Molecular Interactions of Type III Secretion System Transcriptional Regulators in *Pseudomonas aeruginosa*: ExsA and ExsD

Robert Cory Bernhards

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biological Sciences

Florian D. Schubot, Chair
Marcy Hernick
Stephen B. Melville
Ann M. Stevens

May 2, 2013
Blacksburg, Virginia

Keywords: *Pseudomonas aeruginosa*, ExsA, ExsD, type III secretion system, transcriptional regulation

Molecular Interactions of Type III Secretion System Transcriptional Regulators in *Pseudomonas aeruginosa*: ExsA and ExsD

Robert Cory Bernhards

ABSTRACT

The opportunistic pathogen *Pseudomonas aeruginosa* ranks among the leading causes of nosocomial infections. The type III secretion system (T3SS) aids acute *P. aeruginosa* infections by injecting potent cytotoxins (effectors) into host cells to suppress the host's innate immune response. Expression of all T3SS-related genes is strictly dependent upon the transcription factor ExsA. Consequently, ExsA and the biological processes that regulate ExsA function are of great biomedical interest. The ExsA-ExsC-ExsD-ExsE signaling cascade ties host cell contact to the up-regulation of T3SS gene expression. Prior to T3SS induction, the antiactivator protein ExsD binds to ExsA and blocks ExsA-dependent transcription by interfering with ExsA dimerization and promoter interactions. Upon host cell contact, ExsD is sequestered by the T3SS chaperone ExsC, resulting in the release of ExsA and an up-regulation of the T3SS.

ExsA is an AraC/XylS-type transcriptional regulator and belongs to a subfamily of activators that regulate the T3SS in a variety of Gram-negative pathogens. These regulators are characteristically difficult to purify due to the low solubility of their C-terminal DNA binding domains. A new method for purifying ExsA was developed and produced ExsA with improved solubility. The interaction of ExsA and its P_{exsD} promoter was examined using fluorescence anisotropy. An *in vitro* transcription assay was developed and it was determined that ExsA is sufficient to activate T3SS transcription.

Next, the ExsD–ExsA inhibitory mechanism was examined. It was demonstrated for the first time that ExsD alone is sufficient to inhibit ExsA-dependent transcription *in*

vitro without the aid of any other cellular factors. More significantly and contrary to previously published results, it was discovered that independently folded ExsD and ExsA are capable of interacting, but only at 37 °C and not at 30 °C. Guided by the crystal structure of ExsD, a monomeric variant of the protein was designed to demonstrate that ExsD trimerization prevents ExsD from inhibiting ExsA-dependent transcription at 30 °C.

To further elucidate the ExsD-ExsA inhibitory mechanism, the ExsD-ExsA interface was examined. ExsD variants were generated and used to determine which region of ExsD interacts with ExsA. Interestingly, ExsD was also found to bind DNA, although it is unclear whether or not this plays a role in ExsA inhibition. Fully understanding the mechanism by which ExsD inhibits ExsA may enable the development of drugs that target ExsA in order to shut down the T3SS, thereby eliminating *P. aeruginosa* infection.

ACKNOWLEDGEMENTS

First of all, I would like thank my advisor Dr. Florian Schubot for giving me the opportunity to be a part of his lab and for all of his guidance throughout the years. I would also like to thank my committee members, Dr. Ann Stevens, Dr. Stephen Melville, and Dr. Marcy Hernick for all of their advice and support during my graduate studies. Dr. David Popham and Dr. Birgit Scharf have also been very helpful during my time at Virginia Tech.

I would like to thank all of my fellow lab members, Dr. Nancy Vogelaar, Jing Xing, Xiao Yi, Manisha Shrestha, Jordan Mancl, Shannon Esher, Doug Ryan, Sarah Williams, Amber Sholl, Lisa Swallow, Peter Grinc, and Rika Judd for all of their help. I wish you all the best in your future careers. I would particularly like to recognize David Mishkel, who was the first undergraduate that I mentored as well as my friend. He was a brilliant young scientist and his memory will never be forgotten.

Thank you to all of the people who I've met during grad school for your advice and friendship, especially Dr. Trevor Glaros, Dr. Wes Black, Dr. Jared Heffron, Katie Rodgers, Jared Geissinger, Jake Kocher, Sean Mury, Yan Chen, Hualan Liu, Regina Wallace, Revathy Ramachandran, Alison Kernell, Hardik Zatakia, and Ben Webb. A special thanks to all of those who played softball with me. I had a blast playing with you guys.

To my family, you've been an incredible support system for me throughout my entire life, and I am forever grateful for that. Mom and Dad, you've given me the opportunity to pursue my dreams. To my sister Kirsten, you have always been my best friend and your level of kindness is truly unique. Most importantly, to my wife Casey, I

couldn’t have succeeded without your support. You bring out the best in me and always push me to improve. It is such a blessing to have someone who I can talk to at any time about my research. I look forward to seeing where our careers take us.

ATTRIBUTIONS

Several colleagues aided in the research in this dissertation. A brief description of their contributions is included here.

Chapter Two: Characterization of ExsA, the main transcriptional regulator of the type III secretion system in *Pseudomonas aeruginosa*

Nancy Vogelaar, Ph.D. (Department of Biological Sciences) is a former post doctorate in the Schubot Lab at Virginia Tech who helped develop the ExsA purification protocol as well as the *in vitro* transcription assay.

Dr. Marcy Hernick, Ph.D. (Department of Biochemistry) is an assistant professor at Virginia Tech. Dr. Hernick advised on the fluorescence anisotropy experiments.

Chapter Three: Self-trimerization of ExsD limits inhibition of the *Pseudomonas aeruginosa* transcriptional activator ExsA *in vitro*
Appendix: Extension of Chapter Three

Chapter Three was published in *FEBS Journal* in 2013.

Anne Marsden (Department of Microbiology) is a graduate student in the lab of Dr. Timothy Yahr at the University of Iowa. Ms. Marsden was co-author on this paper and performed the bacterial monohybrid experiment.

Shannon Esher (Department of Biological Sciences) is a former undergraduate student in the Schubot Lab. Ms. Esher was co-author on this paper and constructed the $ExsD^{M59R}$ variant expression plasmid.

Timothy Yahr, Ph.D. (Department of Microbiology) is a currently an associate professor at the University of Iowa. Dr. Yahr was co-author on this paper, principal investigator for one of the grants supporting the research, and contributed editorial comments.

Chapter Four: Mapping the ExsD–ExsA interface and further elucidation of the type III secretion system inhibitory mechanism in *Pseudomonas aeruginosa*

Yi Xiao (Department of Biological Sciences) is a former graduate student in the Schubot Lab. Yi constructed, expressed, and purified the ExsDΔC-C variant.

Shannon Esher performed the differential scanning fluorimetry experiments.

Anne Marsden performed the electophoretic mobility shift assay.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER FOUR

CHAPTER ONE

LITERATURE REVIEW

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative, rod-shaped bacterium that is ubiquitous in nature; occurring in soil, water, and on surfaces of plants and the skin of humans and animals. It is free-living, aerobic, and versatile in that it uses a wide range of organic materials for food and can inhabit many natural and artificial environments. *P. aeruginosa* is motile by a single polar flagellum [1] and is distinguishable by its production of pyocyanin, a blue-green pigment, as well as pyoverdin, a yellow fluorescent pigment [2]. It is capable of causing disease not only in humans and animals, but in plants as well [3].

***P. aeruginosa* infection**

P. aeruginosa causes two distinct types of infections in humans: chronic and acute. It causes chronic infections in the lungs of cystic fibrosis (CF) patients by producing a biofilm. As a result, *P. aeruginosa* has been established as a model organism for the study of biofilms. In acute infections, *P. aeruginosa* utilizes a type III secretion system (T3SS) as the main virulence mechanism to infect its host. *P. aeruginosa* is the premier example of an opportunistic pathogen as it exploits a breach in host defenses in order to start an infection. *P. aeruginosa* almost never infects uncompromised tissues. However, it can infect almost any tissue that becomes compromised, causing a wide variety of infections including bone and joint infections, dermatitis, gastrointestinal infections, respiratory system infections, soft tissue infections, and urinary tract infections [4-9]. Infection can progress to endocarditis, peritonitis, meningitis, bacteremia, septicemia, and death [10-13]. Patients susceptible to systemic infections include burn victims, cancer patients, and AIDS patients [8, 14-16].

P. aeruginosa expresses a number of virulence factors including adhesins, invasins, and toxins. The adhesins include type IV pili [17], a polysaccharide capsule or glycocalyx [18], and alginate slime which helps produce the biofilm [19]. Invasins include alkaline protease [20], elastase [21], hemolysins phospholipase C [22] and lecithinase [23], leukocidin [24], pyocyanin [25], and siderophores [26]. Toxins include lipopolysaccharide (LPS) [27], exotoxin A [28], and exoenzymes S, T, U and Y [29-32]. The capsule, slime layers, biofilm, and LPS also contribute to phagocyte resistance [33, 34].

Chronic infection

Infection by *P. aeruginosa* is the leading cause of death in CF patients where a biofilm develops in the lungs [35]. *P. aeruginosa* utilizes the T3SS to initiate these infections, but quickly switches to a biofilm-producing state [36]. CF patients usually become colonized with *P. aeruginosa* during childhood [37]. These chronic infections are minimally invasive and rarely progress to septicemia [35]. Rather, the inflammation and slow deterioration of pulmonary function is the main cause of death in these patients [35].

Acute infection

Acute infection is typically composed of three distinct stages: (1) bacterial attachment and colonization, (2) local invasion and (3) disseminated systemic disease. However, disease progression may stop at any stage. *P. aeruginosa* is a leading cause of nosocomial infections, especially in immunocompromised and critically ill patients [38]. Patient-to-patient transmission and hospital water sources are known to be responsible for spreading infection [39]. The risk factors in a hospital setting include use of steroids or

chemotherapy, and the use of medical devices/procedures such as vascular and urinary catheters, ventilators, drainage tubes, and endotracheal intubation [40-42]. Previous exposure to antimicrobial agents also increases the risk of *P. aeruginosa* infection, due to the development of antibiotic resistance [43]. Underlying diseases/conditions of hospitalized patients are risk factors as well. Cancer, diabetes, renal failure, chronic obstructive pulmonary disease (COPD), solid organ transplantation, and AIDS all increase the risk of infection [14, 15, 44]. According to the Centers for Disease Control and Prevention, *P. aeruginosa* is the leading Gram-negative organism causing pneumonia in intensive care units in the United States. Furthermore, *P. aeruginosa* is the third most frequently isolated Gram-negative pathogen from blood [45]. A prolonged hospital stay increases the risk of infection due to an elevated likelihood of *P. aeruginosa* developing resistance to antibiotics [46].

Antibiotic resistance

Multi-drug resistance is an important clinical feature of *P. aeruginosa* [47]. High resistance has been seen with all major classes of antibiotics especially β-lactams [48], quinolones [49], and aminoglycosides [50]. Resistance is due in part to very low permeability of the *P. aeruginosa* outer membrane to small, hydrophobic molecules [51, 52]. In addition, *P. aeruginosa* is particularly adept at acquiring antibiotic-resistance genes through genetic mutation or horizontal gene transfer [53]. Mechanisms of resistance include production of enzymes such as β-lactamase [48], alterations in target sites [54], loss of outer membrane proteins or porins [55], and production of efflux pumps [56].

Treatment

Appropriate antimicrobial therapy is critical for treating acute *P. aeruginosa* infections, but finding the proper regimen can be problematic due to the wide range of resistance. Typically, a drug cocktail is administered to the patient. For instance, the combination of gentamicin and carbenicillin is frequently used to treat severe *P. aeruginosa* infections [57, 58]. However, if an effective drug cocktail is not given swiftly, high mortality rates ensue. Choosing a proper combination of drugs can be challenging because different strains are resistant to different antibiotics and strains can quickly acquire new antibiotic resistance. There is no current vaccine for *P. aeruginosa* infection, although several types are currently being tested.

Type III secretion system (T3SS)

The main virulence mechanism for *P. aeruginosa* acute infections is the T3SS [59]. The T3SS works as a molecular syringe to inject effector molecules (cytotoxins) directly into the cytoplasm of the host eukaryotic cell [60]. Many different types of Gram-negative pathogens utilize a T3SS including *Salmonella*, *Shigella*, *Vibrio*, and *Yersinia* spp [61, 62]. The components of the T3SS can be divided into five categories: effector proteins, chaperones, needle complex proteins, translocation apparatus proteins and regulatory proteins.

T3SS effector proteins

Only four effector proteins have been identified in *P. aeruginosa* which is less than any other well-characterized T3SS. They are called exoenzymes S, T, U and Y. These effectors have a variety of functions, including promotion of tissue destruction, evasion of phagocytosis, and dissemination from initial sites of colonization [30, 59, 63-

74]. Each effector contains an amino-terminal signal sequence that is presumably used for targeting to the type III secretion apparatus [75]. Different strains of *P. aeruginosa* secrete different combinations of these effectors with ExoS and ExoU being mutually exclusive [76, 77]. Strains that express ExoS cause delayed cell death via apoptosis, while strains possessing ExoU cause quick and robust cell lysis [77, 78].

ExoS

The best characterized effector is exoenzyme S (ExoS). ExoS has GTPase activating protein (GAP) activity as well as ADP ribosyl transferase (ADPRT) activity [29, 79]. The GAP domain of ExoS targets small GTPases that maintain organization of the host cell actin cytoskeleton, specifically Rac, Rho, and cell division cycle 42 (CDC42) [79, 80]. Normally, these regulatory GTPases switch between an inactive GDP-bound form and an active GTP-bound form, but ExoS GAP domain changes the equilibrium to favor the inactive GDP-bound form [79, 80]. This leads to disruption of the host cell actin cytoskeleton [79, 80], which is associated with cell rounding and decreased internalization of *P. aeruginosa* by certain types of cells. This suggests that ExoS GAP activity has a role in preventing phagocytosis [30, 59, 63]. The ADPRT domain of ExoS binds a eukaryotic 14-3-3 protein to activate ADPRT activity [81-84]. The known ExoS ADPRT host cell targets are apolipoprotein A1, CDC42, cyclophilin A, ezrin, IgG3, moesin, radixin, RAB1, 3, 5, 7, 8 and 11, RAC1, RALA, RAP1, RAP2, RAS, and vimentin [85]. Affects of the ExoS ADPRT activity include actin cytoskeleton disruption associated with cell rounding, cell death, and inhibition of DNA synthesis, vesicular trafficking and endocytosis [64-66, 86]. Cytoskeleton disruption may lead to

loss in cell-to-cell adherence, which may facilitate *P. aeruginosa* penetration through epithelial barriers [64-66].

ExoT

The amino acid sequence of ExoT is very similar to that of ExoS, and as a result, ExoT has GAP and ADPRT activity as well [87-89]. In addition to having the same GAP activity as ExoS, the GAP domain of ExoT also contributes to the inhibition of cytokinesis by inactivating Rho [90, 91]. The ADPRT domain of ExoT is also activated by a 14-3-3 protein, but its targets are unique, yet limited. The known host cell target proteins are CRKI, CRKII, and phosphoglycerate kinase. Collectively, the ExoT ADPRT domain acts to alter the host cell actin cytoskeleton and to inhibit cell adhesion, migration and proliferation. These activities help *P. aeruginosa* to disseminate and avoid phagocytosis [63, 67-69]. The enzymatic activities of ExoT have also been correlated with delays in wound healing [92, 93]. ExoT causes apoptotic-like cell death, but it's delayed compared to ExoS cell-mediated killing [94]. The role of ExoT in pathogenesis is modest compared to that of ExoS and ExoU [63, 67-69].

ExoU

ExoU is a potent phospholipase capable of causing rapid eukaryotic cell death [31]. The patatin-like domain of ExoU gives it phospholipase A_2 (PLA_2) activity [95, 96]. ExoU has a wide range of substrates including phospholipids, lysophospholipids, and neutral lipids [95-97]. Similar to other *P. aeruginosa* effector proteins, ExoU requires a eukaryotic factor for activation [95, 96]. In this case, the factor is Cu^{2+}, Zn^{2+}-superoxide dismutase (SOD1) [98]. The C-terminal domain of ExoU is used for targeting to the plasma membrane [99, 100]. ExoU intoxication causes rapid loss of cell membrane

integrity, resulting in cell death consistent with necrosis [31, 101]. ExoU killing is directed against phagocytes and epithelial barriers to prevent phagocytosis and enhance dissemination [67, 69-72].

ExoY

ExoY is an adenylyl cyclase and works by binding ATP [32]. This results in an increase in intracellular cAMP, causing disruption of the actin cytoskeleton [32, 102], inhibition of phagocytosis [73], and increased endothelial permeability [74]. ExoY also requires a host factor for activity, however, the identity of this factor is unknown [32].

T3SS chaperones

In *P. aeruginosa*, some but not all of the T3SS effectors have chaperones to which they bind before secretion. These chaperones allow for proper storage of the effectors in the bacterial cytosol and delivery of the effectors to the secretion apparatus. SpcS serves as the chaperone for both ExoS and ExoT [103, 104], SpcU is the chaperone for ExoU [105], and no chaperone has been identified for ExoY to date. Chaperones also bind secreted proteins that aren't effectors. Translocation proteins PopB and PopD are bound by the chaperone PcrH [106]. PscF, a structural subunit of the needle complex, has two chaperones: PscE and PscG [107]. ExsC is also a chaperone for the secreted regulatory protein ExsE [108, 109].

T3SS needle complex

The structural proteins of the T3SS that make up the needle apparatus are encodedby genes *pscUTSRQPON*, *pcr1234DR,* and *pscBCDEFGHIJKL*. Much of what is known about the structural proteins is from studies in *Yersinia enterocolitica*, which has a needle complex that is homologous to that in *P. aeruginosa* (Fig. 1.1). PscN

encodes an ATPase that uses energy created from the proton motive force to unfold effector proteins and push them through the needle apparatus [110, 111]. PscN is regulated by PscL [111]. PscC is a secretin-like protein that is thought to oligomerize with the help of the lipoprotein PscW in order to form a channel through the bacterial outer membrane [112, 113]. PscP is thought to serve as a molecular ruler to regulate the length of the needle [114]. PscJ is hypothesized to be a lipoprotein component of the basal structure of the needle complex [115]. Further work needs to be conducted to determine the properties and function of all the components of the needle complex and how they work together for secretion.

T3SS translocation apparatus

The translocation apparatus consists of a pore that delivers the effector molecules through the host cell plasma membrane. It is encoded by *pcrGVH* and *popBD*. PopB and PopD are actually secreted themselves and interact with the host cell membrane to form the pore [106]; however, this mechanism is poorly understood. PcrV is also secreted and is thought to form a multimeric scaffold at the tip of the needle to facilitate the assembly of the PopB–PopD translocation pore [116]. Alternatively, PcrV could serve to connect the needle to preformed PopB–PopD pores [117]. PcrV is a potential vaccine candidate, given that it is required for the translocation of effector proteins [116] and has been shown to elicit protection in animal infection models [118, 119].

T3SS regulation

Effector secretion as well as the expression of all T3SS components is tightly controlled by a regulatory mechanism consisting of four interacting proteins (ExsA, ExsC, ExsD and ExsE), with ExsA being the main transcription factor [120]. In addition to the

ExsACDE regulatory cascade, the T3SS is regulated by a multitude of other proteins (Fig. 1.2). These proteins include CpdA, a cAMP phosphodiesterase [121]; Crc, a catabolite repression control protein involved in carbon regulation [122]; adenylate cyclases CyaA and CyaB [123]; FimL, a type IV pili biogenesis protein [124]; FlhA, a transmembrane protein [125]; KynA, a tryptophan dioxygenase [126]; MgtE, a magnesium transporter [127]; MucA/AlgU/AlgR, alginate biosynthesis proteins [128]; NirS, a nitrite reductase [129]; PrpC, a methylcitrate synthase [122]; PsrA, a transcriptional activator [130]; small proteins PtrB and PtrC [131, 132]; RpoS, the stationary-phase sigma factor [133]; RsmA, an RNA-binding protein [134, 135]; two-component response regulatory systems, LadS/RetS, GacA/GacS and GtrS/GltR [36, 136-138]; TrpA, a tryptophan synthase [126]; and Vfr, a cyclic AMP (cAMP)-binding protein [139]. There are also small RNAs, RsmZ and RsmY, which contribute to T3SS regulation [36, 140]. In addition, the T3SS is regulated by a number of small molecules including calcium [141], acetyl-CoA [142], indole acetic acid (IAA), histidine [143], spermidine [144], and the quorum-sensing signals homoserine lactone (HSL) [145] and *Pseudomonas* quinolone signal (PQS) [146]. However, for the most part, it is unclear how these regulatory proteins/molecules exert their influence on the T3SS.

ExsACDE regulatory cascade

The ExsACDE cascade constitutes perhaps the most direct link between effector secretion and upregulation of T3SS genes [109] (Fig. 1.3). ExsA, the main transcriptional regulator, is inhibited by ExsD when ExsD binds to ExsA in a 1:1 complex [147]. Meanwhile, ExsE sequesters ExsC [108, 109]. Upon host cell contact, ExsE is translocated into the host cell cytoplasm [108, 109]. Now ExsC sequesters ExsD

[148], which frees ExsA to bind to the T3SS promoters and recruit RNA polymerase (RNAP), thus initiating T3SS transcription [149].

ExsA

ExsA is the main transcriptional activator of the T3SS and a member of the AraC/XylS family of transcriptional regulators [120]. These regulators are found in bacteria and fungi and are involved in the regulation of virtually all types of cellular processes [150]. AraC/XylS-type transcription factors are characterized by structurally conserved AraC domains that interact with specific DNA sequences near the -35 promoter region [150]. This facilitates the recruitment of RNAP to the transcription initiation site [150]. On the other hand, the amino-terminal domains of the AraC/XylS-type proteins are highly variable and thought to mediate diverse regulatory signals [150-154]. Typical of an AraC/XylS-like activator, ExsA is composed of two functional domains: a carboxy-terminal DNA binding domain (CTD) and an amino-terminal regulatory domain (NTD) [155]. ExsA is 278 amino acids in length and has a molecular weight of 31.6 kDa.

It is estimated that there are at least 830 members in the AraC/XylS family of proteins [150]. However, out of these, only two complete structures have been published, and they are both from *E. coli*: MarA and Rob [156, 157]. The crystal structures were only obtained when these proteins were in complex with DNA to help stabilize their carboxy-terminal domains. Both MarA and Rob activate genes that are involved in antibiotic resistance.

ExsA homologs

There are close homologs to ExsA in a variety of Gram-negative pathogens including InvF from *Salmonella enterica* serovar Typhimurium, LcrF in *Yersinia spp.*, Rns from enterotoxigenic *E. coli* (ETEC), ToxT from *Vibrio cholerae,* and ExsA from *Vibrio parahaemolyticus*. These proteins make up a subfamily of activators that regulate virulence factor expression. InvF from *S. enterica* regulates the T3SS found on the SPI–1 pathogenicity island [158]. LcrF activates the T3SS in the three pathogenic *Yersinia* species: *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* [159, 160]; but it is unknown whether a regulatory protein binds to LcrF. It is known, however, that LcrF is thermoregulated through post-translational mechanisms [161]. Rns regulates fimbriae expression in ETEC, which is required for pathogenesis [162]. ToxT regulates expression of cholera toxin and toxin-coregulated pilus in *V. cholerae* [163]. The most closely related regulatory mechanism to the T3SS in *P. aeruginosa* is found in T3SS1 of *V. parahaemolyticus*. This system is regulated by a similar ExsACDE regulatory cascade [164-166]; however, the main difference in this system is that ExsA does not regulate its own expression [164].

ExsA promoters

ExsA is the transcriptional activator of the T3SS regulon (Fig. 1.4) [120] and is known to bind to all 10 promoters involved in expression of T3SS genes: P_{exsC}, P_{exsD}, P_{exoS}, P_{exoT}, P_{exoU}, P_{exoY}, P_{orf1}, P_{pcrG}, P_{pcrN} *and* P_{popN} [31, 75, 103, 120]. ExsA is monomeric in solution, but it forms a dimer when bound to one of its promoters [167]. This is in contrast to MarA, which lacks a dimerization domain and binds to DNA as a monomer [156]. It is thought that one molecule of ExsA initially binds to the promoter at binding

site 1 and recruits the second ExsA molecule to binding site 2 [167]. Binding site 1, or the promoter-proximal site, contains highly conserved GnC and TGnnA sequences separated by ~10 base pairs [167]. The ExsA-CTD contains two helix-turn-helix (HTH) motifs, and the first HTH interacts with the GnC sequence while the second HTH interacts with the TGnnA sequence [168]. On the other hand, binding site 2, or the promoter-distal site, contains no obvious sequence similarity [167]. Given that ExsA always binds to binding site 1 in the same manner, binding site 2 is responsible for promoter-specific properties [169]. Promoter strength, ExsA-binding affinity, and the degree of promoter bending are properties that are primarily determined by binding site 2 [169]. Cooperative binding has been demonstrated with the P_{exsC} promoter [167], and the way in which ExsA binds to P_{exsC} is distinct from the interactions that occur at the other promoters [169]. For P_{exsC}, there is a GnC located in binding site 2 that interacts with HTH1 of ExsA, and is functionally equivalent to the GnC sequence in binding site 1 [169]. The P_{exsD}, P_{exoT}, and P_{pcrG} promoters also contain GnC sequences, but these sequences are not required for ExsA binding or transcriptional activation [169]. It has been shown that ExsA binds to the P_{exoT} promoter in an ordered fashion in that occupation of binding site 1 is required for efficient occupation of site 2 [167]. This sequential binding mechanism is dependent upon interactions mediated through the amino-terminal/dimerization domain of the ExsA monomers [155]. Recent studies show that ExsA binds to the P_{exsC}, P_{exsD}, P_{exoT}, and P_{pcrG} promoters in a head-to-tail orientation [168]. It has been demonstrated that ExsA induces DNA-bending upon binding [167]. ExsA shows modest DNA-bending with the P_{exsD} and P_{exoT} promoters, whereas the DNA-bending at the P_{exsC} promoter is more pronounced [167]. An electrophoretic mobility

shift assay (EMSA) was used to determine equilibrium constants (K_{eq}) for the interactions between ExsA and three of its promoters [167]. The apparent equilibrium constants were 1.1 ± 0.2 nM, 4.1 ± 0.2 nM and 5.4 ± 0.6 nM for P_{exsC}, P_{exsD} and P_{exoT}, respectively [167]. These high affinities are characteristic of a transcriptional activator. Once ExsA forms the dimer on the promoter, it is able to recruit RNAP so that transcription can be initiated. DNase I footprinting experiments were used to map the regions of the P_{exsC}, P_{exsD}, and P_{exoT} promoters to which ExsA binds [167]. For each promoter, the ExsA binding site overlaps the -35 RNAP binding site and extends upstream for approximately 34 base pairs [167]. The -35 and -10 sites for typical σ^{70}-dependent promoters are separated by ~17 nucleotides. However, for ExsA-dependent promoters, this gap is 21-22 bases [167]. These features suggest that the -35 site is not involved in RNAP binding.

ExsA–RNAP interactions

Recent work suggests that the -35 RNAP binding site of the P_{exsC} promoter does not contribute to RNAP binding [170]. Instead, ExsA recruits σ^{70}-RNAP to an extended -10 promoter [149, 170]. It has been shown that the carboxy-terminal domain of the α subunit of RNAP is not needed for ExsA-dependent transcriptional activation [170]. This is unusual for transcriptional activators. Instead, studies indicate that ExsA interacts with region 4.2 of σ^{70} because this region is required for ExsA-dependent transcription [170]. It is proposed that this is how ExsA makes up for the lack of a -35 RNAP binding site since region 4.2 typically interacts with the -35 site for most bacterial promoters [170].

ExsD

When the T3SS is turned off, ExsA is inhibited by an antiactivator protein ExsD [171]. An antiactivator is distinguished from a repressor in that it does not have a known DNA recognition site (operator). An antiactivator can bind directly to the activator or bind non-specifically to DNA. ExsD–ExsA interactions have been demonstrated in bacterial two-hybrid studies [171]. It has been shown that ExsD binds to ExsA to form a 1:1 complex [147]. In the current model, ExsD inhibits ExsA activity by disrupting the ExsA dimer [172] and preventing ExsA from binding to the promoters [147, 172].

ExsD (Fig. 1.5) is 276 amino acids in length and has a molecular weight of 31.4 kDa. ExsD self-associates into a trimer when not bound to a different molecule [173]. The crystal structure of ExsD [173] reveals surprising structural homology between ExsD and a DNA binding protein in *E. coli* called KorB [174]. KorB is found on a broad-host-range plasmid called RP4 [174]. It is a repressor protein that binds to DNA operator sequences located as far as 500 base pairs away from the regulated promoter and is thought to mediate transcriptional repression through DNA looping [175].

