translational level of T3SS proteins basically agree with each other. For example, both ExsA and ExsD could not be detected by quantitative western blot when the cells are grown 30^{o}C without induction, and the RNA level of *exsA* and *pscB* were the lowest. However, there seemed to be a threshold for protein translation. For example, *exsA*, *exsC* and *pscB* RNA levels increased greatly when T3SS was induced at 37^{o}C, however, the increase was not reflected by the protein levels of ExsA and ExsD in the same condition: the protein levels were close to or even a little lower than those of cells grown at 37^{o}C without induction. From the results we conclude that temperature may have a significant impact on the T3SS regulation. At 37^{o}C, as the overall transcription and expression of T3SS genes increases, T3SS is turned on even under non-inducting condition. When *P. aeruginosa* finds access to host tissue, T3SS of *P. aeruginosa* is also up-regulated so *P. aeruginosa* can attack host cells. As the temperature of the environment also increases, T3SS-related genes are further up-regulated so the virulence of *P. aeruginosa* also increases. While at room temperature (25^{o}C -30^{o}C), T3SS

of *P. aeruginosa* is down-regulated: the transcription of T3SS-related genes is low, and expression of T3SS related proteins is minimal.

In order to model the ExsACDE feedback loop, we need to know the cellular concentration of ExsA, ExsD, ExsC and ExsE under different conditions (considering temperature, inducing/non-inducing, etc.). So far we have determined the cellular concentration of ExsA and ExsD. We would like to apply the quantitative mass spectrometry to detect ExsC and ExsE, as we have no antibodies for them. Quantitative mass spectrometry measures the concentration of proteins in the similar way as quantitative western blot: it creates a standard curve for peptides of the target protein then calculates the concentration of the protein in cell lysate based on the height of its unique peptide peak. Mass spectrometry has several advantages over quantitative western blotting: it is more sensitive than quantitative western blotting and requires no antibodies. The standard curve can be created by using the short peptides generated from the purified target protein or synthesized peptides. After we get the concentration of ExsA, ExsD, ExsC and ExsE in cell under different conditions, this data will be used as parameters to model the process of ExsACDE signaling pathway. We will consider more details into the current ExsACDE model, such as protein-protein dissociation constants, and protein synthesis and degradation rates. With this model, we would be able to predict the behavior of *P. aeruginosa* virulence thus more effective and efficient strategies can be developed to treat *P. aeruginosa* related infections.

Chapter Five: Concluding remarks

This project has studied features of the regulation of type III secretion system of *Pseudomonas aeruginosa* in many aspects. We solved the structure of ExsA N-terminal domain, which is the regulatory domain of the master regulator of type III secretion system. Based on the structure of the ExsA N-terminal domain, we found the dimerization interface of ExsA, as well as the crucial amino acid residues that mediate this dimerization. We also found structural homologues of ExsA using structure alignment against the protein structures in the protein databank. The results of this work shed light on the structural basis of ExsA behavior and the mechanisms under which ExsA may be regulated by other proteins. The structure of the ExsA N-terminal domain also can serve as a guide for the design of drugs against *P. aeruginosa* related infections.

Other than ExsA, this project also contributed to mapping of ExsA-ExsD interface. One variant of ExsD without the coiled-coil region was shown to be a better inhibitor for ExsA-dependent *in vitro* transcription than wild type ExsD. The result did not support our hypothesis about the function of the ExsD coiled-coil region, suggesting this region may facilitate other functions of ExsD as more and more evidence indicates that ExsD has other roles besides being the inhibitor of ExsA.

Our project also looked at some features of type III secretion system regulation, which were ignored by other researchers. The results of our quantitative experiments testing the level of ExsA and ExsD in cell show that temperature has a significant impact on the regulation of type III secretion system of *P. aeruginosa*. The result indicates the expression of T3SS genes is activated at the body temperature of host even without host cell contact. This result supplements the current understanding about the regulation of T3SS of *P. aeruginosa*.