ExsD also shows structural homology to GreB; a RNAP binding protein from *E. coli* [176]. RNAP occasionally loses its grip on the growing mRNA end during elongation and backtracks on the DNA template [176]. GreB binds to RNAP and cleaves the disengaged 3´ RNA segment so that RNAP can proceed with transcription [176].

ExsC

ExsC is the anti-antiactivator composed of 141 amino acids and has a molecular weight of 16.2 kDa. ExsC acts as a positive regulator of the T3SS and is required for T3SS expression [148]. When the T3SS is turned on, ExsC binds to ExsD [148],

allowing ExsA to initiate transcription. A heterocomplex is formed when ExsD associates with ExsC [177]. Isothermal titration calorimetry (ITC) analysis has shown that formation of this complex is exothermic and thermodynamically favorable [177]. There is a 1:1 stoichiometric ratio between ExsD and ExsC molecules in the complex, and the K_d of the complex was determined to be in the 18-nm range [177]. It is believed that ExsC and ExsD form a 2:2 complex with a dimer of ExsC binding two ExsD monomers [178].

ExsE

ExsE is a small protein consisting of 81 amino acids and has a molecular weight of 8.3 kDa. By itself, ExsE is highly disordered and contains no tertiary structure. ExsE is interesting in that it is a secreted regulatory protein. Acting as a negative regulator, ExsE sits at the top of the regulatory cascade [108, 109], and secretion of ExsE into the host cell cytoplasm triggers upregulation of the T3SS [108, 109]. It is unknown whether ExsE has a function inside the host cell. ExsE is not required for type III-mediated cytotoxicity [109]. Prior to host cell contact, ExsE binds to ExsC and inhibits its activity, which ultimately inhibits transcription. The crystal structure of the ExsC–ExsE complex reveals that an ExsC dimer is bound by one molecule of ExsE [178]. ExsE wraps around the ExsC dimer. When ExsE is secreted from the cell, ExsC now binds ExsD, which allows ExsA to initiate transcription (Fig. 1.3). The preferential binding that ExsC has for ExsE has been supported by ITC experiments that show ExsC has a stronger binding affinity for ExsE (1 nM), compared to ExsD (18 nM) [179]. ExsC acts as a chaperone for ExsE in that ExsE requires ExsC for stability and secretion [108]. ExsC is a member of the CesT-like family of chaperones [108]. Interestingly, ExsE is secreted from cells

under low calcium conditions. The link between low calcium and T3SS initiation is currently unknown, but it allows for studies of type III secretion in the laboratory setting.

Medical importance

ExsA is a potential drug target due to the fact that it is absolutely required for type III secretion. An ExsA mutant is unable to secrete type III effectors and unable to initiate an acute infection. Therefore, a drug that inhibits ExsA would be extremely valuable due to the high antibiotic resistance of *P. aeruginosa*. This type of drug would not kill *P. aeruginosa*, but just render it harmless. Due to this, there would be less evolutionary pressure for the organism to develop resistance. Currently, there are no drugs that act on *P. aeruginosa* in this way. An inhibitor of ExsA should also have no harmful effects on human cells because AraC/XylS family members are only found in bacteria [150]. There is much that needs to be discovered about the interaction between ExsD and ExsA and how ExsD inhibits ExsA-dependent transcription. Elucidation of the ExsD–ExsA inhibitory mechanism could result in the development of drugs that mimic ExsD function and inhibit ExsA to shut down the T3SS, thereby eliminating acute infection.

Research objectives

The purpose of the following studies is to characterize ExsA and ExsD, the main transcriptional regulators of the T3SS in *P. aeruginosa*, through a variety of *in vitro* biochemical techniques to learn the properties of ExsA and ExsD individually and also how they interact. If enough is known about these proteins and the way they regulate transcription, then drugs can be designed to shut down the T3SS. In Chapter Two, the goal was to purify a more soluble recombinant ExsA protein and examine its ability to interact with its promoters and initiate transcription. The affinity of ExsA for its P_{exsD}

promoter was obtained using fluorescence anisotropy. An *in vitro* transcription assay was developed to examine the ability of ExsA to initiate transcription from three of its promoters.

Chapter Three examines the interaction between ExsD and ExsA. Previous studies have shown that the ExsD–ExsA interactions are not freely reversible. Because independently folded ExsD and ExsA were not found to interact, it was hypothesized that folding intermediates of the two proteins form the complex. This study demonstrates for the first time that ExsD alone is sufficient to inhibit ExsA-dependent transcription *in vitro* and that no other cellular factors are required. More significantly, it was discovered that independently folded ExsD and ExsA are capable of interacting, but only at 37 °C and not at 30 °C. ExsD is known to form a trimer in solution when not bound to a different protein [173], but the role of ExsD self-trimerization was previously unknown. Guided by the crystal structure of ExsD, a monomeric variant of the protein was designed to demonstrate that ExsD trimerization prevents ExsD from inhibiting ExsA-dependent transcription at 30 °C.

In Chapter Four, the main goal was to map the ExsD–ExsA interface. ExsD variants lacking certain regions of the protein were used in the *in vitro* transcription assay to determine which regions of ExsD are important for ExsA binding and inhibition. The ExsD–ExsA inhibitory mechanism was also further examined. Contrary to the current model, ExsD was also found to bind to DNA. However, it is unclear whether this plays a role in the inhibition of ExsA. Preliminary results show that ExsD also binds to RNAP, but how this fits into the mechanism remains unclear. Overall, this study contributes to the understanding of how ExsD and ExsA regulate the T3SS and adds to the current

model. These studies will pave the way for future work that will lead to a complete understanding of how ExsD and ExsA regulate the T3SS in *P. aeruginosa*. This will not only help in the development of drugs to treat *P. aeruginosa* infection, but other types of infections caused by the many Gram-negative pathogens that depend on the T3SS to infect humans.

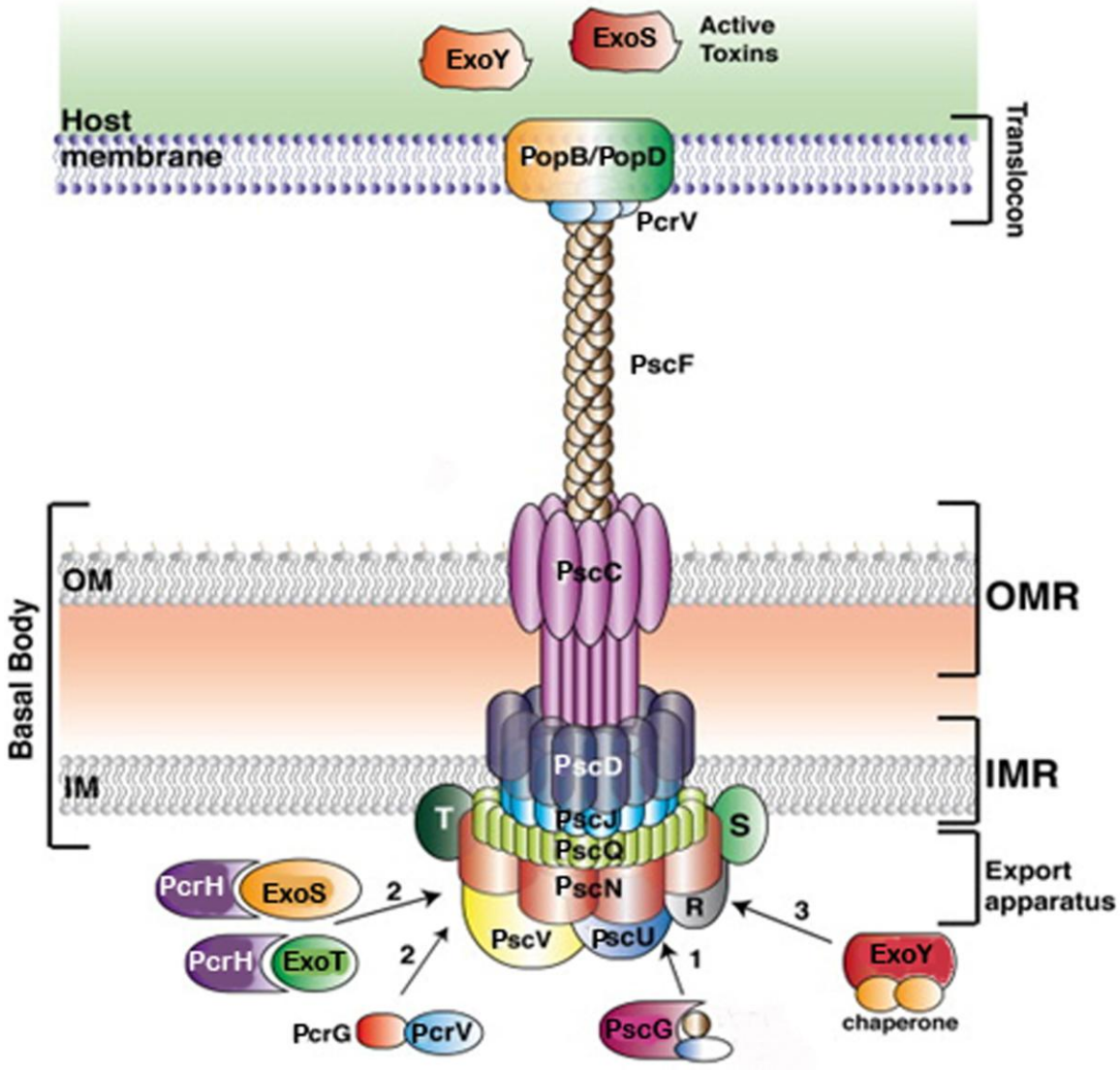


Fig. 1.1. Structural proteins of the T3SS needle apparatus. Figure adapted by author with permission from Izoré et al. *Structure* (2011).

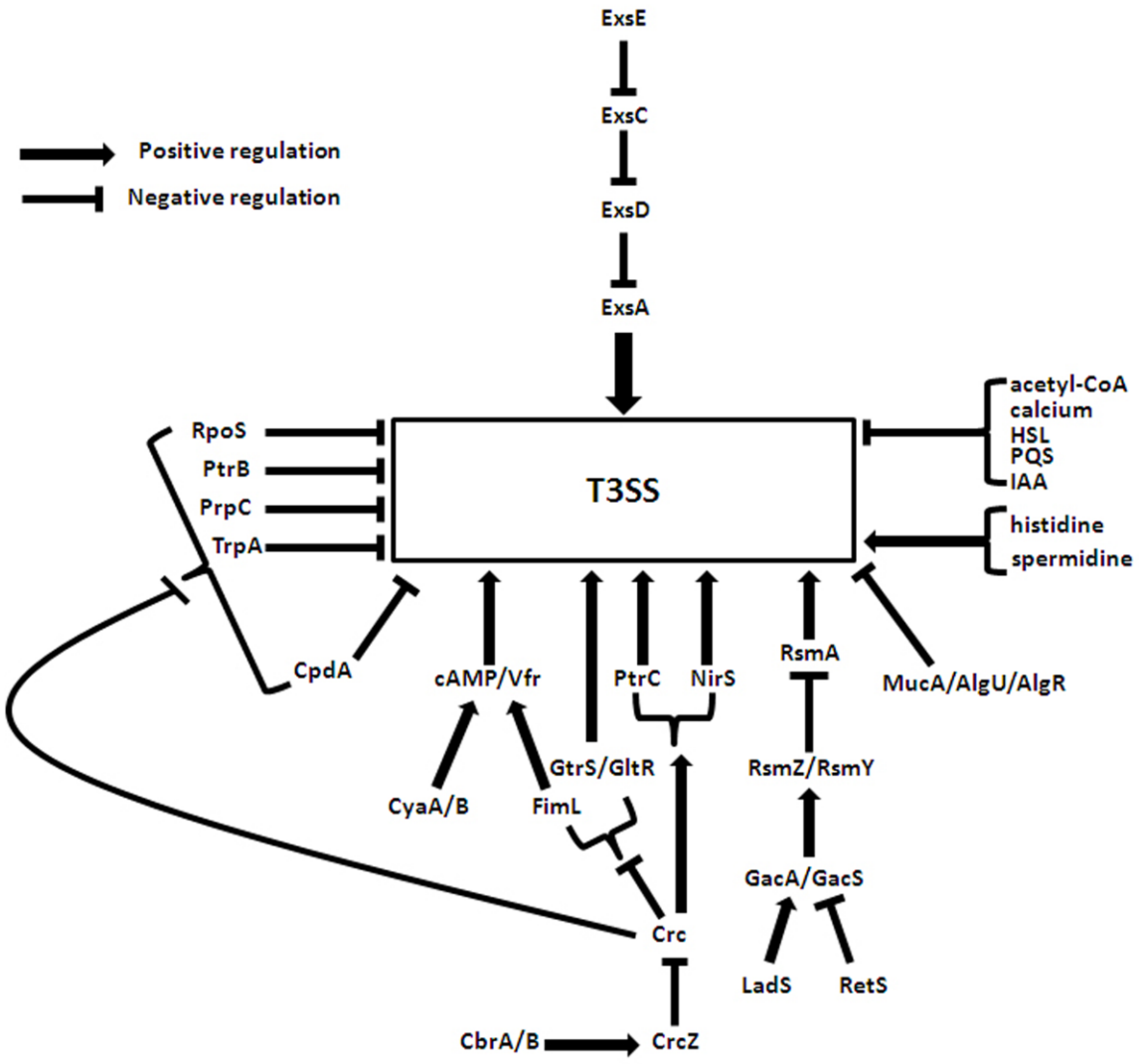


Fig. 1.2. T3SS regulatory network. Model of the known factors that regulate the T3SS.

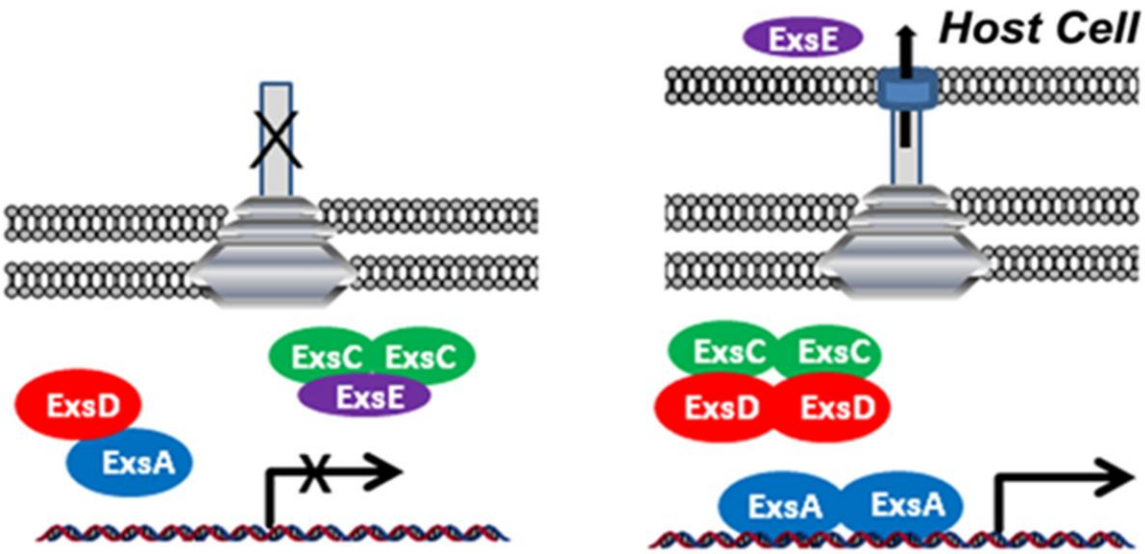


Fig. 1.3. Regulatory cascade of ExsA-dependent activation of the T3SS. Upon host cell contact, ExsE is secreted from the cell, which allows ExsC to bind ExsD. ExsA is now free to initiate transcription.

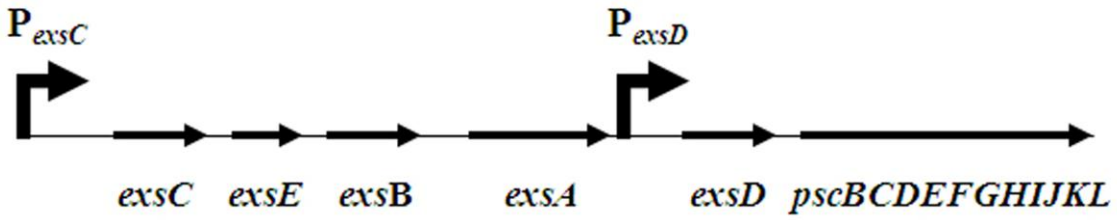


Fig. 1.4. Regulatory and secretory portion of T3SS regulon. ExsA is the transcriptional regulator of the T3SS regulon, which contains the T3SS regulatory genes *exsC*, *exsB*, *exsA*, and *exsD*. The *psc* genes make up part of the T3SS apparatus. ExsA is known to bind to promoters P_{exsC} and P_{exsD} in order to initiate transcription.

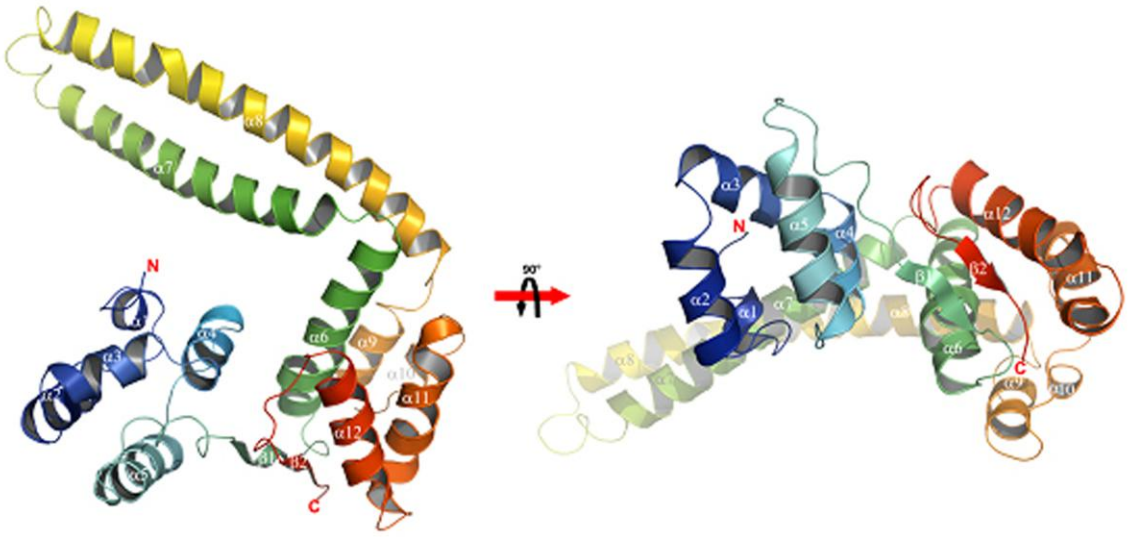


Fig. 1.5. ExsD structure. Two views of the structure of ExsD as a monomer. Bernhards, et al. *Protein Science* (2009).

CHAPTER TWO

Characterization of ExsA, the main transcriptional regulator of the type III secretion system in *Pseudomonas aeruginosa*

Robert C. Bernhards[1], Nancy J. Vogelaar[1], Marcy Hernick[2], Florian D. Schubot[1*]

[1]Virginia Polytechnic Institute and State University
Department of Biological Sciences
125 Life Sciences 1 Building
Washington Street
Blacksburg, VA 24061

[2]Virginia Polytechnic Institute and State University
Department of Biochemistry
305 Engel Hall
West Campus Drive
Blacksburg, VA 24061

***Corresponding author:** Phone: (540) 231-2393
Fax: (540) 231-7126
Email: fschubot@vt.edu

Keywords: ExsA, AraC, *Pseudomonas aeruginosa*, thermoregulation, type III secretion.

Abbreviations: EMSA = electrophoretic mobility shift assay; ExsACDE = ExsA-ExsC-ExsD-ExsE; FA = fluorescence anisotropy; IPTG = isopropyl-β-D-thiogalacto-pyranoside; MBP = maltose binding protein; TCEP = Tris(2-carboxyethyl)phosphine; TEV = tobacco etch virus; T3SS = type III secretion system.

Abstract

ExsA is the main transcription factor or activator of the T3SS system in *Pseudomonas aeruginosa*. ExsA is a member of the AraC/XylS family of transcriptional activators. These proteins are composed of two domains: a C-terminal activation domain and an N-terminal regulatory domain. The activation domain of AraC/XylS-type transcription factors binds to the promoter to initiate transcription. The regulatory domain is the ligand binding domain. In most AraC/XylS-type regulators, small molecules bind to either activate or inhibit the protein. In the case of ExsA, the antiactivator protein ExsD is thought to bind to the regulatory domain and inhibit ExsA function. A variety of ExsA homologs exist in other Gram-negative pathogens of medical relevance, so the study of ExsA could help shed light on the regulatory mechanisms of other T3SS's. Due to this, ExsA is a potential drug target. The activation domain of ExsA homologs is conserved, so initial drug development has targeted the DNA binding domain in hope of finding an inhibitor to DNA binding. Potentially, a drug that effectively inhibits the DNA-binding activity of the ExsA activation domain could be used to treat other types of infections. AraC-type activators have been difficult to purify and crystallize due to their poor solubility. In this study, a new ExsA purification protocol was developed, which led to improved solubility. Fluorescence anisotropy was used to measure the DNA binding activity of ExsA. A dissociation constant (K_d) was determined for ExsA and its P_{exsD} promoter. An *in vitro* transcription assay was also developed to observe ExsA activation with its T3SS promoters. This assay could be used in the future to test drug candidates for their ability to inhibit ExsA-dependent transcription.

Introduction

The type III secretion system (T3SS) is the main virulence mechanism for *Pseudomonas aeruginosa* acute infections. It works as a molecule syringe to inject effector proteins (cytotoxins) directly into the host cell cytoplasm [60]. These effectors aid in infection by preventing phagocytosis by cells of the immune system and allowing for the dissemination of the bacteria throughout the body [30, 59, 63, 74]. *P. aeruginosa* is unable to infect humans without a properly functioning T3SS. ExsA is the main activator of the T3SS in *P. aeruginosa* and is essential for expression of all the genes necessary for type III secretion [31, 75, 103, 120].

ExsA is a member of the AraC/XylS family of transcriptional activators [120]. This family is characterized by the presence of two distinct domains: a C-terminal activation/DNA binding domain and N-terminal regulatory/ligand binding domain. Most AraC/XylS proteins are regulated by the binding of a small molecule. ExsA is different in that its ligand is a protein called ExsD [171]. ExsA sits at the bottom of a regulatory cascade consisting of four interacting proteins: ExsA, ExsD, ExsC, and ExsE [108, 109, 120, 148, 171]. When there is no host cell contact and the T3SS is turned off, ExsA is inhibited by the antiactivator ExsD [171]. Meanwhile, the anti-antiactivator ExsC sequesters ExsE [109, 178]. When host cell contact occurs, ExsE is secreted through the T3SS needle into the host cell [108, 109]. This frees ExsC to now bind to ExsD [148], which allows ExsA to become free and bind to the T3SS promoters as a dimer [167]. ExsA serves to recruit RNA polymerase (RNAP) in order to initiate transcription [149]. ExsA activates transcription from all 10 T3SS promoters, so it is required for the

production of all genes necessary for type III secretion, including the regulators themselves [31, 75, 103, 120].

This study focuses on the interaction between ExsA and its promoters. A new protocol was developed for the purification of recombinant ExsA protein, which greatly improved solubility. This ExsA product did not require a protein fusion tag after purification. The purity and solubility of ExsA was suitable for biochemical testing. First, fluorescence anisotropy was used to measure the binding affinity of ExsA for its P_{exsD} promoter. Then an *in vitro* transcription assay was developed to study T3SS activation by ExsA. ExsA was found to be sufficient to activate T3SS transcription *in vitro* from all the promoters tested.

Results

ExsA purification

Members of the AraC/XylS family of activators are notoriously difficult to purify. This is due to a highly insoluble C-terminal/DNA-binding domain (CTD). When DNA is not present, these proteins have very low solubility, making them extremely difficult to purify. Due to this, we decided to engineer a construct that would fuse maltose binding protein (MBP) to ExsA. MBP is known to help other proteins fold properly, which improves their solubility. To do this, we utilized a His_6-MBP expression plasmid [180]. ExsA was insertionally cloned into the plasmid directly downstream of MBP. This produced a His_6-MBP-ExsA construct, termed pFS-HMBPExsA. This plasmid was transformed into an *E. coli* expression strain. Cultures were grown at 37 °C and overexpression was induced at a low temperature (18 °C) for six hr. Purification

included a Ni^{2+}NTA affinity column, anion exchange, and cation exchange all before removing the His_6-MBP tag. The fusion protein was cut using TEV protease, which recognizes a TEV cleavage sequence in between MBP and ExsA. Then a second Ni^{2+}NTA affinity column was run, this time to purify ExsA away from His_6-MBP. The final purification step was gel filtration. This produced a highly purified ExsA sample with improved solubility (Fig. 2.1). This ExsA sample can be stored at -80 °C for years and still retains activity. Aliquoting and storing at -80 °C is highly recommended because ExsA will start to unfold if left at 4 °C for more than 24 hr. The ExsA sample is highly active and suitable for biochemical assays as long as it is thawed and used within 24 hr to ensure full activity.

ExsA promoter binding

We sought out to measure the affinity of ExsA for its promoters in order to determine if there was a correlation between promoter affinity and transcriptional activity. The first promoter we wanted to test was the P_{exsD} promoter. This promoter is interesting in that it regulates the transcription of the antiactivator ExsD [171, 181]. Fluorescence anisotropy (FA) was used to determine a dissociation constant (K_d) for the P_{exsD} promoter. FA is a technique that utilizes a fluorescent probe attached to a ligand, which in this case is the P_{exsD} promoter. We used a 60 base pair segment of P_{exsD} that was attached to a TAMRA probe. A titration of ExsA was performed, and the concentration of P_{exsD}-TAMRA was held constant. The resulting K_d was 8.5 ± 2 nM (Fig. 2.2). This K_d is indicative of a very tight interaction common to transcriptional activators [182]. During the course of our study, the affinity for ExsA and three of its promoters was determined by another group using an electrophoretic mobility shift assay (EMSA) [167]. Due to

this, we decided not to further test other promoters. However, EMSA is not an equilibrium technique, meaning that ExsA is not free to bind and dissociate from the promoter under the experimental conditions. FA is an equilibrium technique. The K_d we obtained is two-fold higher than the one obtained via EMSA (4.1 ± 0.2 nM) [167]. Our K_d value is actually closer to what was obtained for AraC, using a fluorescence quenching equilibrium technique [182].

ExsA is sufficient to initiate T3SS transcription *in vitro*

An *in vitro* transcription assay was developed in order to see if ExsA alone was sufficient to activate T3SS transcription. Native RNAP from *P. aeruginosa* was purified and used in this assay, along with purified ExsA, a P_{exsD} promoter template, and nucleoside triphosphates (NTPs). Radiolabeled UTP is also added, which gets incorporated into the resulting mRNA transcript. These transcripts were run on a gel and quantified. A 0-15 min time course was performed with each reaction containing 133 nM ExsA (Fig. 2.3). This experiment shows that ExsA is necessary and sufficient for efficient transcriptional activation at the P_{exsC}, P_{exsD}, and P_{exoS} promoter sites. This also further demonstrates that the recombinant ExsA is highly active. The next step is to test whether ExsD is sufficient to inhibit ExsA-dependent transcription in this assay.

Discussion

Proteins belonging to the AraC/XylS family have characteristically low solubility and are extremely difficult to purify. As a result, there are currently only two solved structures out of the 830-plus family members [156, 157]. This highlights the importance of the newly developed purification protocol for ExsA. Furthermore, ExsA belongs to a

medically important subfamily of transcriptional activators that regulate virulence factors in a variety of Gram-negative pathogens. These include ExsA from *Vibrio parahaemolyticus,* InvF from *Salmonella enterica* serovar Typhimurium, LcrF in *Yersinia spp.*, Rns from enterotoxigenic *E. coli* (ETEC), ToxT from *V. cholerae*. T3SS1 of *V. parahaemolyticus* is regulated by a similar ExsACDE regulatory cascade [166]. InvF from *S. enterica* regulates the T3SS located on the SPI–1 pathogenicity island [158]. LcrF activates the T3SS in all three pathogenic *Yersinia* species: *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* [159, 160]; although it is unknown whether a regulatory protein binds to LcrF. However, it is know that LcrF is thermoregulated through post-translational mechanisms [161]. Rns regulates fimbriae expression in ETEC [162], and ToxT controls expression of cholera toxin in *V. cholerae* [163]. As one can anticipate, what is learned about ExsA can be translated to these other important Gram-negative pathogens. Given that ExsA can be purified with relatively good solubility, it can serve as a model protein for this subfamily. Recently, using the expression and purification protocols outlined in this study, the structure of the amino terminal domain of ExsA (ExsA-NTD) was solved, and current work is being done to crystallize full-length ExsA in complex with DNA. This study demonstrates that recombinant ExsA is properly folded and highly active. It was shown to bind to the P_{exsD} promoter with high affinity. ExsA was also shown to be sufficient to activate *P. aeruginosa*-specific T3SS transcription in an *in vitro* transcription assay. This assay serves as a reliable tool and was used in the following chapters to study the ExsD–ExsA inhibitory mechanism.

With a better understanding of how ExsA interacts with its promoters and activates transcription, drugs can be developed to target ExsA and prevent it from binding to the T3SS promoters. These drugs can potentially be used to treat infections from all of the aforementioned bacterial pathogens.

Materials and Methods

ExsA expression and purification

ExsA was overexpressed in *E. coli* from a vector constructed by Gateway recombinational cloning (Invitrogen, Carlsbad, CA, USA). A tobacco etch virus (TEV) protease recognition site and the appropriate att recombination sites (attB1 and attB2) were added to the *exsA* gene during PCR, and the amplicons were subsequently recombined into pDONR201 (Invitrogen). The nucleotide sequences of the ORFs were verified, then recombined into the destination vector pDEST-HisMBP [180] to create the expression vector pFS-HMBPExsA. This vector was designed to produce ExsA as a fusion to the C-terminus of an N-terminally His_6-tagged *E. coli* maltose-binding protein (MBP).

A single colony of *E. coli* BL21(DE3) CodonPlus RIL cells (Stratagene, La Jolla, CA, USA) containing the pFS-HMBPExsA expression plasmid was used to inoculate 125 mL of Luria broth (LB) supplemented with 2 g/L dextrose, 100 µg/mL ampicillin, and 30 µg/mL chloramphenicol. The cultures were grown with shaking (225 rpm) to saturation overnight at 37 °C and then diluted 66-fold into 6 L of fresh medium. They were grown to an OD_{600} of 1.0 then induced with IPTG at a final concentration of 1 mM. The induction temperature was 18 °C, and the cultures were shaken for six hr. Cells were harvested by centrifugation at 5,000 x g for 15 min. The cell pastes were resuspended in

200 mL of 500 mM NaCl, 25 mM imidazole, 50 mM Tris-HCl (pH 7.4), 2 mM DTT (buffer A), along with three tablets of Complete, EDTA-free Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA). The cells were lysed via sonication and centrifuged at 40,000 x g for 25 min. The supernatants were filtered through 0.45-μm polyethersulfone membranes and applied to a 30 mL Ni-NTA Superflow affinity column (Qiagen, Valencia, CA, USA) equilibrated with buffer A. During the run, the column was washed with five column volumes of buffer A, and proteins were eluted with a linear gradient from 25 to 250 mM imidazole (pH 7.4). The His_6-MBP-ExsA fusion protein sample was then dialyzed against a buffer of 50 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM DTT and loaded onto a HiTrap Q HP column (GE Healthcare) that had been equilibrated with the same buffer. The His_6-MBP-ExsA fusion protein was eluted using a linear NaCl gradient from 0.05 M to 1 M. The sample was dialyzed against 2 L of 45 mM NaCl, 25 mM Tris-HCl (pH 7.15), and 2 mM DTT (buffer B) overnight. The sample was then loaded onto a HiTrap Heparin HP column (GE Healthcare) equilibrated in buffer B and eluted with a 0.05 M to 1 M gradient of NaCl. The NaCl concentration in the His_6-MBP-ExsA sample was adjusted to 0.5 M, and the fusion protein was digested with 3 mg of His-tagged TEV(S219V) protease [183] at 4 °C overnight. The TEV protease cleaves at the TEV recognition site located between MBP and ExsA. After digestion, ExsA was run through a second Ni-NTA Superflow affinity column to remove both the His_6-MBP tag and the protease, using the same buffers as the first Ni-NTA column. The ExsA sample was collected in the flow through. Finally, gel filtration using a HighLoad 26/60 Superdex 200 prep grade column (GE Healthcare) was performed with the ExsA sample using 500 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM TCEP

(ExsA storage buffer). The sample was concentrated to 1 mg/mL, flash-frozen using liquid nitrogen, and stored at -80 °C.

Fluorescence anisotropy

A 60 base single strand of the P_{exsD} promoter fragment attached to a 5´–TAMRA probe was purchased from Integrated DNA Technologies, Coralville, IA, USA: 5´–TAMRA–GGAAGGACGAATGCCGGGCTAAAAATAACTGACGTTTTTTGAAAGCCCGGTAGCGGCTGC–3´. This region contains both ExsA binding sites. The complementary strand (5´–GCAGCCGCTACCGGGCTTTCAAAAAACGTCAGTTATTTTTAGCCCGGCATTCGTCCTTCC–3´) was annealed by incubation at 75 °C for 10 min and cooling at room temperature for 5 min. The experiment consisted of individual samples of a titration of ExsA with a final concentration ranging from 0 to 50.7 nM. Each sample also contained a final concentration of 150 mM NaCl, 25 mM Tris-HCl pH 7.4, 0.6 mM TCEP pH 8.0, and 10 nM P_{exsD}-TAMRA. The final volume of each sample was 30 µL. Each sample was pipetted into separate wells in a 384 well, flat bottom, black polystyrene assay plate (Corning, Corning, NY, USA). Measurements at Ex. 559 nm and Em. 600 nm were taken using SPECTRAmax M5e ROM v2.00b255 with SoftMax Pro 5.2 software. The K_d was determined by data analysis using XLfit (IDBS, Bridgewater, NJ, USA). The data points were fitted using the Boltzmann constant. The experimental samples were in triplicate.

RNA polymerase purification and specific activity determination

RNA polymerase (RNAP) was purified from *P. aeruginosa* PAO1 cells following the original procedure of Allan and Kropinski [184]. However, changes were made to the later chromatographic steps. All purification steps were performed at 4 °C. *P. aeruginosa* PAO1 cultures were grown in LB broth to an OD_{600} of 0.8, harvested by centrifugation at 6,000 x g, then lysed by sonication. The cell debris was removed by centrifugation at 35,000 x g for 30 min, and 25% polyethyleneimine (pH 7.5) was added to the supernatant to a final concentration of 0.5% in order to precipitate the RNAP. The supernatant was centrifuged at 35,000 x g for 30 min. The polyethyleneimine precipitate was washed with 10 mM Tris-HCl (pH 8.0), 250 mM NaCl, 5% glycerol, 0.05 mM EDTA, 1 mM DTT, and 0.1 mM PMSF (wash buffer) and centrifuged at 35,000 x g for 30 min. RNAP was released by resuspending the pellet in 10 mM Tris-HCl (pH 8.0), 800 mM NaCl, 5% glycerol, 0.05 mM EDTA, 1 mM DTT, and 0.1 mM PMSF (release buffer) and centrifuged at 25,000 x g for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 30%, followed by gentle stirring for one hr, and centrifugation at 35,000 x g for 30 min. Additional ammonium sulfate was then added to bring the supernatant to 60% saturation. After a second centrifugation at 35,000 x g for 30 min, the pellet was resuspended in 1 mL wash buffer per liter of original culture. The suspension was dialyzed versus 2 L wash buffer overnight. The dialyzed RNAP sample was centrifuged, and the supernatant was filtered in preparation for gel filtration. The sample was run through a Sephacryl S-300 HR column (GE Healthcare) using wash buffer. The fractions were analyzed by SDS-PAGE and collected to run on a Hi-Trap Heparin HP column (GE Healthcare) using a loading buffer composed of 10 mM Tris-

HCl (pH 8.0), 250 mM NaCl, 5% glycerol, 0.05 mM EDTA, and 1 mM TCEP. RNAP was eluted using a linear gradient of 0.25 M to 1 M NaCl. Fractions were analyzed via SDS-PAGE, pooled, and concentrated to 1 mg/mL of total protein. Glycerol was added to a final concentration of 50%. RNAP was aliquoted and stored at -20 °C.

The specific activity of the purified *P. aeruginosa* RNAP was determined by comparing its activity to a standard curve generated with different amounts of *E. coli* RNA polymerase holoenzyme (Epicentre Biotechnologies, Madison, WI, USA) using an ExsA-independent RNA-1 promoter which produces a 108 base transcript [185].

***In vitro* transcription assay**

The P_{exsC} promoter template encompassed positions -268 to 123 of the P_{exsC} promoter, relative to the transcription start site; and from this template, RNA polymerase synthesizes a 154 base mRNA transcript. The P_{exsC} template was produced by PCR using forward primer 5´-CGGGAAGGAGAGGTCAACGC-3´ and reverse primer 5´-CAGGAGGCTCGCCATGC-3´. The P_{exsD} promoter template encompassed positions -207 to 94 of the P_{exsD} promoter and it codes for an 82 base mRNA transcript. The P_{exsD} template was produced by PCR using forward primer 5´-CATCAGTTGCTGCTCAACAGCG-3´ and reverse primer 5´-CACCGCTTCTCGGGAGTACTGC-3´. The P_{exoS} promoter template encompassed positions -126 to 112 of the P_{exoS} promoter and coded for a 137 base mRNA transcript. The P_{exoS} template was produced by PCR using forward primer 5´-CCTCAATCTGTCCCAAACGCCC-3´ and reverse primer 5´-GCAGTGCCAGCCCGGAGAGAC-3´. All three PCR products were run on 2% agarose gels and purified using the Wizard SV Gel and PCR Clean-up System (Promega,

Madison, WI, USA). Each 30 µL transcription assay reaction contained 4.4 fM of promoter template, 3.3 U purified RNA polymerase from *P. aeruginosa* (see above), 1 U RiboGuard RNase Inhibitor (Epicentre Biotechnologies), 15 ng/µL poly(deoxyinosinic-deoxycytidylic) acid (to prevent non-specific transcription initiation), 133 mM NaCl, 32 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 25 µM EDTA, 0.9 mM TCEP, 0.2 mM DTT, and 15.5% glycerol. Each experiment contained 133 nM ExsA. Samples were mixed and allowed to equilibrate at room temperature for five min. To start the reaction, 3 µL NTPs (stock concentrations of 200 µM ATP, CTP, GTP and 40 µM UTP) mixed with 0.2 µL (0.2 µCi) of 3.3 mM P^{32}-alpha UTP was added to each sample, and samples were incubated at 30 °C. The reactions were stopped by adding 12 µL 1X stop solution (3M ammonium acetate, 50 mM EDTA, 0.11 mg/mL glycogen). Then 170 µL 100% cold ethanol was added, and the samples were incubated at -20 °C for one hr. Following centrifugation at 12,000 x g for 15 min, the supernatant was discarded and pellets were resuspended in 12 µL 1X TBE (Tris/Borate/EDTA)-urea sample buffer and heated at 70 °C for five min. After a brief centrifugation, the samples were loaded onto a 10% TBE-urea gel and run at 200 mV for 60 min. Gels were exposed to a storage phosphor screen (GE Healthcare) for 16 hr. The phosphor screen was scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare), and gel bands were quantified using Image Quant TL v2005 (Amersham Biosciences, Piscataway, NJ, USA). Each experiment was performed in triplicate.

Acknowledgements

This study was supported by the American Heart Association (09SDG2260401 to FDS).

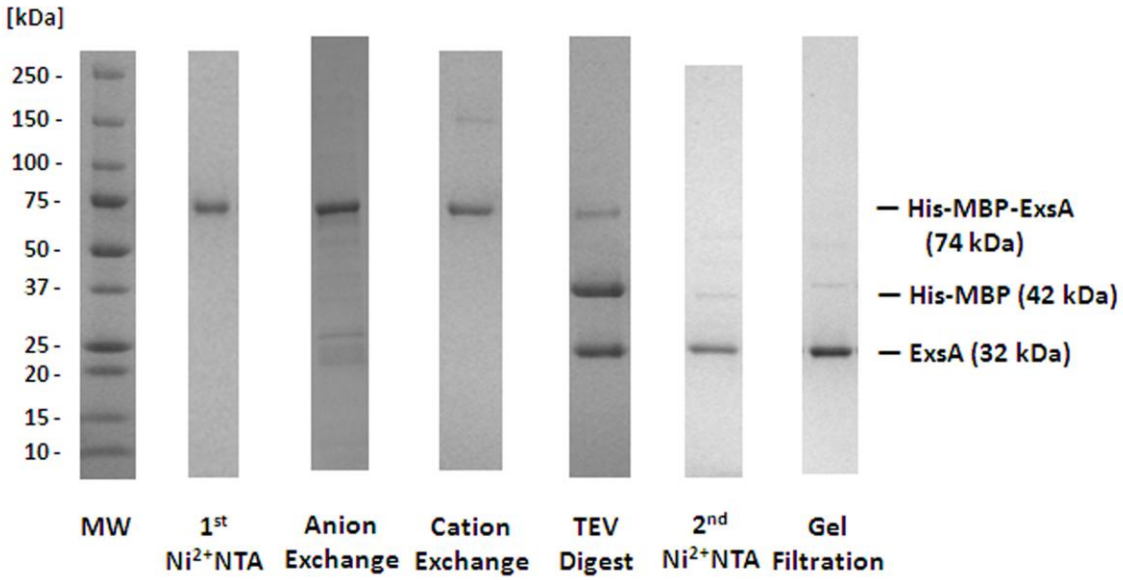


Fig. 2.1. ExsA purification. Each gel strip represents the protein sample after the indicated purification steps.

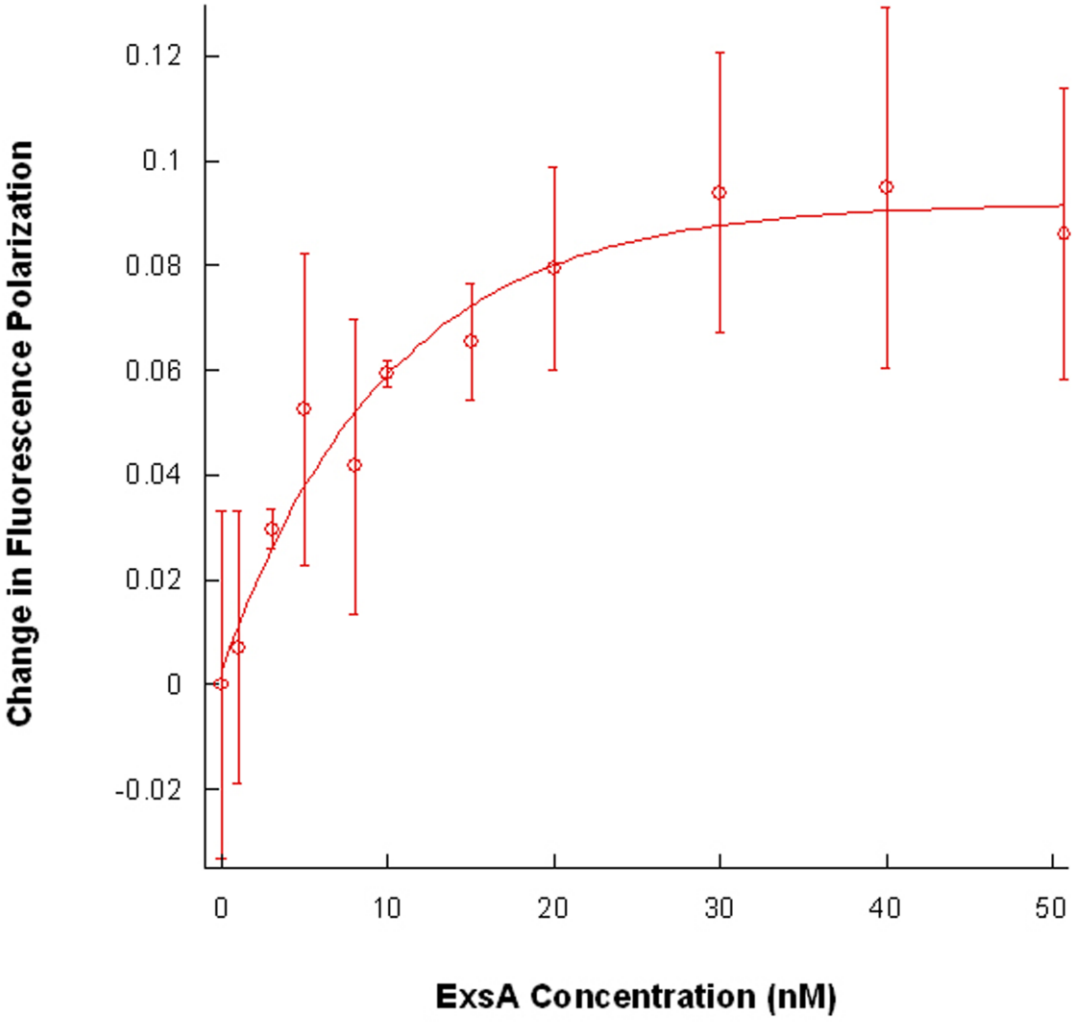


Fig. 2.2. ExsA promoter affinity. Fluorescence anisotropy was used to measure the affinity of ExsA for its P_{exsD} promoter. The resulting K_d was 8.5 ± 2 nM. This experiment was performed in triplicate.

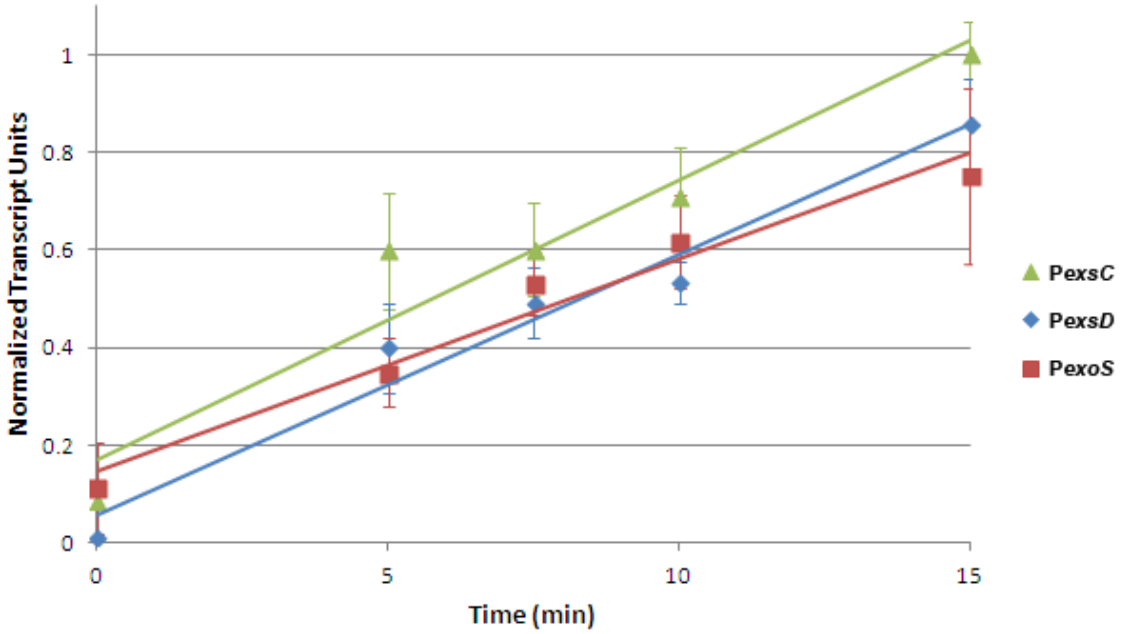


Fig. 2.3. T3SS transcriptional activation by ExsA. Recombinant ExsA is capable of activating T3SS transcription from the P_{exsC}, P_{exsD}, and P_{exoS} promoters in an *in vitro* transcription assay using native RNAP from *P. aeruginosa*.

CHAPTER THREE

Self-trimerization of ExsD limits inhibition of the *Pseudomonas aeruginosa* transcriptional activator ExsA *in vitro*

Robert C. Bernhards[1], Anne E. Marsden[2], Shannon K. Esher[1], Timothy L. Yahr[2], and

Florian D. Schubot[1*]

FEBS Journal **280** (2013) 1084–1094

[1]Virginia Polytechnic Institute and State University
Department of Biological Sciences
125 Life Sciences 1 Building
Washington Street
Blacksburg, VA 24061

[2]University of Iowa
Department of Microbiology
540B Eckstein Medical Research Building
Iowa City, IA 52242

[*] **Corresponding author:** Phone: (540) 231-2393
Fax: (540) 231-7126
Email: fschubot@vt.edu

Keywords: ExsA, ExsD, *Pseudomonas aeruginosa*, thermoregulation, type III secretion.

Abbreviations: CD = circular dichroism; DSF = differential scanning fluorimetry; ITC = isothermal titration calorimetry; DTT = dithiothreitol; EMSA = electrophoretic mobility shift assay; ExsACDE = ExsA-ExsC-ExsD-ExsE; IPTG = isopropyl-β-D-thiogalacto-pyranoside; MBP = maltose binding protein; TCEP = Tris(2-carboxyethyl)phosphine; TEV = tobacco etch virus; T3SS = type III secretion system.

Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* ranks among leading causes of nosocomial infections. The type III secretion system (T3SS) aids acute *P. aeruginosa* infections by injecting potent cytotoxins into host cells to suppress the host's innate immune response. Expression of all T3SS-related genes is strictly dependent upon the transcription factor ExsA. Consequently, ExsA and the biological processes that regulate ExsA function are of great biomedical interest. The presented work focuses on the ExsA-ExsC-ExsD-ExsE signaling cascade that ties host cell contact to the up-regulation of T3SS gene expression. Prior to T3SS induction, the antiactivator protein ExsD binds to ExsA and blocks ExsA-dependent transcription by interfering with ExsA dimerization and promoter interactions. Upon host cell contact, ExsD is sequestered by the T3SS chaperone ExsC resulting in the release of ExsA and an up-regulation of the T3SS. Previous studies have shown that the ExsD-ExsA interactions are not freely reversible. Because independently folded ExsD and ExsA were not found to interact, it has been hypothesized that folding intermediates of the two proteins form the complex. Here we demonstrate for the first time that ExsD alone is sufficient to inhibit ExsA-dependent transcription *in vitro* and that no other cellular factors are required. More significantly, we show that independently folded ExsD and ExsA are capable of interacting, but only at 37 °C and not at 30 °C. Guided by the crystal structure of ExsD, we designed a monomeric variant of the protein and demonstrate that ExsD trimerization prevents ExsD from inhibiting ExsA-dependent transcription at 30 °C. We propose that this unique mechanism plays an important role in T3SS regulation.

Introduction

The opportunistic human pathogen *Pseudomonas aeruginosa* poses a significant medical threat due to its high levels of natural and acquired antibiotic resistance [8, 42, 186-190]. *P. aeruginosa* utilizes a broad array of virulence mechanisms to establish and sustain infections. The type III secretion system (T3SS) is a hallmark of acute infections and aids infection by translocating at least four distinct effector proteins into the eukaryotic host cell [191-193]. Inside the host, these effectors act to subvert the host-immune response by interfering with critical signal transduction pathways [31, 194-200]. The needle complex that constitutes the secretion apparatus is assembled from multiple copies of 27 distinct proteins. Protein translocation through the T3SS is powered by the proton motive force, while a cytoplasmic ATPase (PscN in *P. aeruginosa*) is thought to mediate targeting and unfolding of the transported effectors at the base of the needle complex [201, 202]. Because expression, assembly, and operation of the T3SS are energy-intensive, T3SS-related gene expression is tightly regulated via a number of regulatory pathways and closely tied to host infection [108, 109, 130]. The ExsA-ExsC-ExsD-ExsE (ExsACDE) signaling cascade constitutes perhaps the most direct link between opening of the T3SS channel and activation of T3SS-gene expression [108]. The AraC-type transcriptional activator, ExsA, facilitates the recruitment of RNA polymerase to the transcription initiation site and is required for transcription from all 10 T3SS-related promoters including its own expression as well as genes of the other members of the signaling cascade: *exsC*, *exsE*, and *exsD* [120]. While unusual, the underlying regulatory mechanism appears to be relatively straightforward: Prior to the host cell contact-induced opening of the secretion channel, ExsC and ExsE form a tight

2:1 complex, while the antiactivator ExsD sequesters ExsA to prevent transcription activation [171]. Host cell contact triggers the opening of the T3SS channel. Now, ExsE is secreted thereby releasing ExsC, which in turn binds to ExsD to activate ExsA-mediated transcription [108, 109].

Recent work has focused on the question of how ExsD inhibits ExsA function. Thibault *et al.* determined that ExsD and ExsA form a 1:1 complex which fails to bind to ExsA-dependent promoters *in vitro*, suggesting that ExsD interferes with ExsA-promoter interactions [147]. Brutinel *et al.* subsequently discovered that ExsD also interferes with ExsA self-association using a monohybrid study [172]. While both phenomena have not been connected experimentally, the enhancement of DNA binding affinity through self-association is a widespread feature of DNA binding protein factors. In both studies, researchers reported an unusual feature of the signaling mechanism. ExsD could only bind to ExsA when both proteins were synthesized at the same time. Thibault *et al.* unsuccessfully added ExsD to see if it would interfere with ExsA-DNA interactions in EMSA studies [147]. When attempting to reconstitute the entire signaling cascade *in vitro*, Brutinel *et al.* observed that ExsC was indeed capable of dissociating the ExsD-ExsA complex [172]. Perhaps mirroring a scenario where the bacterial cell loses host cell contact, the addition of ExsE to the sample readily brought about the formation of an ExsC-ExsE complex. However, the released ExsD protein was not able to rebind to ExsA, suggesting that the signaling process is not freely reversible. To explain this phenomenon, it was proposed that concurrent expression of both ExsD and ExsA might be required, because folding intermediates of either ExsD, ExsA, or both proteins might actually associate to form this complex [172]. Under this scenario, dissociation of the

ExsD-ExsA complex would allow for complete folding of the subunit(s) and create a formidable kinetic barrier preventing reassociation of the complex. In the present study, we demonstrate that, rather than the folding of either protein, it is ExsD self-trimerization that accounts for the observed irreversible dissociation of the ExsD-ExsA complex. We also demonstrate that this barrier may be overcome by shifting the temperature from 30 to 37 °C.

Results

ExsA-mediated *in vitro* transcription is not inhibited by wild-type ExsD at 30 °C, but is strongly inhibited at 37 °C

The above cited work demonstrated that ExsD interferes with ExsA dimerization and ExsA-promoter interactions [147]. *In vitro* transcription studies subsequently confirmed that ExsA is necessary and sufficient for activation of T3SS promoters [149]. Yet, what has not been explicitly shown is that ExsD alone can block ExsA-mediated transcription *in vitro*. We believe that this distinction is important, because the interaction of ExsA with RNA polymerase could significantly impact the interactions between ExsD and ExsA; for example, by stabilizing the ExsA dimer or binding of ExsA to the promoter region. We designed and optimized an efficient *in vitro* transcription assay using purified ExsA, ExsD, and *P. aeruginosa* RNA polymerase. In the process, we also developed a new expression and purification protocol for the transcriptional regulator ExsA, which produces a highly homogeneous sample suitable for structural studies. Unlike previous preparations, ExsA purified according to the described protocol does not require detergent and may be concentrated up to 50 μM. Figure S3.1 in the

supplemental material contains the SDS-polyacrylamide gel lanes of all three purified samples. Our initial *in vitro* transcription experiments closely mirrored the protocol used in previously published experiments [149]. As anticipated, activation of the P_{exsD} promoter requires the presence of ExsA, and it was established that transcript production proceeded in a linear fashion up to at least 20 min under the given experimental conditions (Supplementary Fig. S3.2). Also, confirming results of previous studies, even the addition of vast excess of ExsD (up to 50 μM) had no significant effect on the rate of transcription (Fig. 3.1a). We now applied this assay to examine previously unexplored parameters of ExsA-dependent transcription. Although temperature is likely important in the context of infection, to this point all studies of the ExsACDE cascade, including our initial assays, had been performed at or below 30 °C. Because the body temperature of a human host is approximately 37 °C, we conducted a second set of *in vitro* transcription assays at this temperature. At 37 °C, the overall rate of ExsA-dependent transcription increased by about 13% compared to the 30 °C assay experiment (Supplementary Fig. S3.3). When ExsD was included in the assay at the higher temperature, ExsD now strongly inhibited ExsA-dependent transcription, suggesting that the elevated temperature had alleviated the kinetic barrier that had previously prevented ExsD-ExsA interactions (Fig. 3.1a). Dose-response studies yielded a half maximal inhibitory concentration (IC_{50}) value of 7.7 μM for ExsD under the given experimental conditions, which is indicative of a relatively weak inhibitor (Fig. 3.1b). To determine if the observed effect of ExsD is specific to ExsA-dependent transcription, we repeated the *in vitro* transcription reactions, this time using a template containing the constitutively expressed RNA-1 promoter [185]. ExsD had no effect on transcript levels from this promoter at 37 °C (Supplementary Fig.

S3.4), thus demonstrating that the observed inhibition is specific to ExsA-dependent promoters.