References

1. Church, D., et al., *Burn wound infections.* Clin Microbiol Rev, 2006. **19**(2): p. 403-34.
2. Richards, M.J., et al., *Nosocomial infections in combined medical-surgical intensive care units in the United States.* Infect Control Hosp Epidemiol, 2000. **21**(8): p. 510-5.
3. Garau, J. and L. Gomez, *Pseudomonas aeruginosa pneumonia.* Curr Opin Infect Dis, 2003. **16**(2): p. 135-43.
4. Johnson, L.E., et al., *Pseudomonas aeruginosa bacteremia over a 10-year period: multidrug resistance and outcomes in transplant recipients.* Transpl Infect Dis, 2009. **11**(3): p. 227-34.
5. Gaynes, R., J.R. Edwards, and S. National Nosocomial Infections Surveillance, *Overview of nosocomial infections caused by gram-negative bacilli.* Clin Infect Dis, 2005. **41**(6): p. 848-54.
6. Trautmann, M., P.M. Lepper, and M. Haller, *Ecology of Pseudomonas aeruginosa in the intensive care unit and the evolving role of water outlets as a reservoir of the organism.* Am J Infect Control, 2005. **33**(5 Suppl 1): p. S41-9.
7. Depuydt, P., et al., *Outcome in bacteremia associated with nosocomial pneumonia and the impact of pathogen prediction by tracheal surveillance cultures.* Intensive Care Med, 2006. **32**(11): p. 1773-81.
8. Sadikot, R.T., et al., *Pathogen-host interactions in Pseudomonas aeruginosa pneumonia.* Am J Respir Crit Care Med, 2005. **171**(11): p. 1209-23.
9. Wine, J.J., *The genesis of cystic fibrosis lung disease.* J Clin Invest, 1999. **103**(3): p. 309-12.
10. Hauser, A.R., *The type III secretion system of Pseudomonas aeruginosa: infection by injection.* Nat Rev Microbiol, 2009. **7**(9): p. 654-65.
11. Schechter, L.M., et al., *Pseudomonas syringae type III secretion system targeting signals and novel effectors studied with a Cya translocation reporter.* J Bacteriol, 2004. **186**(2): p. 543-55.
12. Hauser, A.R., et al., *Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by Pseudomonas aeruginosa.* Crit Care Med, 2002. **30**(3): p. 521-8.
13. Hsu, D.I., et al., *Fluoroquinolone-resistant Pseudomonas aeruginosa: risk factors for acquisition and impact on outcomes.* J Antimicrob Chemother, 2005. **55**(4): p. 535-41.
14. Roy-Burman, A., et al., *Type III protein secretion is associated with death in lower respiratory and systemic Pseudomonas aeruginosa infections.* J Infect Dis, 2001. **183**(12): p. 1767-74.
15. Coburn, B., I. Sekirov, and B.B. Finlay, *Type III secretion systems and disease.* Clin Microbiol Rev, 2007. **20**(4): p. 535-49.
16. Frank, D.W., *The exoenzyme S regulon of Pseudomonas aeruginosa.* Mol Microbiol, 1997. **26**(4): p. 621-9.
17. Galle, M., I. Carpentier, and R. Beyaert, *Structure and Function of the Type III Secretion System of Pseudomonas aeruginosa.* Curr Protein Pept Sci, 2012. **13**(8):