A single mutation generating the ExsD^{M59R} variant disrupts ExsD trimer formation

The studies described above demonstrate that independently folded ExsD and ExsA interact *in vitro* at 37 °C. While it was still conceivable that the elevated temperature causes partial unfolding of either protein to permit binding as the original model posits, we sought to test an alternative hypothesis. A striking feature of ExsD is the apparent plasticity of its oligomeric state depending on the interacting partner. ExsD forms a 2:2 complex with ExsC and a 1:1 complex with ExsA, while analytical ultracentrifugation studies and the ExsD crystal structure suggest that ExsD self-associates to form a trimer in absence of the other two proteins [173, 177]. Our recent work suggests that the ExsC-ExsD complex actually consists of two ExsD monomers bound to an obligate ExsC dimer, rather than a dimer of dimers [178]. Therefore, ExsD appears to either form a homotrimer or enter 1:1 interactions with ExsC or ExsA, indicating that the mutually exclusive interactions of ExsD with ExsA and ExsC also compete with ExsD self-association. We hypothesized that dissociation of the ExsD trimer at 37 °C prior to ExsA binding might account for the unusual kinetic phenomenon that prevents the association of the two proteins at 30 °C. To test this hypothesis, we sought to engineer a monomeric variant of ExsD by disrupting the trimer through site-directed mutagenesis. We have previously reported the crystal structure of ExsD which contained three molecules in the asymmetric unit related by an almost perfect three-fold symmetry axis [173]. In the trimer, each ExsD molecule forms two distinct protein-protein interfaces, both covering approximately 1200 $Å^2$ of surface area. Guided by the

detailed structural maps of these interfaces, we focused our efforts on a hydrophobic patch formed by residues from helix α1 of one molecule and helices α6 and α9 from the other molecule (Fig. 3.2a). Because Met59 and Met217 are positioned at the heart of this interface, we decided to initially target these two residues. We reasoned that replacing one or both of these residues with amino acids possessing large charged side chains should not only disrupt the interface, but also increase the polarity of this part of the structure to prevent aggregation of the variant protein due to the exposure of a large hydrophobic surface. Both single point mutants were constructed, however, because the ExsD^{M59R} variant already displayed the desired properties, the ExsD^{M217R} variant was not characterized. Following purification of the variant protein, we conducted an analytical gel filtration study to estimate the approximate molecular weight of the ExsD^{M59R} variant (Fig. 3.2b). Using a calibration curve obtained from analyzing a set of standard proteins, the retention time of the variant protein gave an apparent molecular weight of 42 kDa. While this is somewhat larger than the actual 32 kDa mass of an ExsD monomer, the discrepancy is readily explained by the distinctively non-globular shape of the molecule [173]. In contrast, wild-type ExsD (Fig. 3.2b) eluted significantly earlier from the gel filtration column and gave an estimated molecular weight of 102 kDa, consistent with a homotrimeric complex as previously reported [173].

To examine if the ExsD^{M59R} variant had undergone a dramatic conformational change as a result of the mutation, we also compared circular dichroism spectra of wild-type ExsD and the variant. The spectra are virtually identical (Fig. 3.2c), thus indicating the observed difference in elution volumes from the gel filtration column is due to an altered oligomeric state of ExsD^{M59R} rather than a conformational change.

Monomeric ExsD^{M59R} efficiently inhibits ExsA-dependent transcription *in vitro* at 30 °C

After confirming that the engineered ExsD^{M59R} variant was indeed monomeric, we tested this protein in our *in vitro* transcription assay at 30 °C (Fig. 3.3). In agreement with our hypothesis, this variant strongly inhibits ExsA-dependent transcription even at 30˚C. A dose-response curve for ExsD^{M59R} produced an IC_{50} value of approximately 0.5 μM, which is 15-fold lower than that obtained for wild-type ExsD at 37 °C under otherwise identical conditions. This indicates that ExsD^{M59R} is a stronger inhibitor than wild-type ExsD. In order to verify that ExsD^{M59R} is specific for inhibiting ExsA-dependent transcription, an ExsA-independent RNA-1 promoter template was also tested at 30 °C. As anticipated, ExsD^{M59R} had no impact on the transcript levels when this promoter was used (Supplementary Fig. S3.4).

ExsD^{M59R} disrupts ExsA dimerization, but does not interfere with ExsA promoter binding

We replicated two experiments previously conducted with wild-type ExsD to directly examine the effect of ExsD^{M59R} on ExsA dimerization and DNA binding. In these assays, ExsD^{M59R} behaved in a manner that is indistinguishable from the wild-type protein. Using a bacterial monohybrid assay, we were able to demonstrate that ExsD^{M59R} expression efficiently disrupts ExsA dimerization (Fig. 3.4). However, in subsequent EMSA studies, ExsD^{M59R} did not measurably interfere with ExsA-DNA interactions (data not shown). Even pre-incubating ExsA and ExsD^{M59R} at 37 °C prior to running the EMSA gel did not affect the outcome of the assay. Based on the *in vitro* transcription

results, we had anticipated that the ExsD variant would also interfere with ExsA-DNA binding interactions despite not being co-expressed with ExsA.

Discussion

The ExsACDE signaling cascade displays a number of remarkable features, the most striking perhaps being that signal transduction does not involve the type of phosphate transfer events that mediate most of the signaling between extracellular milieu and bacterial gene expression. Instead, signal transduction in the ExsACDE pathway was shown to be based entirely on the competitive association and dissociation of various bimolecular complexes formed by the four involved proteins [109, 148, 171]. The newly discovered role of ExsD trimer formation in the regulation of T3SS gene expression adds an intriguing new wrinkle to the mechanism. Interestingly, as ITC studies have shown, ExsD self-association does not interfere with heterocomplex formation between ExsC and ExsD, suggesting that the affinity of the ExsC-ExsD complex is sufficiently high to overcome this obstacle [177]. The actual K_d for the ExsD-ExsA interactions is not known, however, consistent with its position at the bottom of the regulatory cascade, the complex is readily disrupted by ExsC [172]. Our data suggest that the affinity of the ExsD-ExsA complex falls perhaps in the hundreds of nanomolar range. Therefore, one would anticipate that the requirement for dissociation of the ExsD trimer prior to binding poses a more significant barrier for the ExsD-ExsA complex.

The thermoregulatory effect observed in the *in vitro* transcription assay appears to be produced by the self-association of ExsD into trimers. While the $ExsD^{M59R}$ variant has lost the ability to trimerize, we do not believe that the temperature increase from 30

to 37 °C causes a dramatic shift in the monomer-trimer equilibrium of wild-type ExsD. Rather, we propose that the temperature increase weakens this homotrimer sufficiently to permit the thermodynamically favored association of ExsD and ExsA. Two pieces of experimental data support the idea that ExsD is still primarily trimeric at 37 °C. The IC_{50} value for the inhibition of ExsA-dependent transcription by wild-type ExsD is significantly larger than that observed for the $ExsD^{M59R}$ variant, suggesting that the wild-type protein still has a poorer affinity for ExsA. Second, our differential scanning fluorimetry experiments reveal that the $ExsD^{M59R}$ variant itself is significantly more temperature sensitive than the wild-type protein, presumably because the additional surface areas buried at the trimer interfaces stabilize the wild-type protein (Fig. 3.2d). Even though the melting temperature (T_m) of a protein depends on many factors, such as buffer conditions and the presence of ligands, we observed a striking drop in T_m for the variant, which would indicate that monomeric ExsD is not stable at 37 °C unless it is associated with a different protein, such as ExsA or ExsC.

The observation that $ExsD^{M59R}$ did not affect ExsA-DNA interactions in the EMSA experiments is intriguing. In conjunction with the results of our *in vitro* transcription experiments, which indirectly demonstrate that $ExsD^{M59R}$ does interact with ExsA at 30 °C, these findings appear to suggest that the presence of RNA polymerase is required for the association of ExsD and ExsA. Perhaps the binding of ExsA to RNA polymerase triggers a conformational change in the transcription factor that reveals the otherwise obscured ExsD binding site.

Even though ExsD self-association has been documented in two independent studies, its biological role is not clear [173, 177]. We carried out an initial comparison of

wild-type *P. aeruginosa* and a mutant strain carrying an M59R mutation in the chromosomal *exsD* gene using a $P_{exsD\text{-}lacZ}$ transcriptional reporter [171]. In this study, we found no difference in reporter activity under non-inducing or inducing conditions at 30 °C (data not shown). These results were perhaps not completely unexpected and highlight the fundamental question of when the cellular concentration of free ExsD will most likely reach significant levels. Because ExsD is not a secreted protein and expression is positively regulated by ExsA, the protein may accumulate inside the cell during a prolonged period of T3SS induction. However, the pool of free ExsD is diminished by its associations with ExsA and ExsC. Increases in the cellular levels of ExsE, on the other hand, should cause the concentration of free ExsD to rise. In the context of an infection, this could reflect a scenario where a bacterial cell releases from the host cell at a later stage, causing the closure the T3SS channel, and thereby, an intracellular accumulation of the ExsE-ExsC complex. While the temperature increase causes an overall rise in the expression levels of T3SS genes [203, 204], the ability of ExsD to re-associate with ExsA at 37 °C may serve to dampen this effect at this stage, and thus, fine-tune virulence gene expression. The $ExsD^{M59R}$ variant provides a useful system for our molecular studies, however, due to its temperature sensitivity, it is not well suited for testing this model *in vivo*. Perhaps, a mutation that attenuates, but does not fully disrupt ExsD self-association, would be a better choice for these studies. *In vivo*, the temperature change from 30 °C to 37 °C is likely to affect the expression of multiple *P. aeruginosa* genes, and it might be instructive to examine the role of ExsD self-association in the larger context of these changes through computational modeling studies.

In summary, we have shown *in vitro* that independently expressed ExsD does not inhibit ExsA-dependent transcription at 30 °C, but inhibits efficiently at 37 °C. A monomeric ExsD variant strongly inhibits ExsA-dependent transcription at 30 °C, suggesting that the temperature effect is caused by ExsD self-association. ExsD self-association appears to have no impact on basal T3SS expression levels at 30 or 37 °C. Instead, we propose that trimerization limits the effective concentration of ExsD and stabilizes the protein. Re-association of the accumulated ExsD protein with ExsA may assist in fine-tuning T3SS gene expression at a later stage of an infection.

Materials and Methods

Recombinant protein expression and purification

ExsA and ExsD were overexpressed in *E. coli* from a vector constructed by Gateway recombinational cloning (Invitrogen, Carlsbad, CA, USA). A tobacco etch virus (TEV) protease recognition site and the appropriate att recombination sites (attB1 and attB2) were added to the *exsA* and *exsD* genes during PCR, and the amplicons were subsequently recombined into pDONR201 (Invitrogen). The nucleotide sequences of the ORFs were verified, then recombined into the destination vector pDEST-HisMBP [180] to create the expression vectors pFS-HMBPExsD and pFS-HMBPExsA. These vectors were designed to produce either ExsA or ExsD as a fusion to the C-terminus of an N-terminally His_6-tagged *E. coli* maltose-binding protein (MBP).

Single colonies of *E. coli* BL21(DE3) CodonPlus RIL cells (Stratagene, La Jolla, CA, USA) containing either expression plasmid were used to inoculate 125 mL of Luria broth (LB) supplemented with 2 g/L dextrose, 100 µg/mL ampicillin, and 30 µg/mL

chloramphenicol. The cultures were grown with shaking (225 rpm) to saturation overnight at 37 °C and then diluted 66-fold into 6 L of fresh medium. ExsA cultures were grown to an OD_{600} of 1.0, ExsD cultures were grown to an OD_{600} of 0.5, and $ExsD^{M59R}$ cultures were grown to an OD_{600} of 0.8. All three cultures were induced with IPTG at a final concentration of 1 mM. The induction temperature for the ExsA cultures was 18 °C, and they were shaken for six hr. ExsD cultures were induced at 28 °C for four hr, and $ExsD^{M59R}$ cultures were induced at 17 °C overnight. Cells were harvested by centrifugation at 5,000 x g for 15 min. The cell pastes were resuspended in 200 mL of 500 mM NaCl, 25 mM imidazole, 50 mM Tris-HCl (pH 7.4), 2 mM DTT (buffer A), along with three tablets of Complete, EDTA-free Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA). The cells were lysed via sonication and centrifuged at 40,000 x g for 25 min. The supernatants were filtered through 0.45-μm polyethersulfone membranes and applied to a 30 mL Ni-NTA Superflow affinity column (Qiagen, Valencia, CA, USA) equilibrated with buffer A. For each run, the column was washed with five column volumes of buffer A, and proteins were eluted with a linear gradient from 25 to 250 mM imidazole (pH 7.4).

The His_6-MBP-ExsD protein was digested with 5 mg His-tagged TEV (S219V) protease [183] while being dialyzed overnight in 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 11.6 mM imidazole (pH 7.4), and 1 mM DTT. The sample was then passed through a second Ni-NTA column to remove both the His_6-MBP tag and the protease, using the same buffers as the first Ni-NTA column. The protein sample was collected in the flow through. The sample was diluted with 50 mM Tris-HCl (pH 7.4) and 2 mM DTT in order to lower the NaCl concentration to 50 mM. The ExsD sample was loaded onto a HiTrap

Q HP column (GE Healthcare, Waukesha, WI, USA) that had been equilibrated with 50 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM DTT, and elution was achieved by applying a linear gradient of NaCl from 50 mM to 1 M. Finally, gel filtration was performed using 150 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM TCEP (ExsD storage buffer). ExsD^{M59R} was purified in the same manner as wild-type ExsD, except that no anion exchange was performed. All purification steps were performed at 4 °C. After each purification step, fractions were analyzed via SDS-PAGE and pooled accordingly. ExsD and ExsD^{M59R} were concentrated to 4.5 mg/mL and 6.8 mg/mL, respectively. Protein samples were flash-frozen using liquid nitrogen and stored at -80 °C.

The His_6-MBP-ExsA fusion protein was treated differently. Following the initial Ni-NTA affinity purification step, the fusion protein was dialyzed against a buffer of 50 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM DTT and loaded onto a HiTrap Q HP column (GE Healthcare) that had been equilibrated with the same buffer. The His_6-MBP-ExsA fusion protein was eluted using a linear NaCl gradient from 0.05 M to 1 M. The sample was dialyzed against 2 L of 45 mM NaCl, 25 mM Tris-HCl (pH 7.15), and 2 mM DTT (buffer B) overnight. The sample was then loaded onto a HiTrap Heparin HP column (GE Healthcare) equilibrated in buffer B and eluted with a 0.05 M to 1 M gradient of NaCl. The NaCl concentration in the His-MBP-ExsA sample was adjusted to 0.5 M, and the fusion protein was digested with 3 mg of His-tagged TEV(S219V) protease at 4 °C overnight. Next, ExsA was run through a second Ni-NTA Superflow affinity column, this time collecting ExsA in the flow through. Finally, gel filtration using a HighLoad 26/60 Superdex 200 prep grade column (GE Healthcare) was

performed with the ExsA sample using 500 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM TCEP (ExsA storage buffer). The sample was concentrated to 1 mg/mL, flash-frozen using liquid nitrogen, and stored at -80 °C.

RNA polymerase purification and specific activity determination

RNA polymerase (RNAP) was purified from *P. aeruginosa* PAO1 cells following the original procedure of Allan and Kropinski [184]. However, changes were made to the later chromatographic steps. All purification steps were performed at 4 °C. *P. aeruginosa* PAO1 cultures were grown in LB broth to an OD_{600} of 0.8, harvested by centrifugation at 6,000 x g, then lysed by sonication. The cell debris was removed by centrifugation at 35,000 x g for 30 min, and 25% polyethyleneimine (pH 7.5) was added to the supernatant to a final concentration of 0.5% in order to precipitate the RNAP. The supernatant was centrifuged at 35,000 x g for 30 min. The polyethyleneimine precipitate was washed with 10 mM Tris-HCl (pH 8.0), 250 mM NaCl, 5% glycerol, 0.05 mM EDTA, 1 mM DTT, and 0.1 mM PMSF (wash buffer) and centrifuged at 35,000 x g for 30 min. RNAP was released by resuspending the pellet in 10 mM Tris-HCl (pH 8.0), 800 mM NaCl, 5% glycerol, 0.05 mM EDTA, 1 mM DTT, and 0.1 mM PMSF (release buffer) and centrifuged at 25,000 x g for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 30%, followed by gentle stirring for one hr, and centrifugation at 35,000 x g for 30 min. Additional ammonium sulfate was then added to bring the supernatant to 60% saturation. After a second centrifugation at 35,000 x g for 30 min, the pellet was resuspended in 1 mL wash buffer per liter of original culture. The suspension was dialyzed versus 2 L wash buffer overnight. The dialyzed RNAP sample was centrifuged, and the supernatant was filtered in preparation for gel filtration. The

sample was run through a Sephacryl S-300 HR column (GE Healthcare) using wash buffer. The fractions were analyzed by SDS-PAGE and collected to run on a Hi-Trap Heparin HP column (GE Healthcare) using a loading buffer composed of 10 mM Tris-HCl (pH 8.0), 250 mM NaCl, 5% glycerol, 0.05 mM EDTA, and 1 mM TCEP. RNAP was eluted using a linear gradient of 0.25 M to 1 M NaCl. Fractions were analyzed via SDS-PAGE, pooled, and concentrated to 1 mg/mL of total protein. Glycerol was added to a final concentration of 50%. RNAP was aliquoted and stored at -20 °C.

The specific activity of the purified *P. aeruginosa* RNAP was determined by comparing its activity to a standard curve generated with different amounts of *E. coli* RNA polymerase holoenzyme (Epicentre Biotechnologies, Madison, WI, USA) using an ExsA-independent RNA-1 promoter which produces a 108 base transcript [185].

Site-directed mutagenesis

The $ExsD^{M59R}$ variant was generated by site-directed mutagenesis using Quik-Change (Stratagene) and the manufacturer's suggested protocol. The following primers were used:

5´–CTGCAGCGGCGGCTGCCGCGCCTGCGGCTGGAGC–3´

5´–GGCGCGGCAGCCGCCGCTGCAGCAACGCCAG–3´.

ExsA-dependent *in vitro* transcription assays

The linear DNA template used in each assay encompassed positions -207 to 94 of the P_{exsD} promoter, relative to the transcription start site; and from this template, RNA polymerase synthesizes an 82 base mRNA transcript. The template was produced by PCR using forward primer 5´-CATCAGTTGCTGCTCAACAGCG-3´ and reverse primer 5´-CACCGCTTCTCGGGAGTACTGC-3´. The PCR product was run on a 2% agarose

gel and purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA). Each 30 µL transcription assay reaction contained 4.4 fM of promoter template, 50.4 µM bovine serum albumin (to eliminate non-specific protein-protein interactions), 10 U purified RNA polymerase from *P. aeruginosa* (see above), 1 U RiboGuard RNase Inhibitor (Epicentre Biotechnologies), 15 ng/µL poly(deoxyinosinic-deoxycytidylic) acid (to prevent non-specific transcription initiation), 133 mM NaCl, 32 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 25 µM EDTA, 0.9 mM TCEP, 0.2 mM DTT, and 15.5% glycerol. The time-course experiments contained 64 nM ExsA and either no ExsD or 50 µM ExsD (no ExsA was added for the RNA-1 control experiments). Samples were mixed and allowed to equilibrate at room temperature for five min. Samples were then pre-incubated for 10 min at either 30 or 37 °C, depending on the experiment. Next, 3 µL NTPs (stock concentrations of 200 µM ATP, CTP, GTP and 40 µM UTP) mixed with 0.2 µL (0.2 µCi) of 3.3 mM P^{32}-alpha UTP was added to each sample to start the reaction, and samples were incubated at either 30 or 37 °C, depending on the experiment. After the reactions were stopped by adding 12 µL 1X stop solution (3M ammonium acetate, 50 mM EDTA, 0.11 mg/mL glycogen), 170 µL 100% cold ethanol was added, and the samples were incubated at -20 °C for one hr. Following centrifugation at 12,000 x g for 15 min, the supernatant was discarded and pellets were resuspended in 12 µL 1X TBE (Tris/Borate/EDTA)-urea sample buffer and heated at 70 °C for five min. After a brief centrifugation, the samples were loaded onto a 10% TBE-urea gel and run at 200 mV for 60 min. Gels were exposed to a storage phosphor screen (GE Healthcare) for 16 hr. The phosphor screen was scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare), and gel bands were quantified using Image Quant TL v2005 (Amersham

Biosciences, Piscataway, NJ, USA). Each experiment was performed in triplicate, and curve fits were analyzed with XLfit (IDBS, Bridgewater, NJ, USA).

Analytical size exclusion chromatography

One hundred µL samples of 2.1 µM purified wild-type ExsD and ExsD^{M59R}, each containing 150 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM TCEP, were separately loaded onto a Superdex 200 10/300 GL column (GE Healthcare). The proteins were eluted with 150 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM TCEP. The absorbance at UV_{280} was plotted on the y-axis and V_e/V_o was plotted on the x-axis, where V_e is the elution volume and V_o is the void volume (7.93 mL). Cytochrome C, carbonic anhydrase, bovine serum albumin, and β-amylase standards were individually run using the same elution buffer. These standards were plotted using the log of their known molecular weights on the y-axis and V_e/V_o on the x-axis; from this, a best fit line was determined. The molecular weights of wild-type ExsD and ExsD^{M59R} were subsequently estimated using the fitted linear equation.

Circular dichroism (CD) measurements

Circular dichroism measurements using far-UV (200-240 nm) of wild-type ExsD and ExsD^{M59R}, each at a concentration of 5.3 µM, were separately measured at 4 °C using a JASCO J-815 Circular Dichroism Spectrometer (JASCO, Easton, MD, USA) with a 1 mm pathlength cuvette. Each protein sample contained 150 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM TCEP. The spectra obtained represent an average of three scans that were corrected for the buffer baseline.

Differential scanning fluorimetry (DSF)

The DSF experiments were performed utilizing an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each 30 μL reaction contained 5x Sypro Orange (Ex. 490 nm, Em. 530 nm) (Invitrogen), 10 μM wild-type ExsD or $ExsD^{M59R}$, 150 mM NaCl, 8.3 mM Tris-HCl (pH 7.4), and 0.67 mM TCEP. Starting at 10 °C, the temperature was incrementally increased to 68 °C at a rate of 1 °C per min. Data analysis was conducted using XLfit (IDBS).

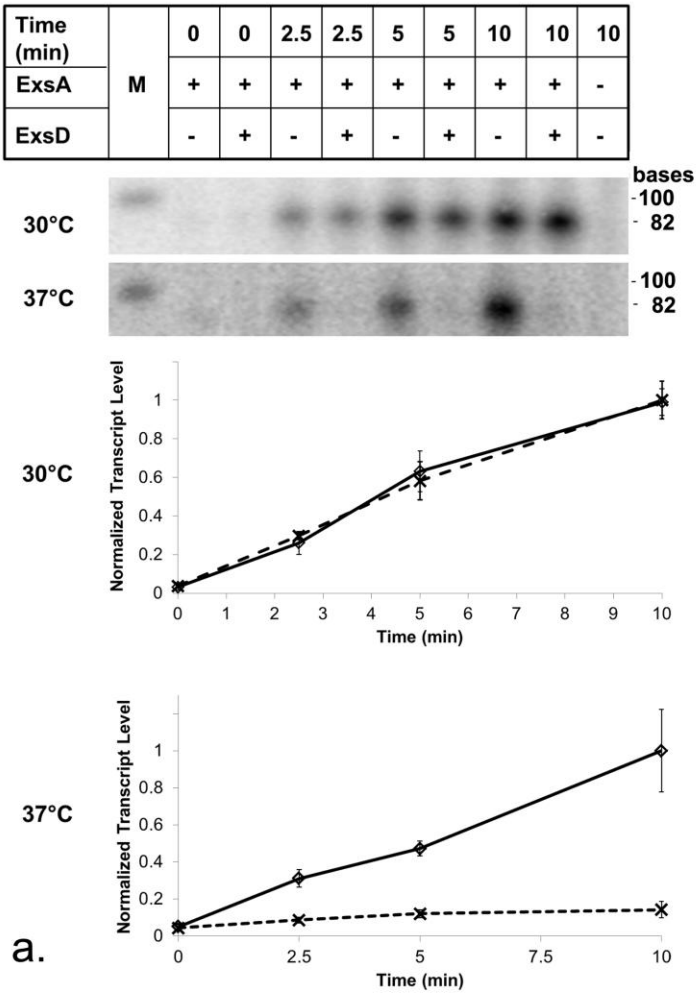
LexA monohybrid assay

Quik-Change (Stratagene) mutagenesis was used to modify the previously constructed arabinose-inducible vector pJN*exsD*Δα [172] by introducing an M59R substitution in *exsD* using primer $ExsD^{M59R}$ (5´-CGTTGCTGCAGCGGCGCCTGCCGCGCCTGC-3´). The resulting plasmid was designated pAM102. *E. coli* strain SU101, which carries a LexA-repressible $P_{sulA\text{-}lacZ}$ reporter, was transformed with pAM102 and the IPTG-inducible pSR658-*exsA* vector [172] and selected on LB agar with gentamicin (15 μg/mL) and tetracycline (12 μg/mL). Self-association of ExsA in the presence of ExsD or $ExsD^{M59R}$ was measured as previously described [172, 205]. Briefly, strains containing the appropriate expression vectors were grown overnight at 30 °C with shaking in 5 mL LB with 50 μM IPTG, 0.5% arabinose, and appropriate antibiotics. The following day, cultures were diluted to OD_{600} = 0.1 in 5 mL trypticase soy broth supplemented with 100 mM monosodium glutamate and 1% glycerol, as well as 50 μM IPTG, 0.5% arabinose, and antibiotics and grown at 30 °C with shaking to OD_{600} = 1.0. β-galactosidase levels were measured as previously described [172]. Miller units were reported as the average of three replicates with error

bars representing the standard error of the mean (SEM). Immunoblots were performed with α-ExsD and α-$LexA_{1-87}$ to confirm the stability of ExsD and ExsA-$LexA_{1-87}$, respectively.

Acknowledgements

This study was supported by the National Institutes of Health (1R21AI101774-01 to FDS and RO1- AI055042 to TLY) and the American Heart Association (09SDG2260401 to FDS).


Time (min)
M
0 0 2.5 2.5 5 5 10 10 10
ExsA + + + + + + + + -
ExsD - + - + - + - + -
bases
100
82
30°C
37°C
Normalized Transcript Level
Time (min)
a.

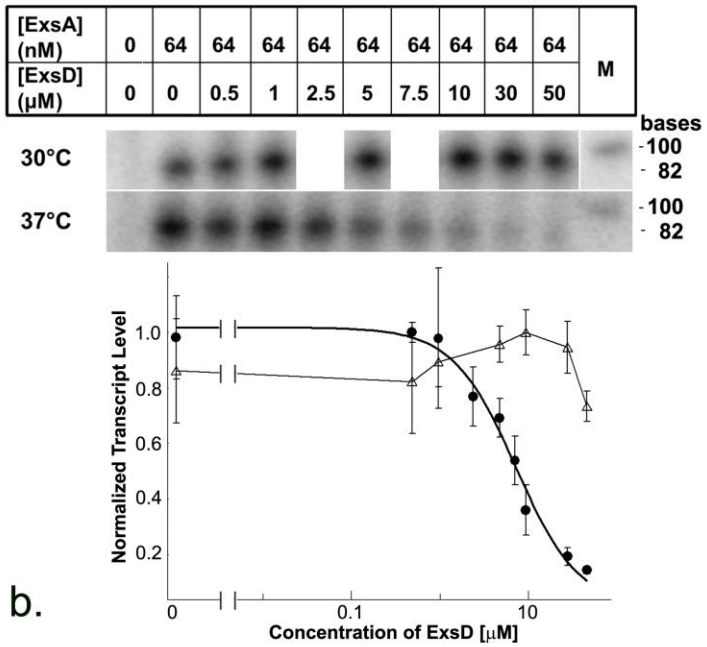

[ExsA] (nM)
[ExsD] (μM)
0 64 64 64 64 64 64 64 64 64
0 0 0.5 1 2.5 5 7.5 10 30 50
M
bases
100
82
30°C
37°C
Normalized Transcript Level
Concentration of ExsD [μM]
b.

Fig. 3.1. Temperature-dependent regulation of ExsA by ExsD. a. Autoradiograms and graphical representations for the *in vitro* transcription of an 86 nucleotide transcript from an ExsA-dependent P_{exsD} promoter template. Assays were performed at 30 °C and 37 °C and in absence and presence of 50 μM ExsD. In both graphs, the ExsD-free data are represented by solid trend lines and empty diamonds, whereas the data obtained in the presence of ExsD are represented by dashed trend lines and crosses. **b.** Dose response data and fit generated by measuring *in vitro* transcription levels at the 10-min time point at 37 °C in the presence of increasing amounts of ExsD protein (filled circles). Data (empty triangles) and dashed trend line obtained when the same experiment was conducted at 30 °C. The 30 °C gel strip has been digitally manipulated to align the fewer data points with those generated in the experiments performed at 37 °C. In order to obtain a reliable dose-response curve, two additional concentrations were included in the 37 °C experiment.

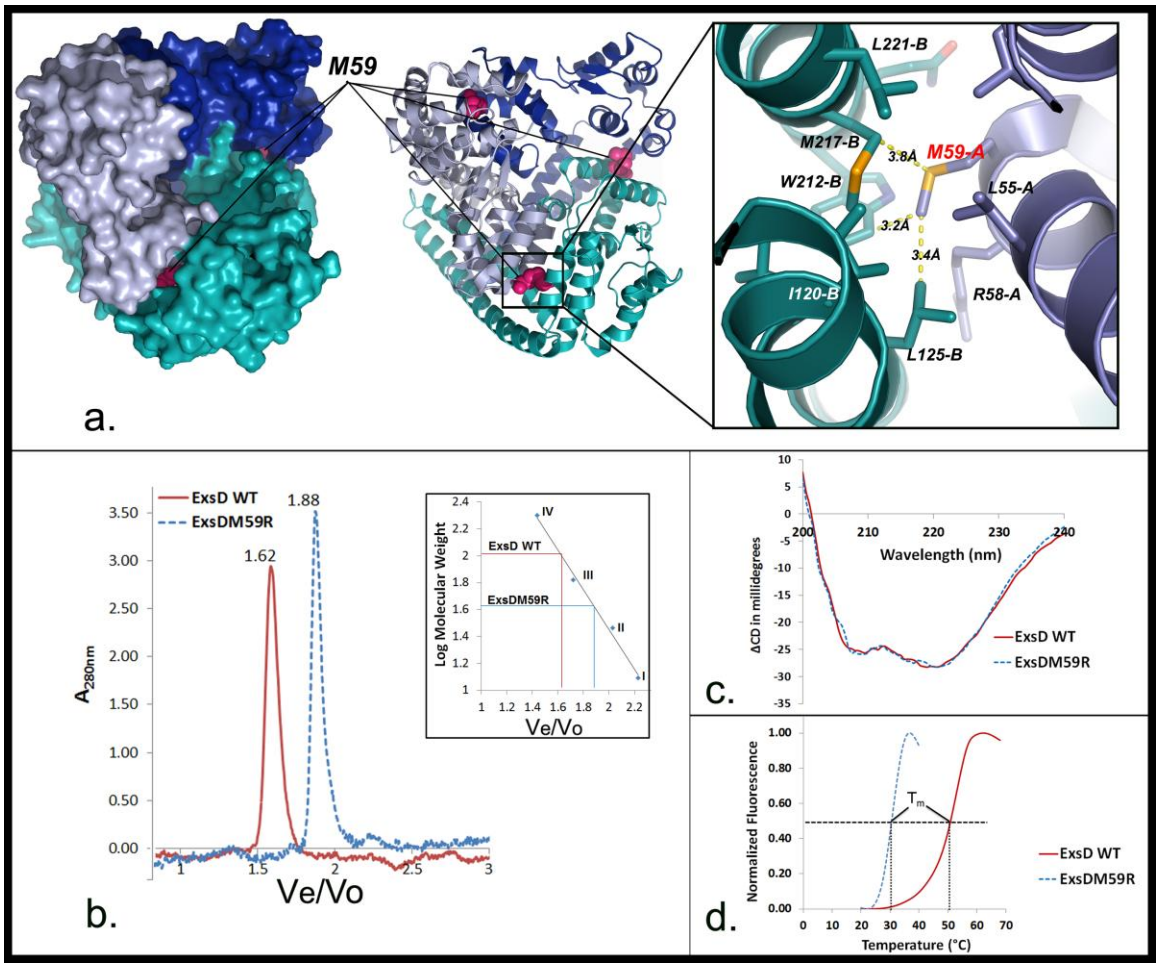


Fig. 3.2. Characterization of the ExsD^{M59R} variant. a. Three views of the ExsD trimer (PDB code 3FD9). The mutated methionine 59 is highlighted at the three interfaces. The rightmost view provides a close-up of the intermolecular contacts of the mutated M59 residue. The letters behind the residue names denote the chain identifications of the different ExsD molecules in the trimer. **b**. Elution profiles for ExsD^{M59R} and wild-type ExsD from an analytical gel filtration column. The inset shows the calibration curve for the column and the resulting apparent molecular weights for the two proteins. The four standards used to calibrate the column were I. cytochrome C (MW = 12.4 kDa), II. carbonic anhydrase (MW = 29 kDa), III. bovine serum albumin (MW = 66 kDa), and IV. β-amylase (MW = 200 kDa). **c.** Overlay of the circular dichroism spectra of ExsD^{M59R} and wild-type ExsD. **d.** Differential scanning fluorimetry profiles for ExsD^{M59R} and wild-type ExsD. The melting temperature (T_m) is defined as the temperature where 50% of the protein is unfolded, i.e., the inflection points of the curves. A T_m of 50.8 °C was obtained for wild-type ExsD, while ExsD^{M59R} had a T_m of 30.7 °C.

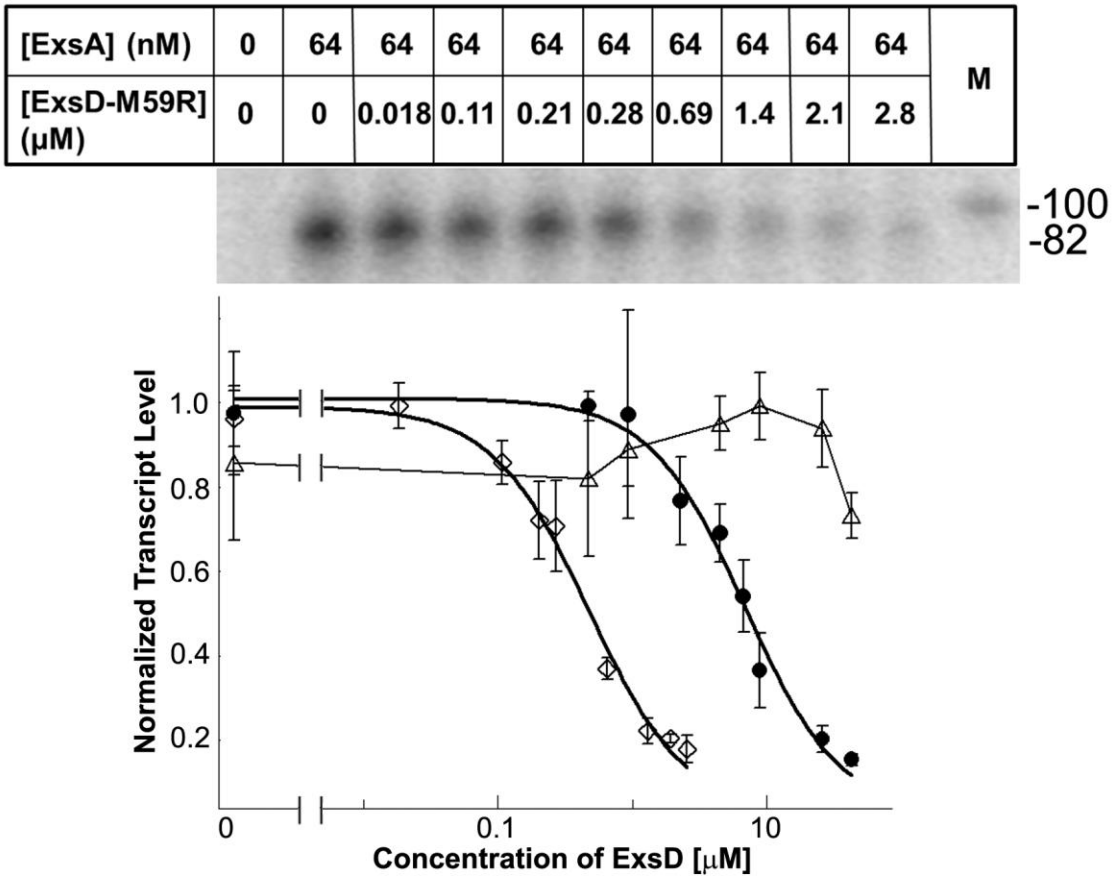


Fig. 3.3. Effect of ExsD^{M59R} on ExsA-dependent transcription. Dose response data and curve fit generated by measuring *in vitro* transcription levels in the presence of increasing amounts of ExsD^{M59R} protein at the 10-min time point and at 30 °C (clear diamonds). To highlight the differences, data shown in figure 3.1b are reproduced. Clear triangles show the results for the titration experiment with wild-type ExsD at 30 °C, while filled circles show data obtained at 37 °C.

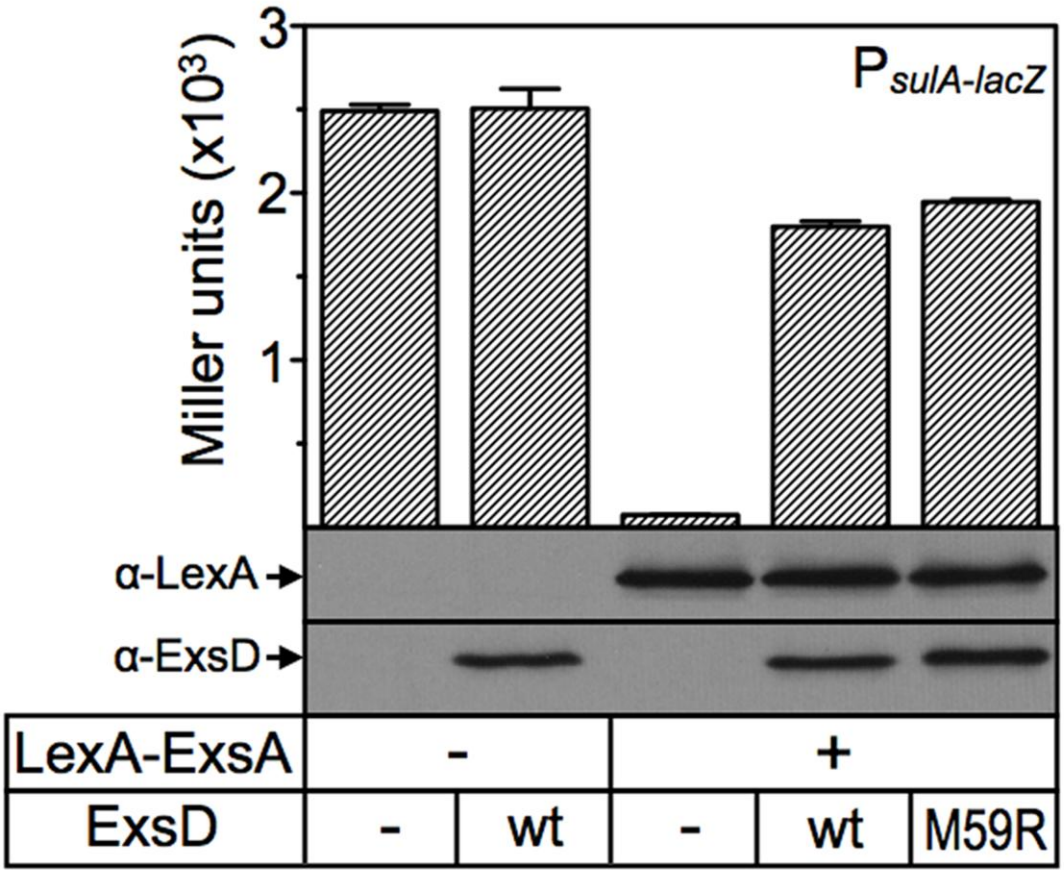


Fig. 3.4. ExsD^{M59R} disrupts ExsA dimerization. *E. coli* strain SU101 carrying a $P_{sulA\text{-}lacZ}$ transcriptional reporter was transformed with two different plasmids, as previously described [172]. The first plasmid was either a vector control (-, pSR658) or pSR658 expressing a LexA-ExsA fusion protein. The second plasmid was either a vector control (-, pJN105Δα) or pJN105Δα expressing either wild-type ExsD or the M59R variant. The resulting strains were cultured in LB medium containing the appropriate antibiotics and 50 μM IPTG to A_{600} = 1.0 and assayed for β-galactosidase activity. The reported values represent the average of three independent experiments.

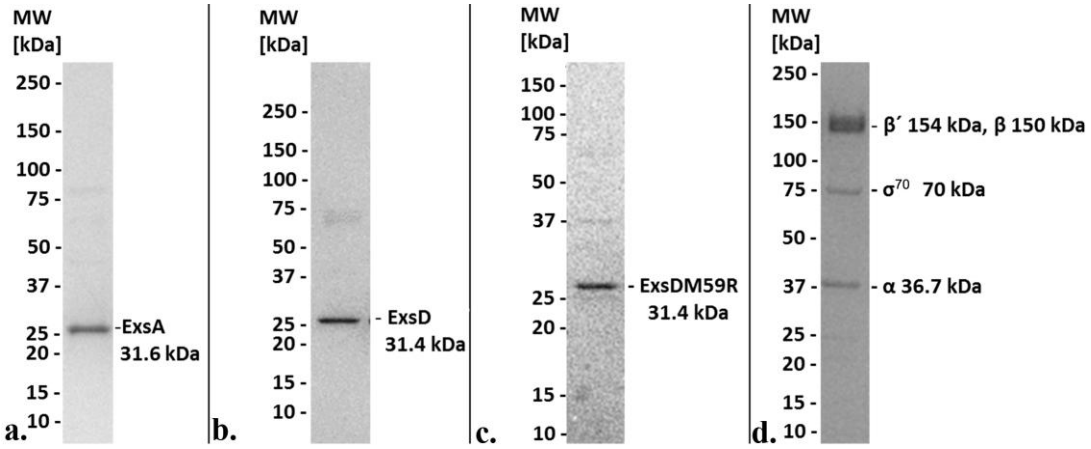


Fig. S3.1. SDS-PAGE images for the purified proteins used in the different assays. **a.** ExsA, **b.** ExsD, **c.** ExsD^{M59R}, and **d.** *P. aeruginosa* RNA polymerase.

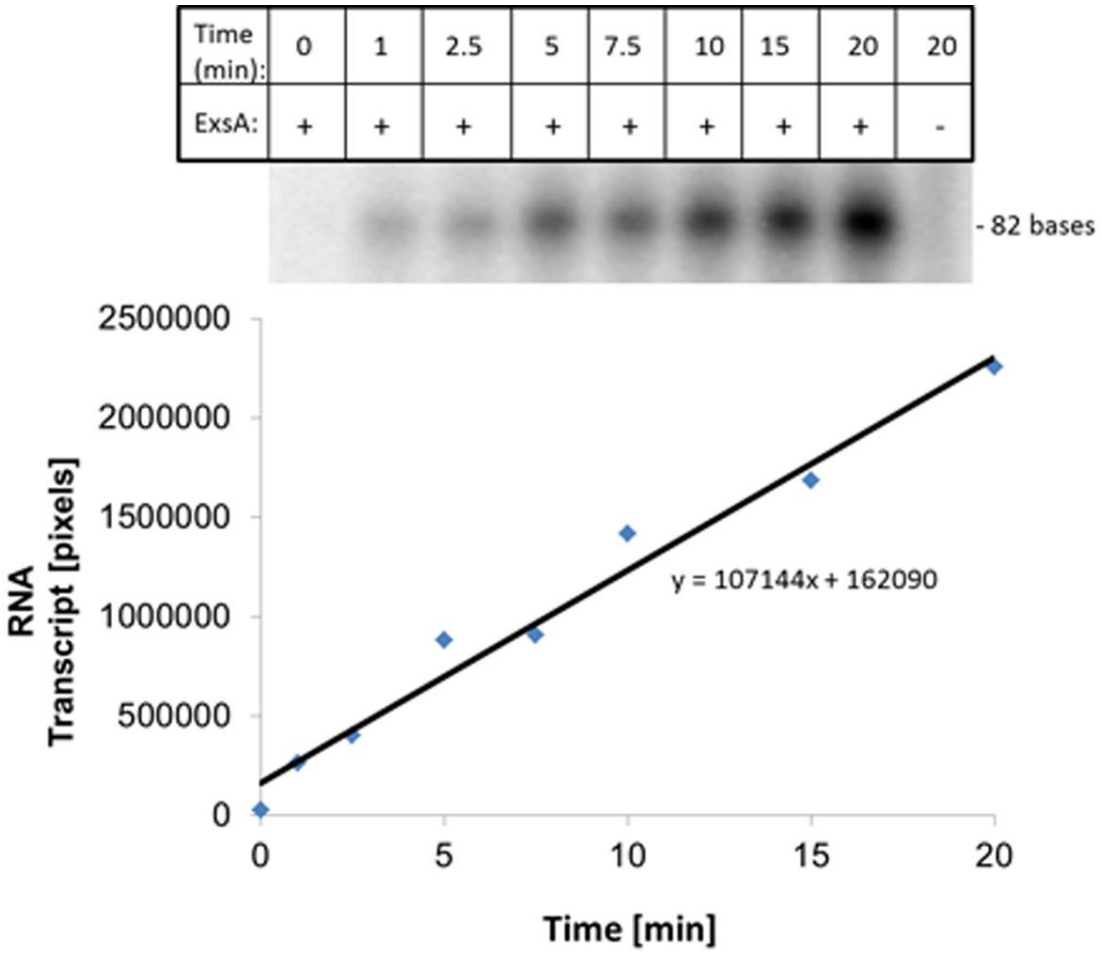


Fig. S3.2. *In vitro* transcription assay time-course at 37 °C. Initial time-course study performed to determine a suitable time-point for the dose response experiments. Under the described experimental conditions, the rate of transcription remains linear for at least 20 min.

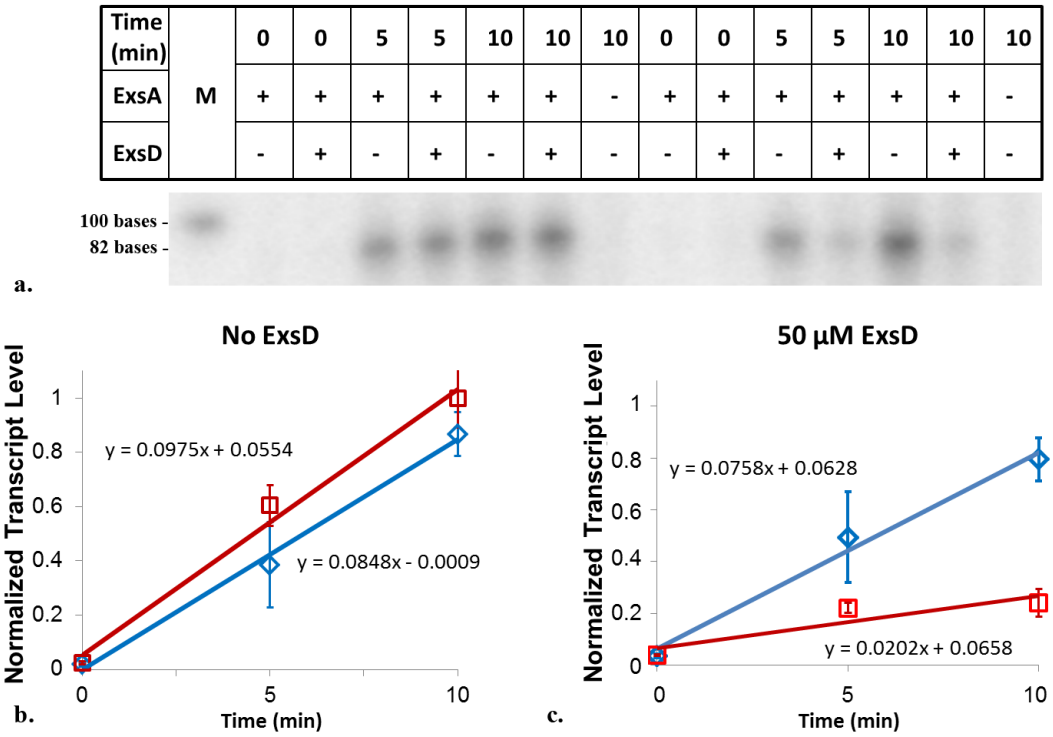


Fig. S3.3. a. Temperature dependence of ExsA-dependent transcription, both in the absence and presence of 50 µM ExsD, at 30 °C versus 37 °C. Assays were performed as described in Experimental procedures. Both experiments were analyzed on the same gel. **b.** Trend line fit to band intensity data for experiments performed in the absence of ExsD at 30 °C (blue line, clear diamonds) and at 37 °C (red line, clear squares). In the absence of ExsD, *in vitro* transcription proceeds at a 13% higher rate at 37 °C. **c.** Same experiment as shown in **b**, except the assay was performed in the presence of 50 µM wild-type ExsD.

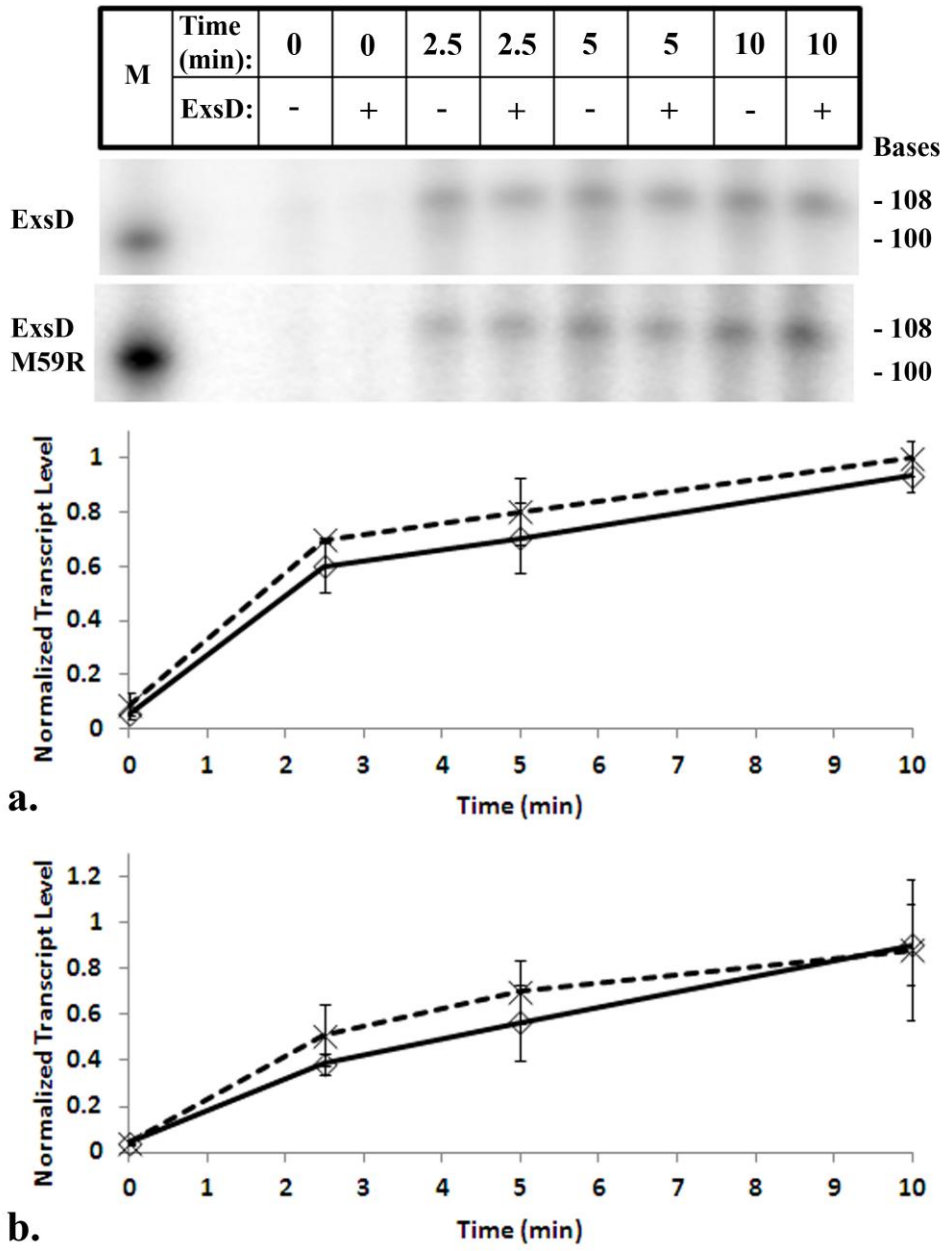


Fig. S3.4. Neither wild-type ExsD nor ExsD^{M59R} affects ExsA-independent transcription. Autoradiograms and graphical representations for the *in vitro* transcription of a 108 nucleotide transcript from an ExsA-independent RNA-1 promoter template with and without 50 μM ExsD at 37 °C **(a)**, and with and without 2.8 μM ExsD^{M59R} at 30 °C **(b)**. In both graphs, the ExsD/ ExsD^{M59R}-free data are represented by solid trend lines and empty diamonds, whereas the data obtained in the presence of ExsD/ExsD^{M59R} are represented by dashed trend lines and crosses.

CHAPTER FOUR

Mapping the ExsD–ExsA interface and further elucidation of the type III secretion system inhibitory mechanism in *Pseudomonas aeruginosa*

Robert C. Bernhards[1], Yi Xiao[1], Anne E. Marsden[2], Shannon K. Esher[1], Timothy L. Yahr[2], and Florian D. Schubot[1*]

Manuscript in Preparation

[1]Virginia Polytechnic Institute and State University
Department of Biological Sciences
125 Life Sciences 1 Building
Washington Street
Blacksburg, VA 24061

[2]University of Iowa
Department of Microbiology
540B Eckstein Medical Research Building
Iowa City, IA 52242

***Corresponding author:** Phone: (540) 231-2393
Fax: (540) 231-7126
Email: fschubot@vt.edu

Keywords: ExsA, ExsD, *Pseudomonas aeruginosa*, type III secretion.

Abbreviations: DSF = differential scanning fluorimetry; EMSA = electrophoretic mobility shift assay; FA = fluorescence anisotropy; IPTG = isopropyl-β-D-thiogalacto-pyranoside; MBP = maltose binding protein; TCEP = Tris(2-carboxyethyl)phosphine; TEV = tobacco etch virus; T3SS = type III secretion system.

Abstract

Pseudomonas aeruginosa is an emerging opportunistic human pathogen that is problematic due to its resistance to a multitude of common antibiotics. *P. aeruginosa* utilizes a type III secretion system (T3SS) to cause acute infections. The T3SS is regulated by a cascade of four interacting proteins: ExsA, ExsD, ExsC and ExsE. ExsA is the main transcriptional activator and regulates transcription of all the genes necessary for the T3SS. ExsD is the antiactivator and inhibits ExsA when there is no contact of the T3SS needle and the host cell. Meanwhile, ExsC sequesters ExsE. Upon host cell contact, ExsE is secreted into the host cell cytoplasm, which frees ExsC to bind to ExsD. ExsA is now free to activate transcription. The way in which ExsD inhibits ExsA is largely unknown. The current model posits that ExsD inhibits ExsA by binding in a 1:1 complex, which interrupts ExsA dimerization and prevents ExsA from binding to the T3SS promoters. This study reveals that there could be more to this inhibitory mechanism. We show conclusive evidence that ExsD is a DNA-binding protein through differential scanning fluorimetry (DSF) and electrophoretic mobility shift assays (EMSAs). We also begin to map the ExsD–ExsA binding interface by testing an assortment of ExsD variants in an *in vitro* transcription assay to determine which regions of ExsD are important for ExsA binding and inhibition. A complete understanding of how ExsD inhibits ExsA would allow for the development of drugs that mimic ExsD function and target ExsA to shut down the T3SS.

Introduction

Pseudomonas aeruginosa utilizes a type III secretion system (T3SS) to infect its host and cause acute infections [191, 193]. The T3SS is regulated by the ExsA-ExsC-ExsD-ExsE cascade, which works by direct protein-protein interactions [179]. In this cascade, ExsA is main transcriptional activator and controls expression of all the genes necessary for type III secretion [120]. ExsA is a member of the AraC/XylS family of transcriptional activators [120]. When the T3SS is turned off, ExsA is inhibited by the antiactivator protein ExsD [171]. ExsD binds to ExsA in a 1:1 complex [147]. Meanwhile, ExsC sequesters ExsE [108, 109]. Upon host cell contact, ExsE is secreted through the T3SS needle apparatus into the host cell cytoplasm [108, 109]. This now allows for ExsC to bind to ExsD [148, 177], which frees ExsA to bind to the T3SS promoters as a dimer and recruit RNA polymerase (RNAP) to activate T3SS transcription [149].