p. 831-42.
18. Matz, C., et al., *Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae.* ISME J, 2008. **2**(8): p. 843-52.
19. Angus, A.A., et al., *The ADP-ribosylation domain of Pseudomonas aeruginosa ExoS is required for membrane bleb niche formation and bacterial survival within epithelial cells.* Infect Immun, 2010. **78**(11): p. 4500-10.
20. Maresso, A.W., et al., *Pseudomonas aeruginosa ExoS ADP-ribosyltransferase inhibits ERM phosphorylation.* Cell Microbiol, 2007. **9**(1): p. 97-105.
21. Pederson, K.J., et al., *The amino-terminal domain of Pseudomonas aeruginosa ExoS disrupts actin filaments via small-molecular-weight GTP-binding proteins.* Mol Microbiol, 1999. **32**(2): p. 393-401.
22. Garrity-Ryan, L., et al., *The ADP ribosyltransferase domain of Pseudomonas aeruginosa ExoT contributes to its biological activities.* Infect Immun, 2004. **72**(1): p. 546-58.
23. Cowell, B.A., et al., *ExoT of cytotoxic Pseudomonas aeruginosa prevents uptake by corneal epithelial cells.* Infect Immun, 2000. **68**(1): p. 403-6.
24. Phillips, R.M., et al., *In vivo phospholipase activity of the Pseudomonas aeruginosa cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors.* J Biol Chem, 2003. **278**(42): p. 41326-32.
25. Sato, H. and D.W. Frank, *ExoU is a potent intracellular phospholipase.* Mol Microbiol, 2004. **53**(5): p. 1279-90.
26. Finck-Barbancon, V., et al., *ExoU expression by Pseudomonas aeruginosa correlates with acute cytotoxicity and epithelial injury.* Mol Microbiol, 1997. **25**(3): p. 547-57.
27. Yahr, T.L., et al., *ExoY, an adenylate cyclase secreted by the Pseudomonas aeruginosa type III system.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13899-904.
28. Yahr, T.L. and M.C. Wolfgang, *Transcriptional regulation of the Pseudomonas aeruginosa type III secretion system.* Mol Microbiol, 2006. **62**(3): p. 631-40.
29. Lee, E.J., D.J. Evans, and S.M. Fleiszig, *Role of Pseudomonas aeruginosa ExsA in penetration through corneal epithelium in a novel in vivo model.* Invest Ophthalmol Vis Sci, 2003. **44**(12): p. 5220-7.
30. Gallegos, M.T., et al., *Arac/XylS family of transcriptional regulators.* Microbiol Mol Biol Rev, 1997. **61**(4): p. 393-410.
31. Gallegos, M.T., C. Michan, and J.L. Ramos, *The XylS/AraC family of regulators.* Nucleic Acids Res, 1993. **21**(4): p. 807-10.
32. Egan, S.M., *Growing repertoire of AraC/XylS activators.* J Bacteriol, 2002. **184**(20): p. 5529-32.
33. Wickstrum, J.R. and S.M. Egan, *Amino acid contacts between sigma 70 domain 4 and the transcription activators RhaS and RhaR.* J Bacteriol, 2004. **186**(18): p. 6277-85.
34. Dhiman, A. and R. Schleif, *Recognition of overlapping nucleotides by AraC and the sigma subunit of RNA polymerase.* J Bacteriol, 2000. **182**(18): p. 5076-81.
35. Griffith, K.L. and R.E. Wolf, Jr., *A comprehensive alanine scanning mutagenesis of the Escherichia coli transcriptional activator SoxS: identifying amino acids important for DNA binding and transcription activation.* J Mol Biol, 2002. **322**(2):