ExsD is a dynamic protein in that it interacts with different protein binding partners in different ways. ExsD exists in a 2:2 complex with ExsC [177], where a dimer of ExsC is bound to two ExsD monomers [178]. In addition to ExsD binding to ExsA in a 1:1 complex [147], ExsD also forms a self-trimer when not bound to a different protein [173]. Furthermore, structural evidence as well as preliminary experimental evidence suggests that ExsD is a DNA-binding protein and binds to DNA as a monomer [173]. The plasticity of ExsD gives it the ability to function in many different ways. ExsD is of significant medical importance given that it is capable of inhibiting the T3SS in *P. aeruginosa* [171]. Much can be gained by studying ExsD and how it is able to inhibit ExsA to shut down the T3SS.

This study examines the interaction between ExsD and ExsA. The current model proposes that ExsD disrupts ExsA dimerization and prevents ExsA from binding to the T3SS promoters [172]. ExsD does not interact with DNA in this model. However, based on the crystal structure of ExsD, which was previously solved by our lab, ExsD resembled a DNA-binding protein [173]. Here we further examine the ability of ExsD to bind DNA. Wild type ExsD as well as an $ExsD^{M59R}$ monomeric variant were examined using differential scanning fluorimetry (DSF) to demonstrate that ExsD is a DNA-binding protein. ExsD DNA-binding was confirmed using $ExsD^{M59R}$ in an electrophoretic mobility shift assay (EMSA).

The main focus of this study was to map the ExsD–ExsA binding interface. A group of ExsD variants lacking certain regions of the protein were examined in an *in vitro* transcription assay to determine which regions of ExsD are important for ExsA binding and inhibition. It was determined that the coiled-coil region of ExsD is not important for ExsA binding. On the other hand, we determined that the amino terminus of ExsD is important, but not sufficient to inhibit ExsA-dependent transcription.

Results

ExsD is a DNA-binding protein

The crystal structure of ExsD shows that it resembles a DNA binding protein from *E. coli* called KorB [173, 174]. Initial examination by DSF provided preliminary evidence that ExsD binds nonspecifically to DNA [173]. DSF measures the melting temperature of a protein. If a protein binds to DNA, the protein typically becomes more stable, which results in an increase in melting temperature. However, when DNA is

added to ExsD, it results in a decrease in melting temperature [173]. The ExsD structure suggests that ExsD binds to DNA as a monomer [173], and this could explain the decrease in melting temperature. The ExsD trimer without DNA could be more stable than an individual monomer bound to DNA. To further test this hypothesis, a monomeric ExsD variant called ExsD^{M59R} [206] was examined using DSF for its ability to bind DNA (Fig. 4.1). A portion of the P_{exsD} promoter was used as the dsDNA. Without DNA, the melting temperature of ExsD^{M59R} was 27.64 ± 0.06 °C, and when DNA was added the ExsD^{M59R} melting temperature increased to 31.57 ± 0.22 °C. Along with the wild type ExsD data, this supports the hypothesis that ExsD binds to DNA as a monomer.

The lab of Dr. Timothy Yahr at the University of Iowa used EMSA to determine whether ExsD and ExsD^{M59R} disrupt ExsA promoter binding (Fig. 4.2). In this assay, ExsA forms two distinct shift products when it binds to one of its promoters. The ExsA-dependent P_{exsC} promoter was used in this experiment. The lower shift product corresponds to one ExsA molecule bound to the promoter, and the higher and more pronounced shift product occurs when two ExsA molecules bind to the promoter [167]. If ExsD inhibits ExsA DNA binding then they expected to see a lower amount of shifted DNA when ExsD is added. Although they were not able to see an effect at these particular protein concentrations and temperature (30 °C), they were able to examine the ability of ExsD and ExsD^{M59R} to bind to DNA. At 30 °C, ExsD wild type was unable to bind to P_{exsC}. This is most likely due to the fact that the ExsD trimer is very stable at this temperature and does not dissociate to bind to DNA. This could also explain why there was no disruption of ExsA DNA binding by wild type ExsD. However, ExsD^{M59R} shifted the P_{exsC} as well as the nonspecific DNA control (*pscF*) at 30 °C (Fig. 4.2). This

confirms that ExsD is a DNA binding protein. However, since ExsD^{M59R} nonspecifically binds to DNA, it is not known whether or not ExsA-dependent promoters are specific targets for ExsD.

The coiled-coil region of ExsD is not important for ExsA binding

In order to map the ExsD–ExsA interface, we designed a variety of ExsD variants. The first variant was ExsD lacking the coiled-coil region (ExsDΔC-C). The ExsDΔC-C variant was constructed using site-directed mutagenesis and lacked residues 138-202. The two separated regions were fused by a linker consisting of four glycines. Following overexpression and purification, the ExsDΔC-C variant (Fig. 4.3) was examined using an *in vitro* transcription assay. We hypothesized that if the coiled-coil region was important for ExsA binding, then we would see less ExsA-dependent inhibition in the assay. A titration of ExsDΔC-C was performed at 37 °C (Fig. 4.4). To our surprise, ExsDΔC-C turned out to be a more potent inhibitor than wild type ExsD (IC_{50} = 0.97 ± 0.4 μM). We reasoned that deleting the coiled-coil region made the protein primarily monomeric. We previously demonstrated that the monomeric ExsD variant (ExsD^{M59R}) is a more potent inhibitor of ExsA dependent transcription [206]. At 37 °C, ExsDΔC-C had an IC_{50} value of 0.97 ± 0.4 μM, which closely resembles the IC_{50} value of ExsD^{M59R} (1.3 ± 0.1 μM) at 37 °C. The ExsDΔC-C titration was also performed at 30 °C, and it produced an IC_{50} value of 4.3 ± 0.4 μM (Fig. 4.4). We suspect that at the lower temperature, ExsDΔC-C is still capable of forming trimers, so a certain percentage is trimeric. This would explain the increase in IC_{50} value. Nevertheless, we concluded that the coiled-coil region of ExsD is not important for ExsA binding or inhibition.

The amino terminus of ExsD is important for ExsA binding

The next region of ExsD we wanted to examine was the N-terminus, since it was structurally located on the opposite side of the protein from the coiled-coil region. We also suspected that the ExsD N-terminus of ExsD could be important for ExsA binding, because the N-terminus of ExsD is predicted to be important for binding to ExsC [178]. It would be reasonable to assume that the binding of ExsD to either ExsC or ExsA involves a competitive ExsD binding region. In order to test this hypothesis, a variant of ExsD lacking the first 20 amino acids on the N-terminus was made by limited proteolysis. A simple thermolysin digest results in a highly stable ExsDΔ20 product [173]. ExsDΔ20 was tested in the *in vitro* transcription assay at 37 °C using the same titration as for ExsDΔC-C. The results show a decrease in ExsA-inhibition, indicating that the ExsD N-terminus is important for ExsA binding and inhibition (Fig 4.5). To confirm that the observed effect of ExsDΔ20 is specific to inhibit ExsA-dependent transcription, we performed an *in vitro* transcription assay time course using a DNA template containing the ExsA-independent RNA-1 promoter [185] (Fig 4.6). ExsDΔ20 showed no significant inhibition, indicating that ExsD1-46 is specific to inhibit ExsA.

To confirm that the ExsD N-terminus is important for ExsA binding, we ordered a synthetically made polypeptide of the first 46 amino acids of the ExsD N-terminus (ExsD1-46). A titration in the *in vitro* transcription assay at 37 °C shows ExsD1-46 does inhibit ExsA-dependent transcription, albeit weakly (Fig. 4.7). Since the inhibition was weak, we wanted to confirm that the observed effect of ExsD1-46 is specific to ExsA-dependent transcription, so we performed an *in vitro* transcription assay time course using a DNA template containing the ExsA-independent RNA-1 promoter (Fig. 4.8).

ExsD1-46 showed no significant inhibition, indicating that ExsD1-46 is specific to inhibit ExsA. Overall, these results suggest that the amino terminus of ExsD is important for ExsA binding, but is not sufficient to inhibit ExsA-dependent transcription.

Discussion

In *P. aeruginosa*, ExsA-dependent T3SS transcription is inhibited by the antiactivator ExsD [171]. ExsD is a dynamic protein with many different binding partners and most likely multiple functions [147, 148, 173, 178]. This study confirms that ExsD is a DNA binding protein. However, the role of ExsD DNA binding is unclear. Even though ExsD exists as a trimer, we discovered that ExsD can only bind to DNA as a monomer. Thus, one of the functions of the ExsD trimer could be to prevent ExsD from binding to DNA non-specifically. The ExsD trimer also most likely serves to stabilize the protein, given that the ExsD monomer is significantly less stable. It is unclear whether ExsD has a specific DNA binding site. A ChIP-sequencing study is currently underway to investigate this question.

The main goal of this study was to begin to map the ExsD–ExsA interface, as no information on the way ExsD and ExsA interact currently exists. ExsD variants were constructed and purified, and an *in vitro* transcription assay was utilized to determine which region of ExsD is important for ExsA-binding. The results are shown together in Fig. 4.9 for comparison. It was determined that the ExsD coiled-coil region is not important for ExsA binding. However, the ExsD amino terminus is important for ExsA binding, but not sufficient to inhibit ExsA-dependent transcription. We suspect that another portion of ExsD somewhere between the amino terminus and the coiled-coil

region is also important for ExsA-binding. Future studies will further map the ExsD–ExsA interface by testing amino acid substitution mutants in the *in vitro* transcription assay to determine which amino acids are critical for ExsA-binding. We are also attempting to crystallize the ExsD–ExsA complex.

The way in which ExsD inhibits ExsA remains a mystery. We propose three possible scenarios. The first is the current model where ExsD disrupts ExsA dimerization and promoter interactions by binding to ExsA in a 1:1 complex [147, 172]. The second scenario is ExsD binds to ExsA while ExsA is bound to the promoter and disrupts ExsA dimer formation. ExsD may or may not be binding to DNA under this scenario. The third possibility is that ExsD binds to ExsA when the dimer is formed on the promoter and disrupts ExsA–RNAP interactions. ExsD shows structural similarity to GreB, which is a RNAP binding protein in *E. coli* [176]. During transcription, RNAP occasionally loses its grip on the growing mRNA end during elongation and backtracks on the DNA template [176]. GreB binds to RNAP and cleaves the disengaged 3´ RNA segment, so that RNAP can proceed with transcription [176]. The coiled-coil region of GreB inserts into RNAP [176], and given that the coiled-coil regions of ExsD and GreB are very similar structurally, one could imagine that ExsD interacts with RNAP in the same manner. Given that the coiled-coil region of ExsD does not interact with ExsA, ExsD could be binding to both ExsA and RNAP at the same time. ExsD could function to stall RNAP, thereby inhibiting T3SS transcription.

Insights into the way ExsD inhibits ExsA could lead to the discovery of drugs that mimic ExsD function and target ExsA to shut down the T3SS in *P. aeruginosa*. Drugs that shut down the T3SS would eliminate acute *P. aeruginosa* infection. These drugs

would also induce less selective pressure than current antibiotics, because *P. aeruginosa* can survive without a T3SS outside the host. Drugs that do not induce selective pressure are of particular interest for bacteria such as *P. aeruginosa*, which have the ability to develop quick and wide-ranging resistance to first generation antibiotics. These drugs could also potentially be used to treat human infection from other organisms that contain close homologs to ExsA, such as *Vibrio parahaemolyticus* and *Yersinia pestis*.

Materials and Methods

Recombinant protein expression and purification

ExsA and ExsD were overexpressed in *E. coli* from a vector constructed by Gateway recombinational cloning (Invitrogen, Carlsbad, CA, USA). A tobacco etch virus (TEV) protease recognition site and the appropriate att recombination sites (attB1 and attB2) were added to the *exsA* and *exsD* genes during PCR, and the amplicons were subsequently recombined into pDONR201 (Invitrogen). The nucleotide sequences of the ORFs were verified, then recombined into the destination vector pDEST-HisMBP [180] to create the expression vectors pFS-HMBPExsD and pFS-HMBPExsA. These vectors were designed to produce either ExsA or ExsD as a fusion to the C-terminus of an N-terminally His_6-tagged *E. coli* maltose-binding protein (MBP).

Single colonies of *E. coli* BL21(DE3) CodonPlus RIL cells (Stratagene, La Jolla, CA, USA) containing either expression plasmid were used to inoculate 125 mL of Luria broth (LB) supplemented with 2 g/L dextrose, 100 µg/mL ampicillin, and 30 µg/mL chloramphenicol. The cultures were grown with shaking (225 rpm) to saturation overnight at 37 °C and then diluted 66-fold into 6 L of fresh medium. ExsA cultures

were grown to an OD_{600} of 1.0, ExsD cultures were grown to an OD_{600} of 0.5, and $ExsD^{M59R}$ cultures were grown to an OD_{600} of 0.8. All three cultures were induced with IPTG at a final concentration of 1 mM. The induction temperature for the ExsA cultures was 18 °C, and they were shaken for six hr. ExsD cultures were induced at 28 °C for four hr, and $ExsD^{M59R}$ cultures were induced at 17 °C overnight. Cells were harvested by centrifugation at 5,000 x g for 15 min. The cell pastes were resuspended in 200 mL of 500 mM NaCl, 25 mM imidazole, 50 mM Tris-HCl (pH 7.4), 2 mM DTT (buffer A), along with three tablets of Complete, EDTA-free Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA). The cells were lysed via sonication and centrifuged at 40,000 x g for 25 min. The supernatants were filtered through 0.45-µm polyethersulfone membranes and applied to a 30 mL Ni-NTA Superflow affinity column (Qiagen, Valencia, CA, USA) equilibrated with buffer A. For each run, the column was washed with five column volumes of buffer A, and proteins were eluted with a linear gradient from 25 to 250 mM imidazole (pH 7.4).

The His_6-MBP-ExsD protein was digested with 5 mg His-tagged TEV (S219V) protease [183], while being dialyzed overnight in 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 11.6 mM imidazole (pH 7.4), and 1 mM DTT. The sample was then passed through a second Ni-NTA column to remove both the His_6-MBP tag and the protease, using the same buffers as the first Ni-NTA column. The protein sample was collected in the flow through. The sample was diluted with 50 mM Tris-HCl (pH 7.4) and 2 mM DTT in order to lower the NaCl concentration to 50 mM. The ExsD sample was loaded onto a HiTrap Q HP column (GE Healthcare, Waukesha, WI, USA) that had been equilibrated with 50 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM DTT, and elution was achieved by

applying a linear gradient of NaCl from 50 mM to 1 M. Finally, gel filtration was performed using 150 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM TCEP (ExsD storage buffer). $ExsD^{M59R}$ was purified in the same manner as wild-type ExsD, except that no anion exchange was performed. All purification steps were performed at 4 °C. After each purification step, fractions were analyzed via SDS-PAGE and pooled accordingly. ExsD and $ExsD^{M59R}$ were concentrated to 4.5 mg/mL and 6.8 mg/mL, respectively. Protein samples were flash-frozen using liquid nitrogen and stored at -80 °C.

The His_6-MBP-ExsA fusion protein was treated differently. Following the initial Ni-NTA affinity purification step, the fusion protein was dialyzed against a buffer of 50 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM DTT and loaded onto a HiTrap Q HP column (GE Healthcare) that had been equilibrated with the same buffer. The His_6-MBP-ExsA fusion protein was eluted using a linear NaCl gradient from 0.05 M to 1 M. The sample was dialyzed against 2 L of 45 mM NaCl, 25 mM Tris-HCl (pH 7.15), and 2 mM DTT (buffer B) overnight. The sample was then loaded onto a HiTrap Heparin HP column (GE Healthcare) equilibrated in buffer B and eluted with a 0.05 M to 1 M gradient of NaCl. The NaCl concentration in the His-MBP-ExsA sample was adjusted to 0.5 M, and the fusion protein was digested with 3 mg of His-tagged TEV(S219V) protease at 4 °C overnight. Next, ExsA was run through a second Ni-NTA Superflow affinity column, this time collecting ExsA in the flow through. Finally, gel filtration using a HighLoad 26/60 Superdex 200 prep grade column (GE Healthcare) was performed with the ExsA sample using 500 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2

mM TCEP (ExsA storage buffer). The sample was concentrated to 1 mg/mL, flash-frozen using liquid nitrogen, and stored at -80 °C.

RNA polymerase purification and specific activity determination

RNA polymerase (RNAP) was purified from *P. aeruginosa* PAO1 cells following the original procedure of Allan and Kropinski [184]. However, changes were made to the later chromatographic steps. All purification steps were performed at 4 °C. *P. aeruginosa* PAO1 cultures were grown in LB broth to an OD_{600} of 0.8, harvested by centrifugation at 6,000 x g, then lysed by sonication. The cell debris was removed by centrifugation at 35,000 x g for 30 min, and 25% polyethyleneimine (pH 7.5) was added to the supernatant to a final concentration of 0.5% in order to precipitate the RNAP. The supernatant was centrifuged at 35,000 x g for 30 min. The polyethyleneimine precipitate was washed with 10 mM Tris-HCl (pH 8.0), 250 mM NaCl, 5% glycerol, 0.05 mM EDTA, 1 mM DTT, and 0.1 mM PMSF (wash buffer) and centrifuged at 35,000 x g for 30 min. RNAP was released by resuspending the pellet in 10 mM Tris-HCl (pH 8.0), 800 mM NaCl, 5% glycerol, 0.05 mM EDTA, 1 mM DTT, and 0.1 mM PMSF (release buffer) and centrifuged at 25,000 x g for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 30%, followed by gentle stirring for one hr, and centrifugation at 35,000 x g for 30 min. Additional ammonium sulfate was then added to bring the supernatant to 60% saturation. After a second centrifugation at 35,000 x g for 30 min, the pellet was resuspended in 1 mL wash buffer per liter of original culture. The suspension was dialyzed versus 2 L wash buffer overnight. The dialyzed RNAP sample was centrifuged, and the supernatant was filtered in preparation for gel filtration. The sample was run through a Sephacryl S-300 HR column (GE Healthcare) using wash

buffer. The fractions were analyzed by SDS-PAGE and collected to run on a Hi-Trap Heparin HP column (GE Healthcare) using a loading buffer composed of 10 mM Tris-HCl (pH 8.0), 250 mM NaCl, 5% glycerol, 0.05 mM EDTA, and 1 mM TCEP. RNAP was eluted using a linear gradient of 0.25 M to 1 M NaCl. Fractions were analyzed via SDS-PAGE, pooled, and concentrated to 1 mg/mL of total protein. Glycerol was added to a final concentration of 50%. RNAP was aliquoted and stored at -20 °C.

The specific activity of the purified *P. aeruginosa* RNAP was determined by comparing its activity to a standard curve generated with different amounts of *E. coli* RNA polymerase holoenzyme (Epicentre Biotechnologies, Madison, WI, USA) using an ExsA-independent RNA-1 promoter which produces a 108 base transcript [185].

Site-directed mutagenesis

The ExsD^{M59R} variant was generated by site-directed mutagenesis using Quik-Change (Stratagene) and the manufacturer's suggested protocol. The following primers were used:

5′–CTGCAGCGGCGGCTGCCGCGCCTGCGGCTGGAGC–3′

5′–GGCGCGGCAGCCGCCGCTGCAGCAACGCCAG–3′. The ExsDΔC-C variant was created by two sequential rounds of PCR. The first set of primers was 5′–GTGGAGAACCTGTACTTCCAGGGTATGGAGCAGGAAGAC–3′ and 5′–GTGGAGAACCTGTACTTCCAGGGTGCGATCCCCGGCTGG–3′. The second set was 5′–CGGGTCAACCTCGGAGGAGGAGGATCGGCACTGGCG–3′ 5′–GGGGACAACTTTGTACAAGAAAGTTGCTCATACTGGCAGAGCTGA–3′

Differential scanning fluorimetry (DSF)

The DSF experiments were performed utilizing a C1000^{TM} Thermal Cycler with a CFX96^{TM} Real-Time System (Bio-Rad, Hercules, CA, USA). The DNA used was a 24 base pair segment of the P_{exsD} promoter made by annealing the following primers: 5′–CGAATGCCGGGCTAAAAATAACTG–3′ and 5′–CAGTTATTTTTAGCCCGGCATTCG–3′. The primers were annealed by incubating at 67 °C for 3 min and cooling at room temperature for 5 min. Each 30 µL reaction contained 5x Sypro Orange (Ex. 490 nm, Em. 530 nm) (Invitrogen), 10 µM wild-type ExsD or ExsD^{M59R}, 50 µM dsDNA (P_{exsD}), 150 mM NaCl, 8.3 mM Tris-HCl (pH 7.4), and 0.67 mM TCEP. Starting at 10 °C, the temperature was incrementally increased to 68 °C at a rate of 1 °C per min, and readings were taken every 30 sec. Data analysis was conducted using XLfit (IDBS, Bridgewater, NJ, USA).

Limited proteolysis to obtain ExsDΔ20

ExsDΔ20 was made by performing limited proteolysis on wild type ExsD using thermolysin. The digest contained 500 µL of 2.5 mg/mL ExsD in 150 mM NaCl, 25 mM Tris-HCl pH 7.4 and 2 mM TCEP as well as 500 µL 2× thermolysin buffer (20 mM Tris-HCl pH 8.0, 4 mM $CaCl_2$, 400 mM NaCl, and 10% glycerol). To start the reaction, 10 µL of 0.25 mg/mL thermolysin in 1 × thermolysin buffer was added and the sample was incubated at 37 °C for 1 hr. The reaction was stopped by adding 10 µL of 0.5 M EDTA pH 8.0. In order to remove precipitated protein, the sample was centrifuged at 10,500 x g at 4 °C for 10 min then spin filtered at 10,000 x g at 4 °C for 3 min. The sample was then loaded onto a HighLoad 26/60 Superdex 200 prep grade column (GE Healthcare). The column was washed with 150 mM NaCl, 25 mM Tris-HCl pH 7.4, and 2 mM TCEP pH

8.0. Sample fractions were run on SDS-PAGE. Fractions containing ExsDΔ20 were pooled then the protein sample was aliquoted, flash-frozen, and stored at -80 °C.

***In vitro* transcription assay**

The linear DNA template used in each assay encompassed positions -207 to 94 of the P_{exsD} promoter, relative to the transcription start site; and from this template, RNA polymerase synthesizes an 82 base mRNA transcript. The template was produced by PCR using forward primer 5´-CATCAGTTGCTGCTCAACAGCG-3´ and reverse primer 5´-CACCGCTTCTCGGGAGTACTGC-3´. The PCR product was run on a 2% agarose gel and purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA). Each 30 µL transcription assay reaction contained 4.4 fM of promoter template, 50.4 µM bovine serum albumin (to eliminate non-specific protein-protein interactions), 10 U purified RNA polymerase from *P. aeruginosa* (see above), 1 U RiboGuard RNase Inhibitor (Epicentre Biotechnologies), 15 ng/µL poly(deoxyinosinic-deoxycytidylic) acid (to prevent non-specific transcription initiation), 133 mM NaCl, 32 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 25 µM EDTA, 0.9 mM TCEP, 0.2 mM DTT, 15.5% glycerol, 64 nM ExsA, and the specified concentration of ExsD variant. Samples were mixed and allowed to equilibrate at room temperature for five min. Samples were then pre-incubated for five min at either 30 or 37 °C, depending on the experiment. Next, 3 µL NTPs (stock concentrations of 200 µM ATP, CTP, GTP and 40 µM UTP) mixed with 0.2 µL (0.2 µCi) of 3.3 mM P^{32}-alpha UTP was added to each sample to start the reaction, and samples were incubated for 10 min at either 30 or 37 °C, depending on the experiment. The reactions were stopped by adding 12 µL 1X stop solution (3M ammonium acetate, 50 mM EDTA, 0.11 mg/mL glycogen). Then 170 µL 100% cold

ethanol was added, and the samples were incubated at -20 °C for one hr. Following centrifugation at 12,000 x g for 15 min, the supernatant was discarded and pellets were resuspended in 12 μL 1X TBE (Tris/Borate/EDTA)-urea sample buffer and heated at 70 °C for five min. After a brief centrifugation, the samples were loaded onto a 10% TBE-urea gel and run at 200 mV for 60 min. Gels were exposed to a storage phosphor screen (GE Healthcare) for 16 hr. The phosphor screen was scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare), and gel bands were quantified using Image Quant TL v2005 (Amersham Biosciences, Piscataway, NJ, USA). Each experiment was performed in triplicate, and curve fits were analyzed with XLfit (IDBS, Bridgewater, NJ, USA).

Acknowledgements

This study was supported by the National Institutes of Health (1R21AI101774-01 to FDS and RO1- AI055042 to TLY) and the American Heart Association (09SDG2260401 to FDS).

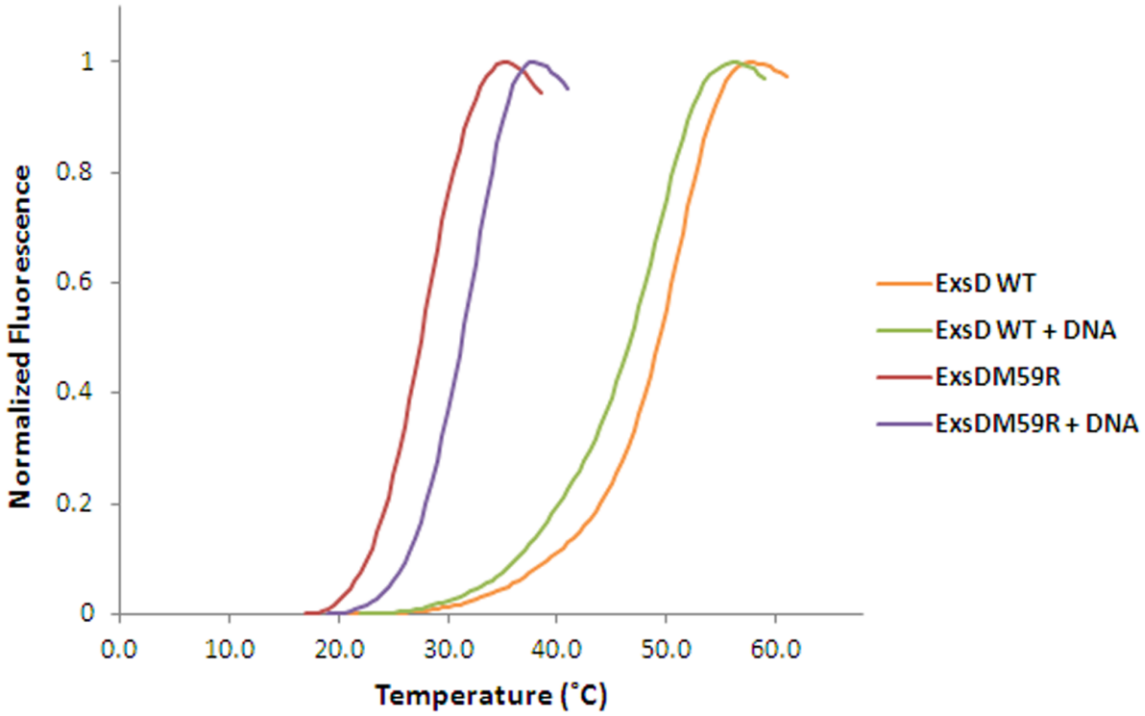


Fig. 4.1. ExsD binds to DNA as a monomer. Differential scanning fluorimetry profiles for wild type ExsD and ExsD^{M59R}. The melting temperature (T_m) is defined as the temperature where 50% of the protein is unfolded, i.e., the inflection points of the curves. The T_m of ExsD with and without DNA was 47.71 ± 0.43 °C and 50.22 ± 0.23 °C, respectively. The T_m of ExsD^{M59R} with and without DNA was 31.57 ± 0.22 °C and 27.64 ± 0.06 °C, respectively.