p. 237-57.
36. Lobell, R.B. and R.F. Schleif, *DNA looping and unlooping by AraC protein.* Science, 1990. **250**(4980): p. 528-32.
37. Niland, P., R. Huhne, and B. Muller-Hill, *How AraC interacts specifically with its target DNAs.* J Mol Biol, 1996. **264**(4): p. 667-74.
38. Soisson, S.M., et al., *Structural basis for ligand-regulated oligomerization of AraC.* Science, 1997. **276**(5311): p. 421-5.
39. Skurnik, M. and P. Toivanen, *LcrF is the temperature-regulated activator of the yadA gene of Yersinia enterocolitica and Yersinia pseudotuberculosis.* J Bacteriol, 1992. **174**(6): p. 2047-51.
40. Vakulskas, C.A., K.M. Brady, and T.L. Yahr, *Mechanism of transcriptional activation by Pseudomonas aeruginosa ExsA.* J Bacteriol, 2009. **191**(21): p. 6654-64.
41. Vakulskas, C.A., E.D. Brutinel, and T.L. Yahr, *ExsA recruits RNA polymerase to an extended -10 promoter by contacting region 4.2 of sigma-70.* J Bacteriol, 2010. **192**(14): p. 3597-607.
42. Brutinel, E.D., et al., *Characterization of ExsA and of ExsA-dependent promoters required for expression of the Pseudomonas aeruginosa type III secretion system.* Mol Microbiol, 2008. **68**(3): p. 657-71.
43. Brutinel, E.D., C.A. Vakulskas, and T.L. Yahr, *Functional domains of ExsA, the transcriptional activator of the Pseudomonas aeruginosa type III secretion system.* J Bacteriol, 2009. **191**(12): p. 3811-21.
44. Zhou, X., M.E. Konkel, and D.R. Call, *Regulation of type III secretion system 1 gene expression in Vibrio parahaemolyticus is dependent on interactions between ExsA, ExsC, and ExsD.* Virulence, 2010. **1**(4): p. 260-72.
45. McCaw, M.L., et al., *ExsD is a negative regulator of the Pseudomonas aeruginosa type III secretion regulon.* Mol Microbiol, 2002. **46**(4): p. 1123-33.
46. Bernhards, R.C., et al., *Self-trimerization of ExsD limits inhibition of the Pseudomonas aeruginosa transcriptional activator ExsA in vitro.* FEBS J, 2013. **280**(4): p. 1084-94.
47. Bernhards, R.C., et al., *Structural evidence suggests that antiactivator ExsD from Pseudomonas aeruginosa is a DNA binding protein.* Protein Sci, 2009. **18**(3): p. 503-13.
48. Dasgupta, N., et al., *A novel anti-anti-activator mechanism regulates expression of the Pseudomonas aeruginosa type III secretion system.* Mol Microbiol, 2004. **53**(1): p. 297-308.
49. Horsman, S.R., R.A. Moore, and S. Lewenza, *Calcium chelation by alginate activates the type III secretion system in mucoid Pseudomonas aeruginosa biofilms.* PLoS One, 2012. **7**(10): p. e46826.
50. Doublie, S., *Preparation of selenomethionyl proteins for phase determination.* Methods Enzymol, 1997. **276**: p. 523-30.
51. Hendrickson W, O.C., *Phase determination from multiwavelength anomalous diffraction measurements.* Methods in Enzymology, 1997(276): p. 494–523.
52. Emsley, P. and K. Cowtan, *Coot: model-building tools for molecular graphics.* Acta Crystallogr D Biol Crystallogr, 2004. **60**(Pt 12 Pt 1): p. 2126-32.
53. Adams, P.D., et al., *PHENIX: building new software for automated*

crystallographic structure determination. Acta Crystallogr D Biol Crystallogr, 2002. **58**(Pt 11): p. 1948-54.
54. Berman, H.M., et al., *The Protein Data Bank.* Nucleic Acids Res, 2000. **28**(1): p. 235-42.
55. Zhang, Y. and J. Skolnick, *The protein structure prediction problem could be solved using the current PDB library.* Proc Natl Acad Sci U S A, 2005. **102**(4): p. 1029-34.
56. Holm, L. and C. Sander, *Mapping the protein universe.* Science, 1996. **273**(5275): p. 595-603.
57. DiRita, V.J., et al., *Regulatory cascade controls virulence in Vibrio cholerae.* Proc Natl Acad Sci U S A, 1991. **88**(12): p. 5403-7.
58. Miyada, C.G., L. Stoltzfus, and G. Wilcox, *Regulation of the araC gene of Escherichia coli: catabolite repression, autoregulation, and effect on araBAD expression.* Proc Natl Acad Sci U S A, 1984. **81**(13): p. 4120-4.
59. Lowden, M.J., et al., *Structure of Vibrio cholerae ToxT reveals a mechanism for fatty acid regulation of virulence genes.* Proc Natl Acad Sci U S A, 2010. **107**(7): p. 2860-5.
60. Dotsch, A., et al., *The Pseudomonas aeruginosa transcriptome in planktonic cultures and static biofilms using RNA sequencing.* PLoS One, 2012. **7**(2): p. e31092.