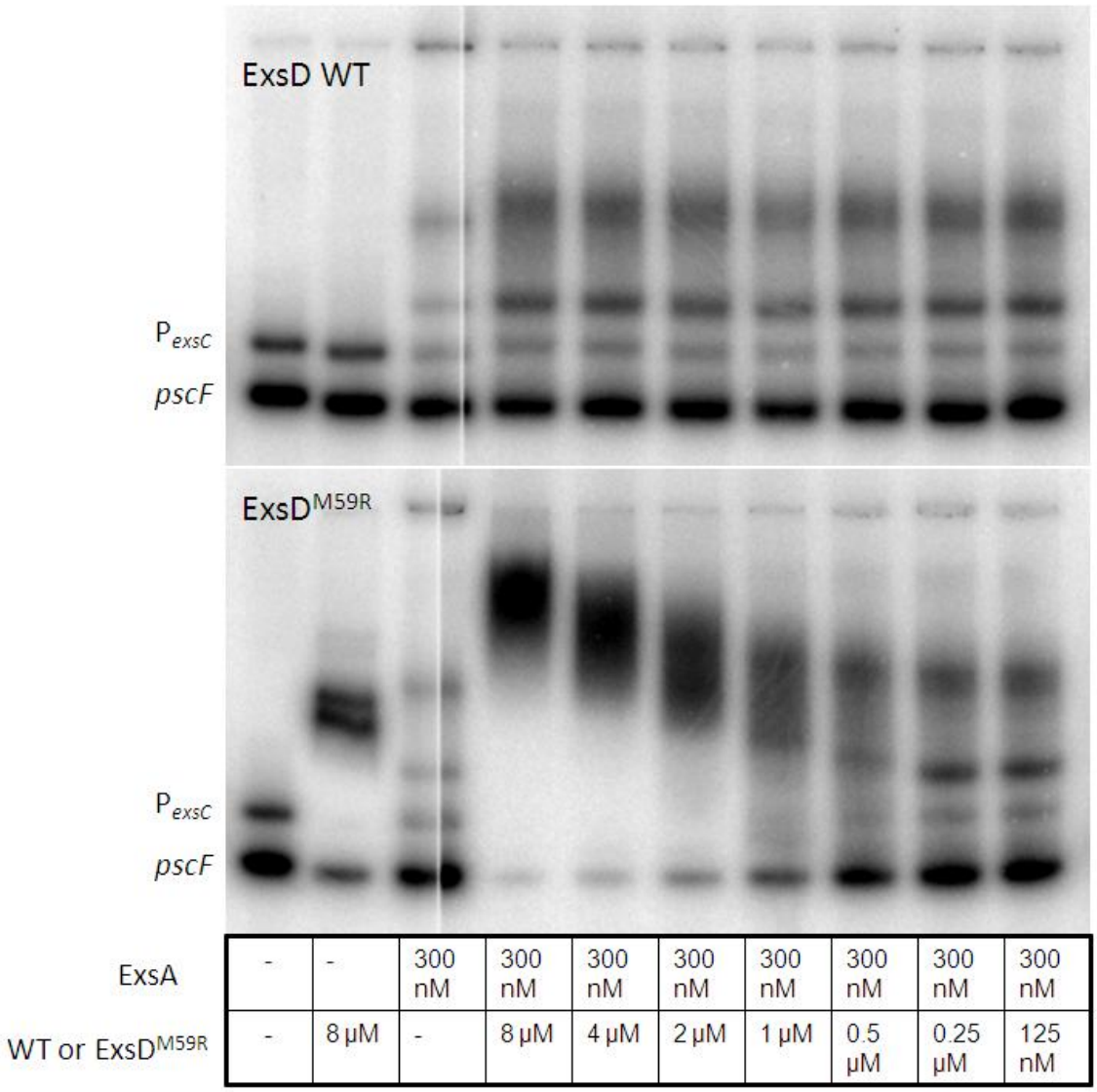


Fig. 4.2. Does ExsD^{M59R} disrupt ExsA-binding activity? EMSA with wild type ExsD (upper panel) and ExsD^{M59R} (lower panel) at 30 °C. The P_{exsC} promoter was used as the template as well as the *pscF* gene which is known to be nonspecific.

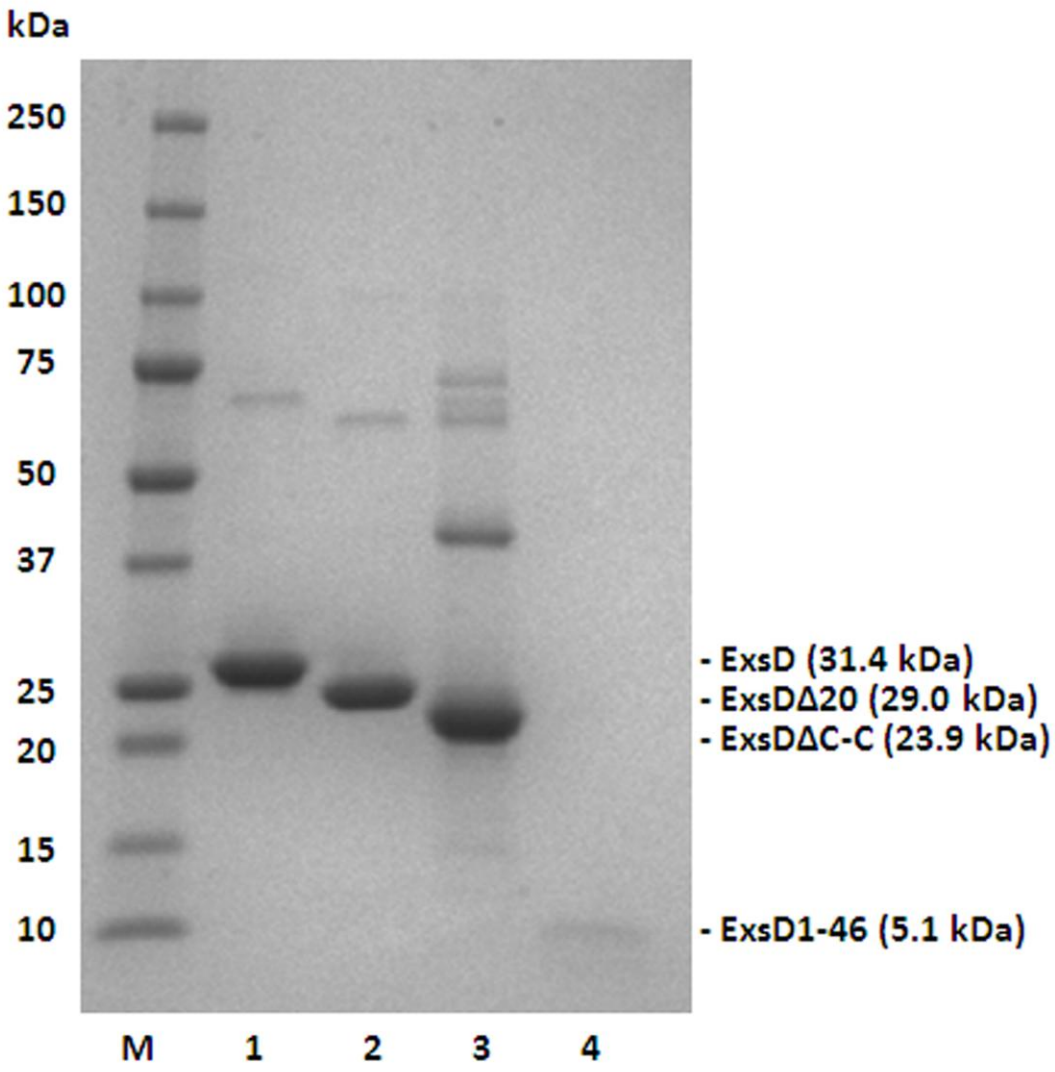


Fig. 4.3. ExsD variant purification. Purified wild type ExsD (lane 1), ExsDΔ20 (lane 2), ExsDΔC-C (lane 3), and ExsD1-46 (lane 4).

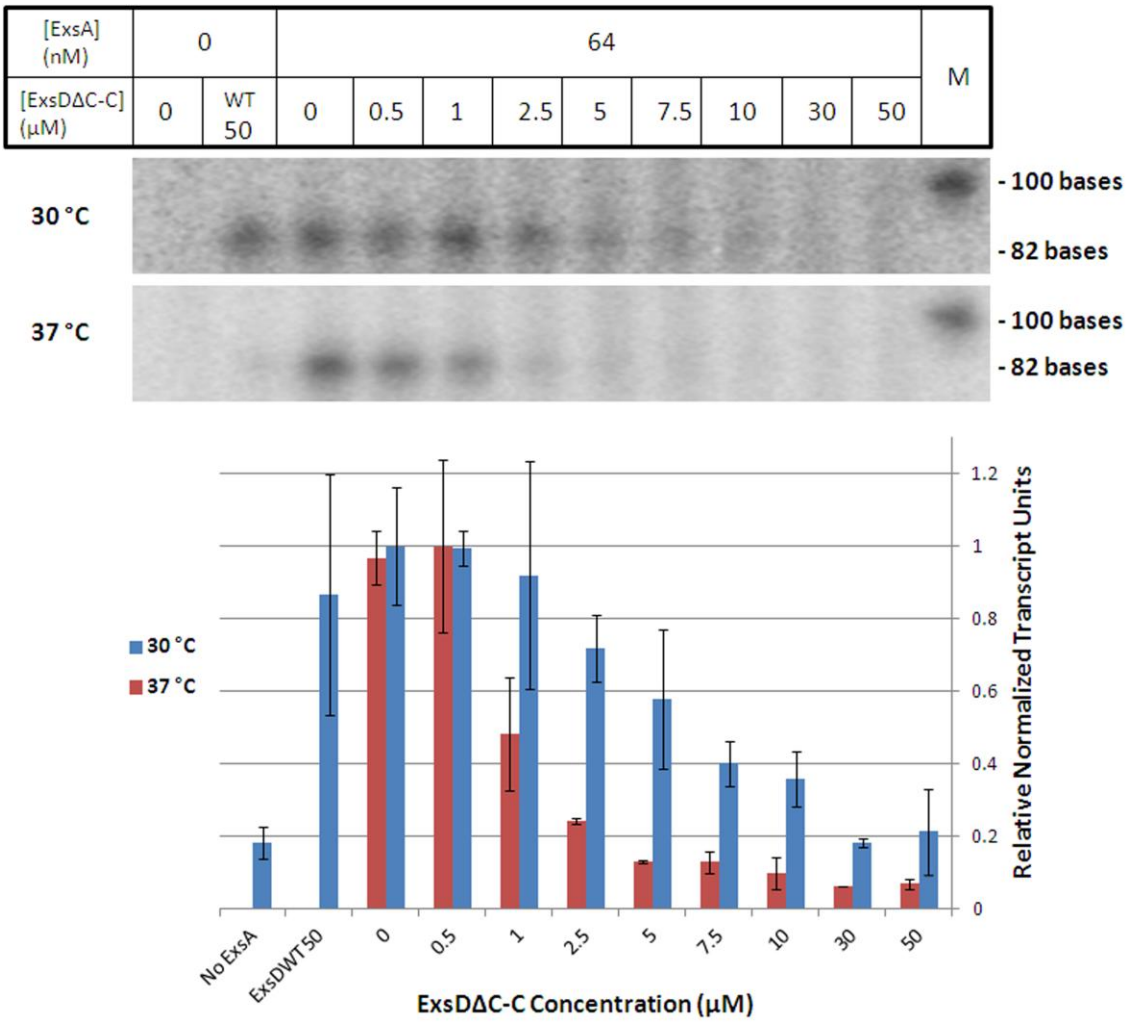


Fig. 4.4. Effect of ExsDΔC-C on ExsA-dependent transcription at 30 and 37 °C. Autoradiograms and graphical representations for the *in vitro* transcription of an 82 base nucleotide transcript from an ExsA-dependent P_{exsD} promoter template with 64 nM ExsA and a titration of ExsDΔC-C at 30 and 37 °C. Wild type ExsD at 50 μM served as a control. Each experiment was performed in duplicate. The IC_{50} values were 4.3 ± 0.4 μM and 0.97 ± 0.4 μM at 30 and 37 °C, respectively.

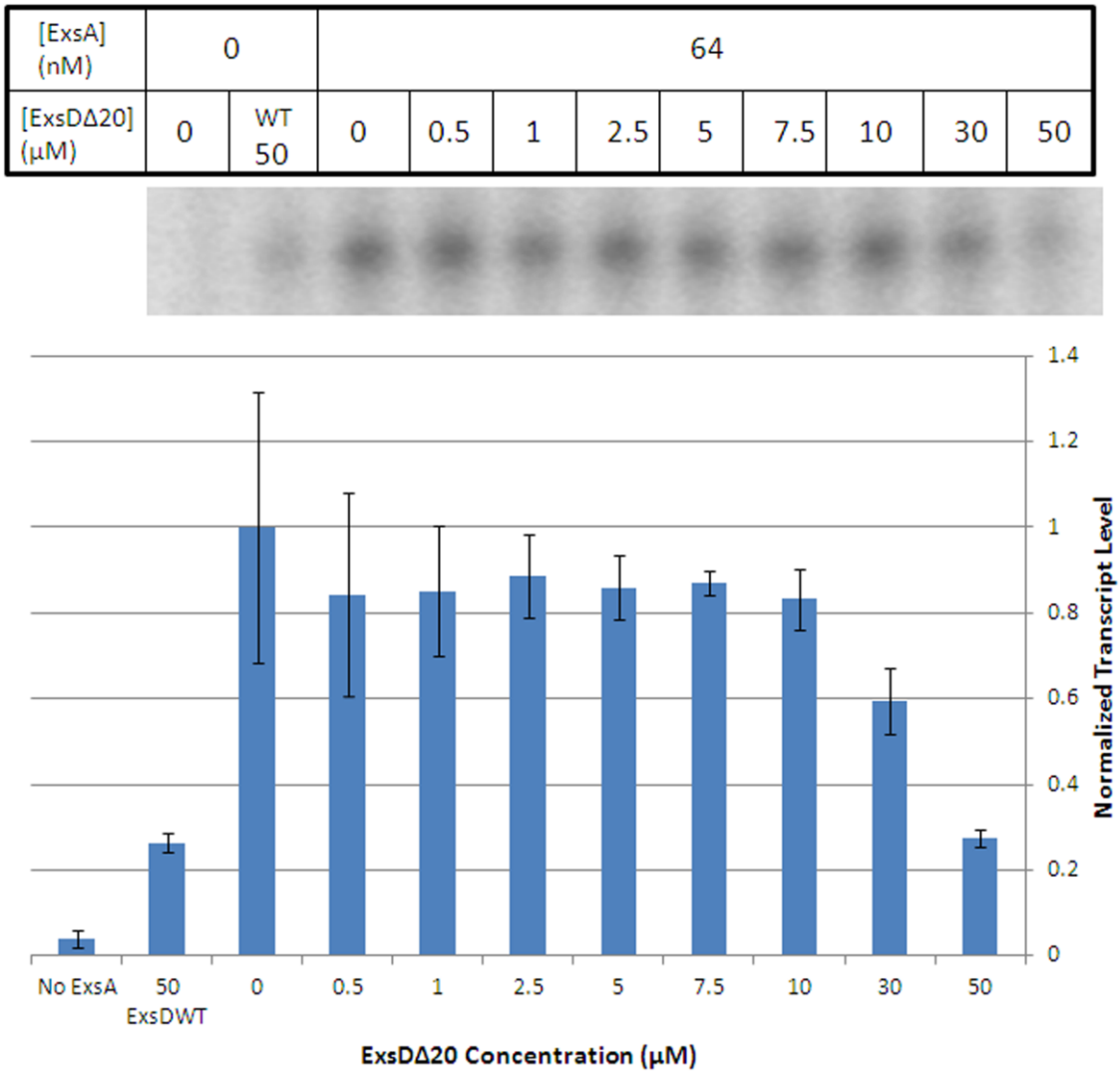


Fig. 4.5. Effect of ExsDΔ20 on ExsA dependent transcription at 37 °C. Autoradiogram and graphical representation for the *in vitro* transcription of an 82 base nucleotide transcript from an ExsA-dependent P_{exsD} promoter template with 64 nM ExsA and a titration of ExsDΔ20 at 37 °C. Wild type ExsD at 50 μM served as a control. This experiment was performed in triplicate.

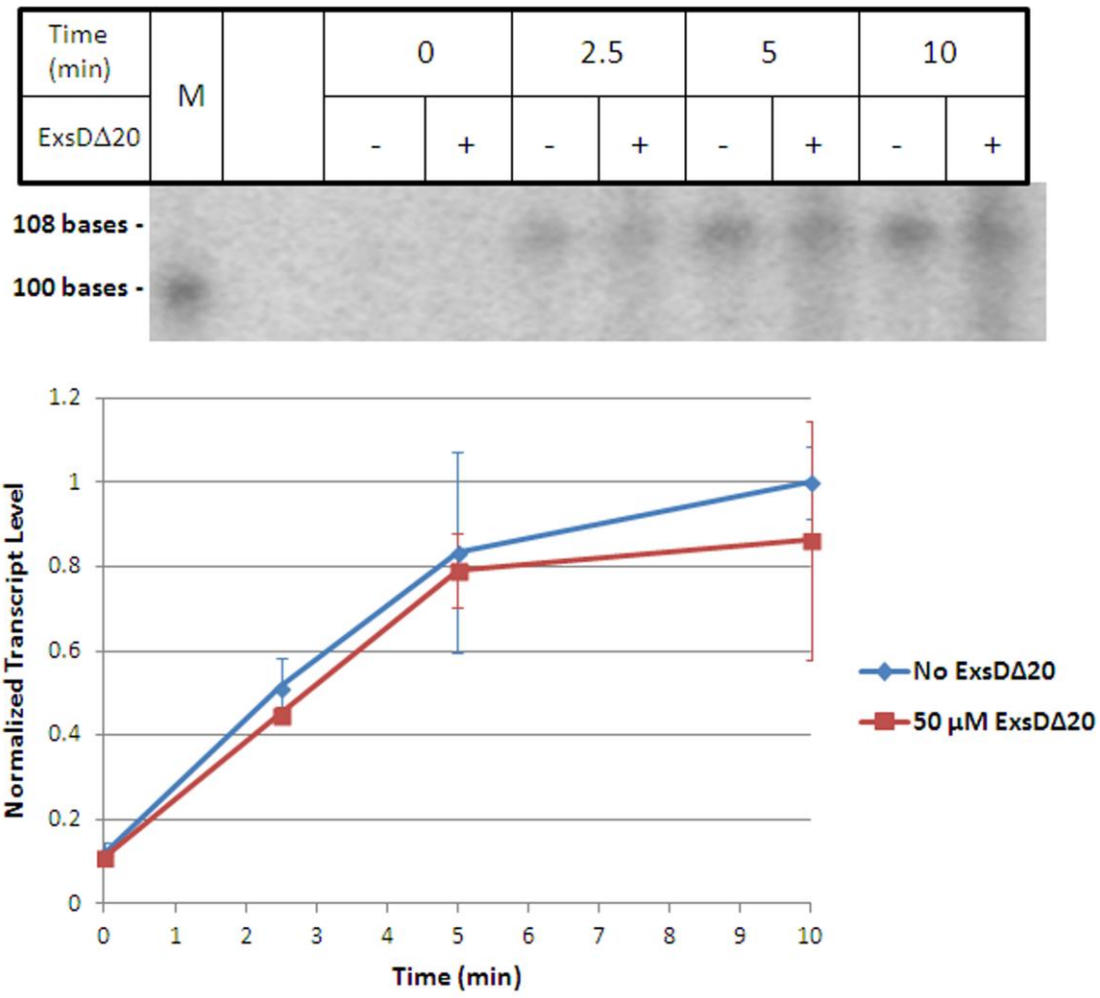


Fig. 4.6. ExsDΔ20 has no effect on ExsA-independent transcription. Autoradiogram and graphical representation for the *in vitro* transcription of a 108 nucleotide transcript from an ExsA-independent RNA-1 promoter template with and without 50 μM ExsDΔ20 at 37 °C. This experiment was performed in duplicate.

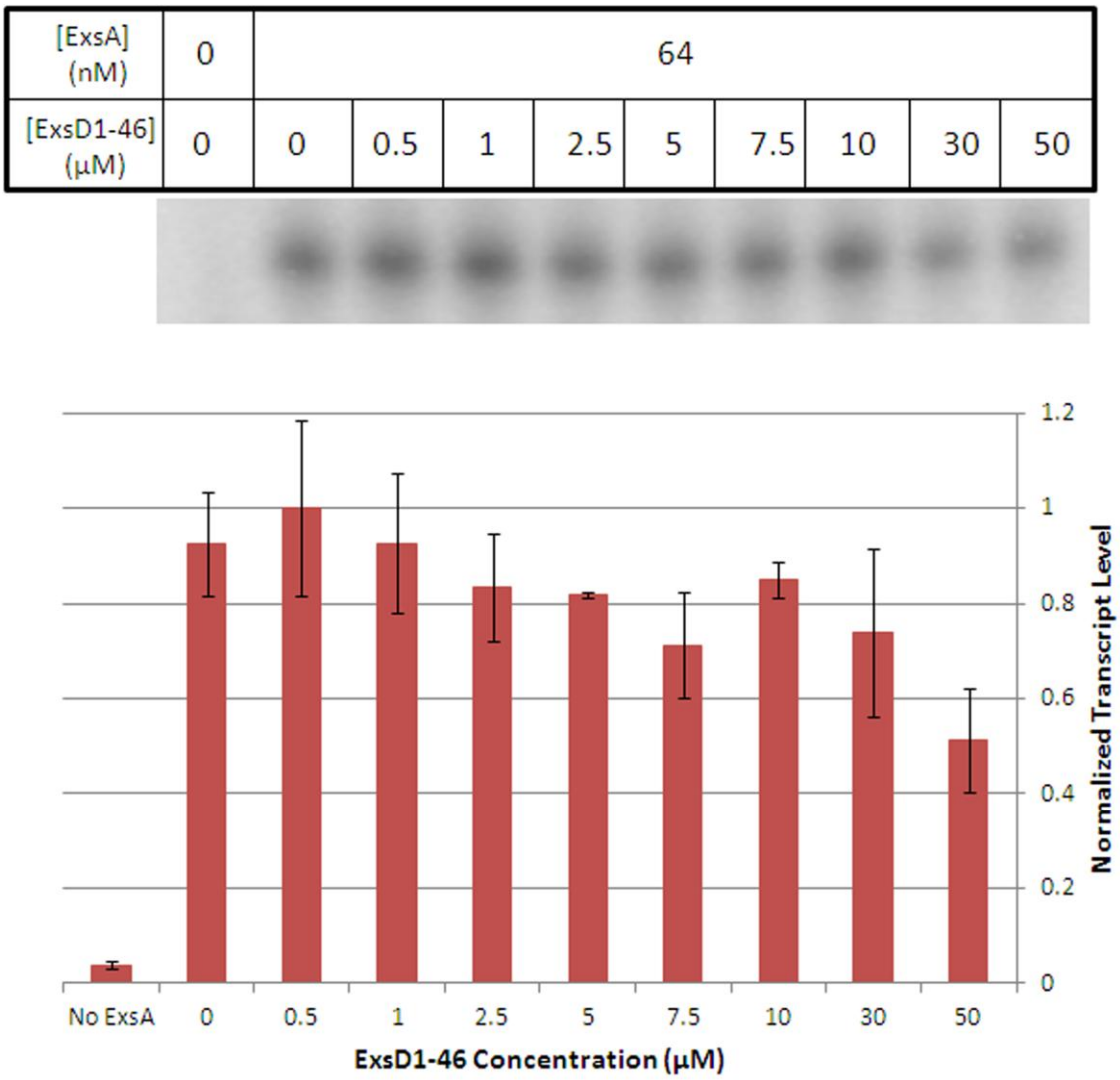


Fig. 4.7. Effect of ExsD1-46 on ExsA dependent transcription at 37 °C. Autoradiogram and graphical representation for the *in vitro* transcription of an 82 base nucleotide transcript from an ExsA-dependent P_{exsD} promoter template with 64 nM ExsA and a titration of ExsD1-46 at 37 °C. This experiment was performed in triplicate.

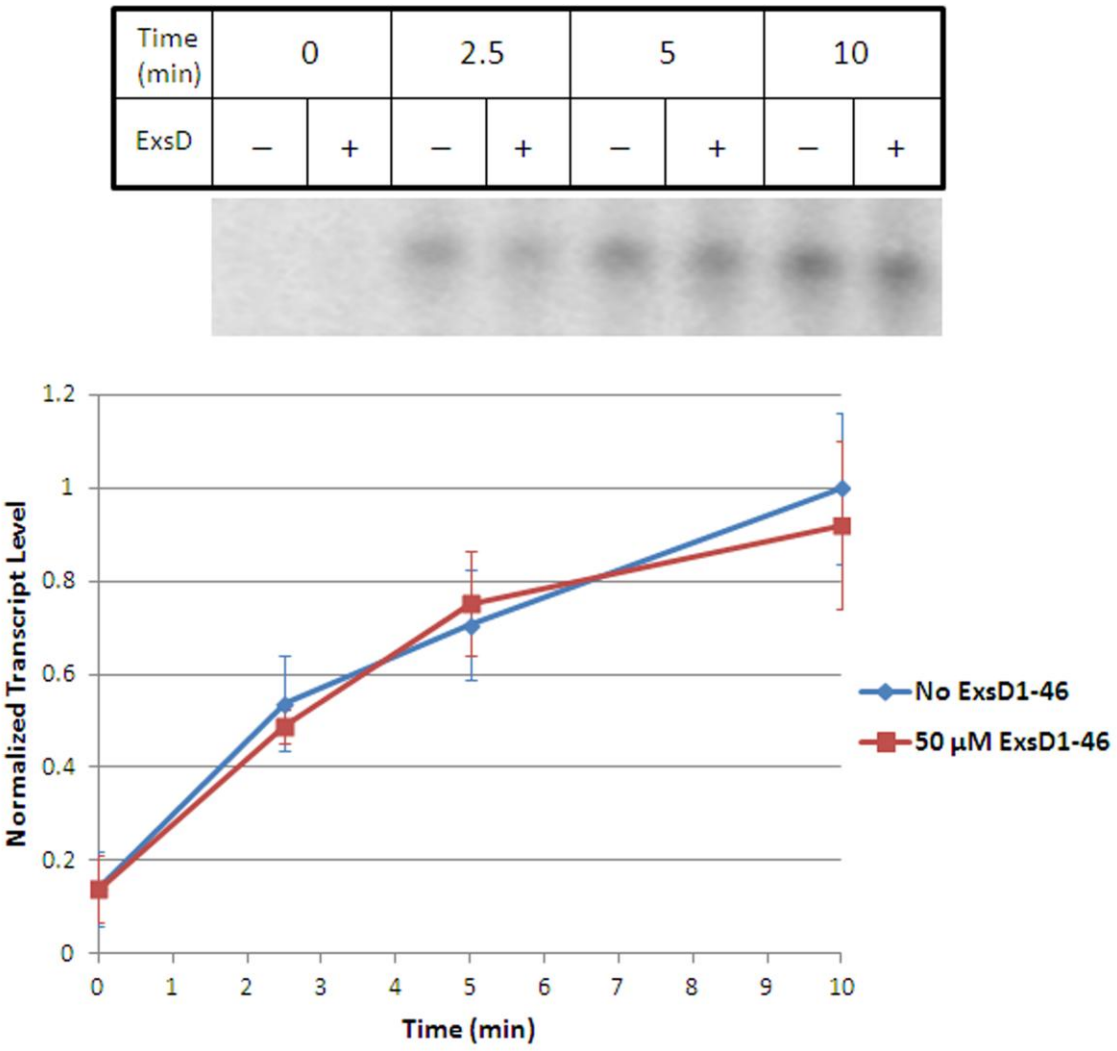


Fig. 4.8. ExsD1-46 has no effect on ExsA-independent transcription. Autoradiogram and graphical representation for the *in vitro* transcription of a 108 nucleotide transcript from an ExsA-independent RNA-1 promoter template with and without 50 µM ExsD1-46 at 37 °C. This experiment was performed in triplicate.

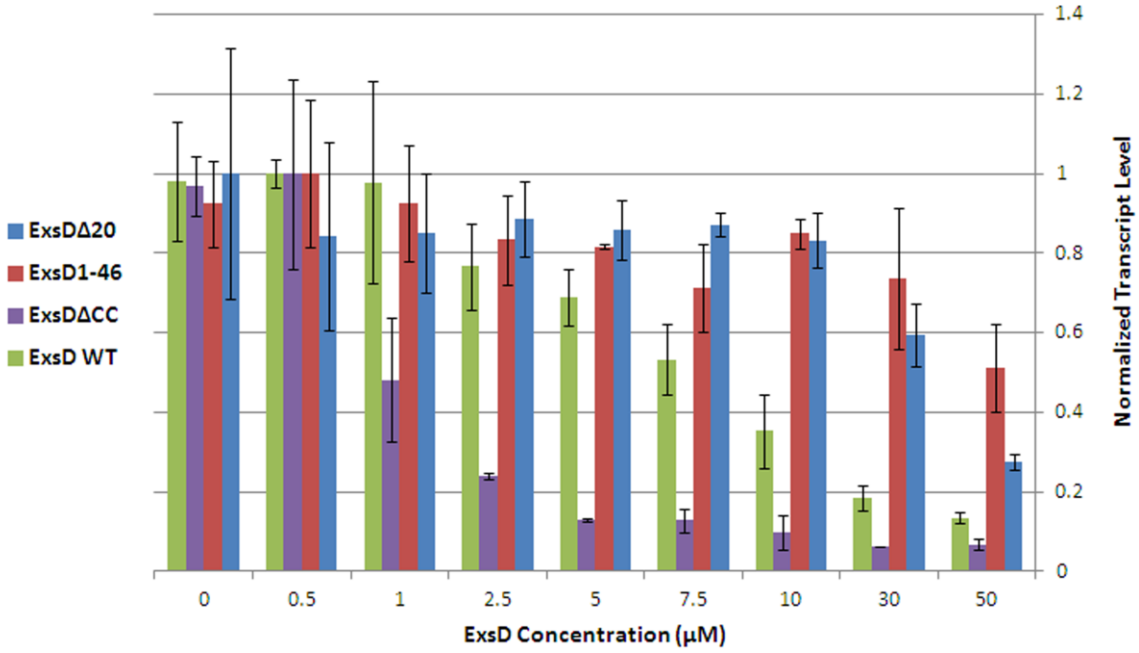


Fig. 4.9. Effect of ExsD variants on ExsA dependent transcription at 37 °C. Comparison of previously shown *in vitro* transcription assays of the ExsD variants to wild type ExsD.

Table 1. IC_{50} values of ExsD variants at 30 and 37 °C.

	30 °C	37 °C
Wild type ExsD	N/A	6.6 ± 0.9
$ExsD^{M59R}$	0.37 ± 0.1	1.3 ± 0.1
ExsDΔC-C	4.3 ± 0.4	0.97 ± 0.4

CHAPTER FIVE

Overall Conclusions

Pseudomonas aeruginosa is a major public health concern due to the increasing number of immunocompromised patients in the United States and around the world as well as its growing resistance to common antibiotics. The T3SS is the main virulence mechanism of *P. aeruginosa* acute infection; therefore it is of great medical importance. The ExsA-ExsC-ExsD-ExsE regulatory cascade in *P. aeruginosa* is highly unusual in that it functions solely on protein–protein interactions to regulate the T3SS. Understanding the way in which these regulatory proteins interact is essential to developing drugs to shut down the T3SS. The ExsD–ExsA interaction is of critical importance, because it sits at the bottom of the regulatory cascade. ExsA is the main transcriptional activator and is absolutely essential for every aspect of the T3SS. ExsD directly binds to ExsA and inhibits the T3SS. Without ExsD, the T3SS is constitutively expressed. Understanding how ExsA and ExsD function will lay the groundwork for the development of novel therapeutics to combat *P. aeruginosa* infection.

Chapter Two examines the T3SS activator ExsA. We detail a procedure for greatly improving the solubility of recombinant ExsA utilizing a His-MBP tag and TEV protease during the purification process. The end result is a highly purified ExsA that is soluble without any tag. An *in vitro* transcription assay was developed and it was determined that the recombinant ExsA is able to activate T3SS transcription. This proves that the recombinant ExsA is highly functional. The affinity of ExsA for its P_{exsD} promoter was also determined by fluorescence anisotropy which is an equilibrium technique, and thus more reliable than EMSA. Considering that ExsA is a member of the AraC/XylS family of transcriptional activators, it can be used as a model since these family members are known for their low solubility. The ability to produce soluble and

highly active ExsA allows for biochemical analyses of the protein which can reveal information on these types of activators. Furthermore, ExsA is a member of a subfamily of AraC/XylS activators that regulate virulence factor transcription in a variety of Gram-negative pathogens. *P. aeruginosa* ExsA shares close homology with many of the activators of this subfamily, especially ExsA in *Vibrio parahaemolyticus* and LcrF in *Yersinia pestis*. Our lab has recently solved the structure of the amino-terminal/regulatory domain of ExsA. Future work will be to crystallize full-length ExsA in complex with DNA and solve its structure. This crystal structure would reveal how ExsA interacts with DNA. With this information, drugs can be designed to target regions of ExsA that interact with DNA. Disrupting ExsA DNA binding would shut down the T3SS. These drugs would also have the potential to shut down the T3SS in other pathogenic species, such as *Vibrio parahaemolyticus* and *Yersinia pestis*.

ExsD is the antiactivator protein that inhibits the function of ExsA in order to shut down the T3SS. ExsD is a dynamic protein that is able to exist as a monomer or a trimer under different conditions. Chapter Three examines the ExsD–ExsA inhibitory mechanism. We show that ExsD is sufficient to inhibit ExsA-dependent transcription *in vitro*, and no other cellular factors are required. We also demonstrate that ExsD inhibits ExsA in a temperature-dependent manner *in vitro*. ExsD inhibits ExsA at 37 °C, but not 30 °C. By engineering an ExsD monomeric variant, we demonstrate that this thermoregulation is due to the ExsD trimer. ExsD inhibits ExsA by binding in a 1:1 complex [147], therefore, the trimer prevents ExsD from binding to ExsA. When the temperature is increased, the ExsD trimer becomes less stable, allowing for ExsD to bind to ExsA in the 1:1 complex that is needed for inhibition. T3SS transcription is known to

be upregulated at 37 °C [203], so it seems contradictory that a shift from 30 °C to 37 °C would inhibit T3SS transcription. We hypothesize that this mechanism could serve to downregulate T3SS gene expression in the lungs of cystic fibrosis patients. In this environment, *P. aeruginosa* switches from an acute form of infection to a chronic/biofilm-producing state in which the T3SS is not expressed [36]. Future work will be to determine the role the ExsD trimer plays *in vivo* and during the course of *P. aeruginosa* infection. A cystic fibrosis mouse model could be used to determine if the PA103 *exsD*^{M59R} strain has a different effect during pathogenesis compared to PA103 wild type [207]. One could hypothesize that the *exsD*^{M59R} mutation would reduce T3SS activity. This could lead to a faster switch to a biofilm-producing state. An alternative outcome is that the mutation could prevent *P. aeruginosa* from establishing an infection since the T3SS is required to initiate infection [68]. Given there was no difference in transcriptional activity between PA103 wild type and PA103 *exsD*^{M59R} in a β-galactosidase assay (Fig. A.3), possibly due to the temperature sensitivity of ExsD^{M59R}, a difference in pathogenesis might not be observed with this strain in the mouse model. A PA103 *exsDΔC-C* strain might be a better choice for this study since this mutation would attenuate but does not fully disrupt ExsD self-association.

Chapter Four provides evidence that ExsD is a DNA-binding protein, which adds to its complexity. We demonstrate that ExsD binds to DNA as a monomer. However, we believe that this DNA binding is non-specific. ChIP-sequencing will be used to determine if ExsD has any specific DNA-binding sites. It is unclear whether ExsD DNA-binding plays a role in the inhibition of ExsA. Future work will investigate this possibility.

The ExsD–ExsA interface is also examined in Chapter Four. It was determined that the coiled-coil region of ExsD is not important for ExsA-binding. Removal of the coiled-coil region greatly reduces the stability of the ExsD trimer, so the coiled-coil region is important for ExsD trimer formation. We found that the amino terminus of ExsD is important for ExsA-binding, but is not sufficient to inhibit ExsA-dependent transcription. Future work will be to determine the key residues that are important for ExsA-binding.

The way in which ExsD inhibits ExsA remains ambiguous. The current model holds that ExsD disrupts ExsA dimerization and promoter interactions by binding to ExsA in a 1:1 complex. We propose two other possible scenarios, as outlined in Chapter Four. The first is ExsD binds to ExsA in a 1:1 complex, while ExsA is bound to the promoter and disrupts ExsA dimer formation. ExsD may or may not be binding to DNA under this scenario. The other possibility is that ExsD binds to ExsA when the ExsA dimer is formed on the promoter and disrupts ExsA–RNAP interactions. Given the structural similarity of ExsD to RNAP binding protein GreB from *E. coli* [176], one could imagine a scenario where ExsD interacts with RNAP in the same manner where the coiled-coil region inserts into RNAP. Seeing as the coiled-coil region of ExsD does not interact with ExsA, ExsD could be binding to both ExsA and RNAP at the same time. ExsD could inhibit T3SS transcription by stalling RNAP. Cryo-electron microscopy is currently being used to determine if ExsD does indeed interact with RNAP.

Given that *P. aeruginosa* is highly resistant to the majority of current classes of antibiotics, new drug treatments are of critical importance. Drugs that inhibit the T3SS would eliminate acute *P. aeruginosa* infection. They could also prevent *P. aeruginosa*

from establishing infection in the lungs of cystic fibrosis patients. In addition, these drugs would place less selective pressure on the organism to develop resistance, because *P. aeruginosa* does not require a T3SS to survive outside of the host. This dissertation contributes to the groundwork for the understanding of the key regulators of the T3SS in *P. aeruginosa*. It highlights the complexity of ExsD and the way it interacts with ExsA. This work reveals new insights that challenge the current ExsD–ExsA inhibitory model. More work will need to be done to fully understand the way in which ExsD inhibits ExsA. Future work will include using mass spectrometry to determine how many molecules of ExsA, ExsD, ExsC, and ExsE are present in the cell under inducing and non-inducing conditions for type III secretion. This could shed light on how the regulatory cascade works to turn on and off the T3SS. We suspect that the concentrations of the regulators play a key role. A small shift in the equilibria may be all that it takes to turn the T3SS on or off in this finely tuned mechanism. Once the ExsD–ExsA inhibitory mechanism is fully elucidated, drugs can be designed to mimic ExsD function in order to inhibit ExsA and shut down the T3SS, thereby eliminating acute *P. aeruginosa* infection.

APPENDIX

Extension of Chapter Three: Self-trimerization of ExsD limits inhibition of the *Pseudomonas aeruginosa* transcriptional activator ExsA *in vitro*

Robert C. Bernhards, Shannon K. Esher, Florian D. Schubot

Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

Abbreviations: MSG = monosodium glutamate; ONPG = ortho-Nitrophenyl-β-galactoside; T3SS = type III secretion system; TBE = Tris/borate/EDTA; TSB = trypticase soy broth; VB = Vogel-Bonner minimal medium.

Abstract

Pseudomonas aeruginosa uses a type III secretion system (T3SS) to cause acute infection in humans. This T3SS is regulated by a cascade of four interacting proteins: ExsA, ExsC, ExsD, and ExsE. ExsA is the main transcriptional activator and is inhibited by the antiactivator ExsD. In Chapter Three, it was observed that ExsD is capable of inhibiting ExsA *in vitro* at 37 °C but not 30 °C. It was discovered that this thermoregulation was a result of ExsD self-trimerization. Here, we further investigate this phenomenon. An *in vitro* transcription assay was performed at 33 °C, and it was determined that ExsD inhibits ExsA based on a temperature gradient, rather than a switch between 30 and 37 °C. We also examined the $ExsD^{M59R}$ monomeric variant at 37 °C. As expected, the inhibition by $ExsD^{M59R}$ decreases at this temperature, because $ExsD^{M59R}$ is unstable at 37 °C. Given that the observed thermoregulation was *in vitro*, we wanted to examine whether ExsD trimerization plays a role *in vivo*. A β-galactosidase assay was performed to compare wild type ExsD to an $ExsD^{M59R}$ mutant strain under inducing and non-inducing conditions for type III secretion.

Introduction

The type III secretion system (T3SS) of *P. aeruginosa* is essential for causing acute infections in immunocompromised patients. Regulation of the T3SS is coordinated by a set of four proteins: ExsA, ExsC, ExsD, and ExsE. ExsA is the main transcription activator [120], and when the T3SS is turned off, ExsA is inhibited by the antiactivator ExsD [147, 171]. When the T3SS is turned on, ExsE is secreted through the T3SS needle [108, 109], which frees ExsC to bind to ExsD [148, 177]. ExsA is now free bind to the T3SS promoters and recruit RNA polymerase to activate T3SS transcription [149].

Chapter Three investigated the ExsD–ExsA inhibitory mechanism, and here, we further investigate this mechanism. Chapter Three demonstrated that ExsD is able to inhibit ExsA-dependent transcription *in vitro* at 37 °C but not 30 °C [206]. Here, an *in vitro* transcription assay was performed at an intermediate temperature (33 °C) to determine if the inhibition by ExsD corresponds to a temperature gradient or simply an on/off switch between 30 and 37 °C. The thermoregulation described in Chapter Three was due to ExsD self-trimerization, because a monomeric ExsD variant ($ExsD^{M59R}$) was able to strongly inhibit ExsA-dependent transcription *in vitro* at 30 °C [206]. Differential scanning fluorimetry was used in Chapter Three to demonstrate that the $ExsD^{M59R}$ variant is not as stable as wild type ExsD [206]. This is most likely due to a greater amount of exposed surface area. We wanted to determine if $ExsD^{M59R}$ is still able to inhibit ExsA at 37 °C, because this is the temperature where *P. aeruginosa* infects its human host. This would serve as a way to test the suitability of $ExsD^{M59R}$ for use *in vivo*. In order to do this, we performed an *in vitro* transcription assay to examine the effect of $ExsD^{M59R}$ on ExsA-dependent transcription at 37 °C. We found that $ExsD^{M59R}$ lost some activity at

this temperature, so we thought it was best to examine the effect of ExsD^{M59R} *in vivo* using a β-galactosidase assay at 30 °C. A chromosomal ExsD^{M59R} mutation was made in the *P. aeruginosa* strain PA103, and this mutant was compared to wild type PA103 under inducing and non-inducing conditions for type III secretion.

Results

Inhibition by ExsD is based on a temperature gradient

It was determined in Chapter Three that ExsD inhibits ExsA-dependent transcription *in vitro* at 37 °C but not 30 °C. We wanted to determine if this temperature-dependent inhibition corresponded to a temperature gradient or simply an on/off switch between 37 and 30 °C. In order to do this, an *in vitro* transcription assay was performed at 33 °C (Fig. A.1). In this 0-10 min time course experiment, each sample contained 64 nM ExsA without or without 50 μM ExsD. There was a 1.72x level of inhibition by ExsD, which is in between what was previously seen at 30 °C (no inhibition) and 37 °C (~3.75x inhibition). This demonstrates that the observed thermoregulation corresponds to a temperature gradient.

Effect of ExsD^{M59R} on ExsA-dependent transcription at 37 °C

In Chapter Three, the ExsD^{M59R} monomeric variant was utilized to determine that the observed thermoregulation was due to ExsD self-trimerization. ExsD^{M59R} was found to strongly inhibit ExsA-dependent transcription in an *in vitro* transcription assay at 30 °C. Here, we wanted to examine ExsD^{M59R} in the *in vitro* transcription assay at 37 °C. Based on a previous differential scanning fluorimetry experiment, ExsD^{M59R} would likely be unstable at this higher temperature. A titration of ExsD^{M59R} was performed keeping

the ExsA concentration constant at 64 nM (Fig. A.2). The IC_{50} value was measured to be 1.3 ± 0.1 μM, which is slightly higher than the IC_{50} value at 30 °C (0.37 ± 0.1 μM). This indicates that a certain percentage of the $ExsD^{M59R}$ protein is becoming denatured at the higher temperature, as expected. However, $ExsD^{M59R}$ at 37 °C is still a more potent inhibitor than wild type ExsD at the same temperature (IC_{50} = 6.6 ± 0.9 μM). This suggests that most of the $ExsD^{M59R}$ is stable enough to still be active at 37 °C.

Effect of $ExsD^{M59R}$ *in vivo*

Given that the thermoregulation due to ExsD trimerization was observed *in vitro*, we wanted to examine if the effect is observed *in vivo*. A PA103 $ExsD^{M59R}$ chromosomal mutant strain was compared to PA103 wild type in a β-galactosidase assay (Fig. A.3). The cultures were grown under inducing and non-inducing conditions for type III secretion at 30 °C, and T3SS transcriptional activation was measured from the P_{exsD} promoter. We hypothesized that the $ExsD^{M59R}$ strain would inhibit ExsA more strongly compared to wild type, because the monomeric $ExsD^{M59R}$ variant is able to strongly inhibit ExsA-dependent transcription at 30 °C *in vitro*. However, there was no significant difference in the level of T3SS transcription under inducing or non-inducing conditions.

Discussion

Here we demonstrate that the observed thermoregulation by ExsD corresponds to a temperature gradient. This suggests that as the temperature increases, the ExsD trimer becomes less stable, which allows for ExsD to bind ExsA in the 1:1 complex that is needed for inhibition [147]. Chapter Three reveals that the $ExsD^{M59R}$ monomeric variant is capable of inhibiting ExsA-dependent transcription at 30 °C, which demonstrates that

the ExsD trimer is reason for the thermoregulation seen with wild type ExsD [206]. Here, we wanted to examine the ability of ExsD^{M59R} to inhibit ExsA-dependent transcription at 37 °C. ExsD^{M59R} in the absence of ExsA or DNA is unstable at 37 °C, as shown by the differential scanning fluorimetry experiment in Chapter Three, which revealed a melting temperature of 30.7 °C [206]. However, in the presence of binding partners ExsA and DNA, the overall stability of ExsD^{M59R} should increase. The *in vitro* transcription assay at 37 °C showed only a slight decrease in inhibition. The IC_{50} value was 1.3 ± 0.1 μM, compared to 0.37 ± 0.1 μM at 30 °C. This suggests that a certain amount of ExsD^{M59R} is denatured, and thus inactive at the higher temperature. However, the inhibition is still stronger than wild type ExsD (IC_{50} = 6.6 ± 0.9 μM) [206], indicating that a high percentage of ExsD^{M59R} is still stable at the increased temperature. In Chapter Four, it was demonstrated that the melting temperature of ExsD^{M59R} does increase when DNA is added. We also assume that the stability of ExsD^{M59R} improves upon binding to ExsA.

Our initial *in vivo* analysis of ExsD^{M59R} was inconclusive. In the β-galactosidase assay, there was no difference between the ExsD^{M59R} mutant and wild type under inducing or non-inducing conditions for type III secretion at 30 °C. As this is our first attempt, the effect of ExsD self-trimerization *in vivo* will need to be further investigated. Outside of the ExsA-ExsC-ExsD-ExsE cascade, regulation of the T3SS is very complex. There are many other factors that influence the T3SS (Fig. 1.2), however the mechanisms are poorly understood. These regulatory mechanisms could be masking the effect of ExsD^{M59R}. Therefore, the ExsD^{M59R} mutant should be tested under a variety of growth conditions. An alternative explanation is that ExsD^{M59R} is not stable enough for use *in vivo*. A more stable ExsD monomeric variant could be a better option. Chapter Four

describes an ExsD variant lacking the coiled-coil region (ExsDΔC-C). This ExsD variant showed strong, monomeric activity in the *in vitro* transcription assay. However, ExsDΔC-C is not believed to be completely monomeric. At 30 °C, the activity of ExsDΔC-C indicates that it is partially trimeric. Therefore, ExsDΔC-C could be more stable than $ExsD^{M59R}$. A differential scanning fluorimetry experiment could be used to determine the stability of ExsDΔC-C. If it shows increased stability, a chromosomal ExsDΔC-C mutant could be constructed and used in β-galactosidase assays. We also demonstrated in Chapter Four that the coiled-coil region is not important for ExsA binding, so the mutation should not affect the ability of ExsD to inhibit ExsA.

Materials and Methods

In vitro transcription assay

The linear DNA template used in each assay encompassed positions -207 to 94 of the P_{exsD} promoter, relative to the transcription start site; and from this template, RNA polymerase synthesizes an 82 base mRNA transcript. The template was produced by PCR using forward primer 5´-CATCAGTTGCTGCTCAACAGCG-3´ and reverse primer 5´-CACCGCTTCTCGGGAGTACTGC-3´. The PCR product was run on a 2% agarose gel and purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA). Each 30 µL transcription assay reaction contained 4.4 fM of promoter template, 50.4 µM bovine serum albumin (to eliminate non-specific protein-protein interactions), 10 U purified RNA polymerase from *P. aeruginosa* (see Chapter Three), 1 U RiboGuard RNase Inhibitor (Epicentre Biotechnologies), 15 ng/µL poly(deoxyinosinic-deoxycytidylic) acid (to prevent non-specific transcription initiation),

133 mM NaCl, 32 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 25 µM EDTA, 0.9 mM TCEP, 0.2 mM DTT, and 15.5% glycerol. The time-course experiments contained 64 nM ExsA and either no ExsD or 50 µM ExsD (no ExsA was added for the RNA-1 control experiments). Samples were mixed and allowed to equilibrate at room temperature for five min. Samples were then pre-incubated for 10 min at either 33 or 37°C, depending on the experiment. Next, 3 µL NTPs (stock concentrations of 200 µM ATP, CTP, GTP and 40 µM UTP) mixed with 0.2 µL (0.2 µCi) of 3.3 mM P^{32}-alpha UTP was added to each sample to start the reaction, and samples were incubated at either 33 or 37°C, depending on the experiment. After the reactions were stopped by adding 12 µL 1X stop solution (3M ammonium acetate, 50 mM EDTA, 0.11 mg/mL glycogen), 170 µL 100% cold ethanol was added, and the samples were incubated at -20 °C for one hr. Following centrifugation at 12,000 x g for 15 min, the supernatant was discarded and pellets were resuspended in 12 µL 1X TBE (Tris/borate/EDTA)-urea sample buffer and heated at 70 °C for five min. After a brief centrifugation, the samples were loaded onto a 10% TBE-urea gel and run at 200 mV for 60 min. Gels were exposed to a storage phosphor screen (GE Healthcare) for 16 hr. The phosphor screen was scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare), and gel bands were quantified using Image Quant TL v2005 (Amersham Biosciences, Piscataway, NJ, USA). The IC_{50} value for the $ExsD^{M59R}$ experiment was determined by analysis using Kaleidograph (Synergy Software).

β-galactosidase assay

PA103-miniCTX-P_{exsD}-lacZ (wild type) and PA103$ExsD^{M59R}$-miniCTX-P_{exsD}-*lacZ* strains were grown on Vogel-Bonner (VB) minimal medium plates with 4 µg/mL

triclosan overnight at 37 °C. Cells were scraped from the plates and resuspended in 2 mL trypticase soy broth (TSB). The broths were shaken at 300 rpm at 37 °C for 5 min to resuspend the cells. OD_{600} measurements were taken and adjusted amounts were added to 10 mL of TSB with 1% glycerol and 100 mM monosodium glutamate (MSG) so that each culture started at an OD_{600} of 0.1. EGTA pH 8.0 was added to both of the (+) EGTA cultures to obtain a final concentration of 2 mM. The cultures were incubated at 30 °C with shaking at 250 rpm. 100 µl samples were taken for each time point and stored at -20 °C. 900 µl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 x H_2O, 10 mM KCl, 1 mM $MgSO_4$ x 7 H_2O) with 50 mM β-mercaptoethanol was added to the frozen samples, and they were thawed in a 28 °C water bath. Once thawed, 30 µl of chloroform and 30 µl of 0.1% SDS were added, and the samples were vortexed for 10 sec. Each sample was pre-incubated at 28 °C for 5 min. Then 0.2 mL *ortho*-Nitrophenyl-β-galactoside (ONPG) solution (4 mg/mL in Z-buffer containing β-mercaptoethanol) was added to start the reaction, and they were incubated immediately at 28 °C. The reactions were stopped once they turned yellow by adding 0.5 mL of 1 M Na_2CO_3. The samples were centrifuged for 5 min at 10,000 rpm to pellet the lysed cells, and the OD_{420} of the supernatant was measured. Miller units were calculated using the following formula: Miller units = (1000 x OD_{400}) / (incubation time in min x volume of cell culture in mL x OD_{600}).

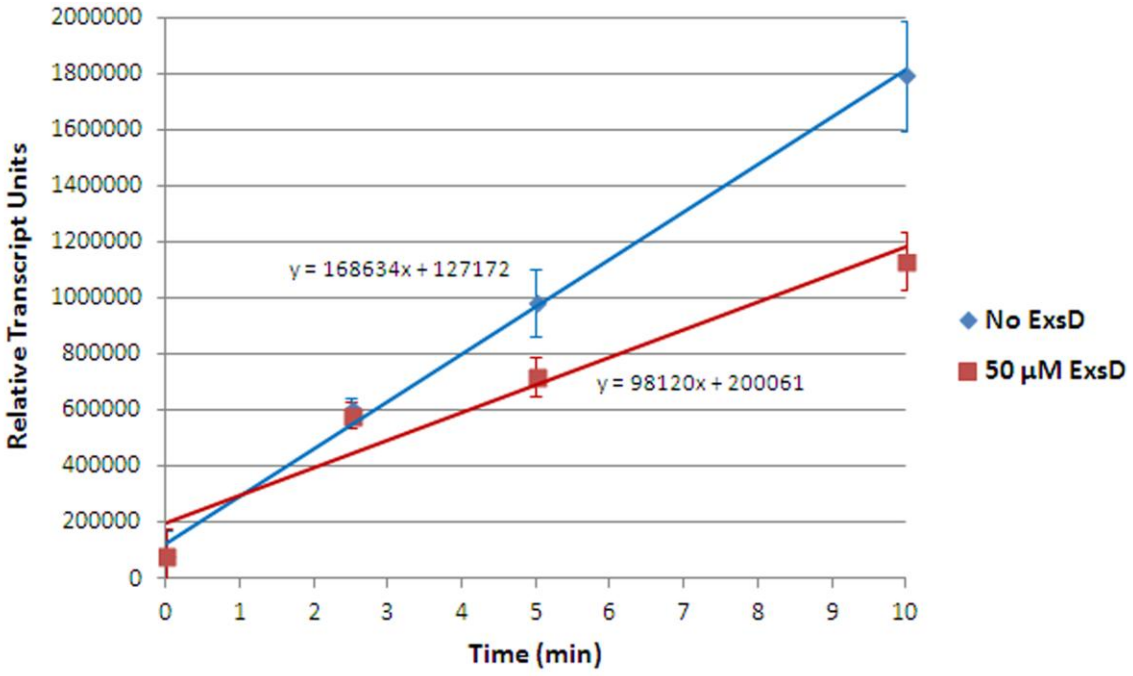


Fig. A.1. Effect of ExsD on ExsA-dependent transcription at 33 °C. A 0-10 min time course was performed with 64 nM ExsA with or without 50 µM ExsD. This experiment was performed in triplicate. ExsD provides a 1.72x level of inhibition at this temperature.

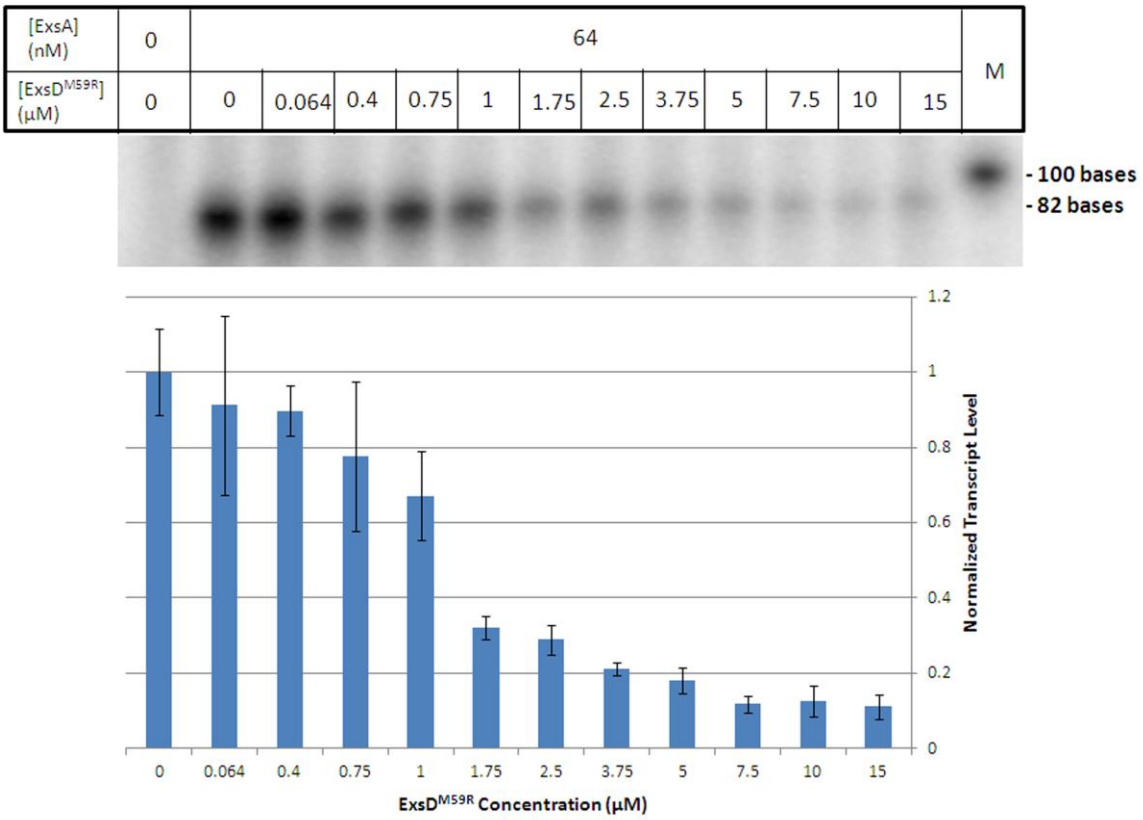


Fig. A.2. Effect of ExsD^{M59R} on ExsA-dependent transcription at 37 °C. A titration of ExsD^{M59R} was performed at 37 °C. Each sample contained 64 nM ExsA. Each reaction was allowed to proceed for 10 min. This experiment was performed in quadruplicate. The resulting IC_{50} value was 1.3 ± 0.1 μM.

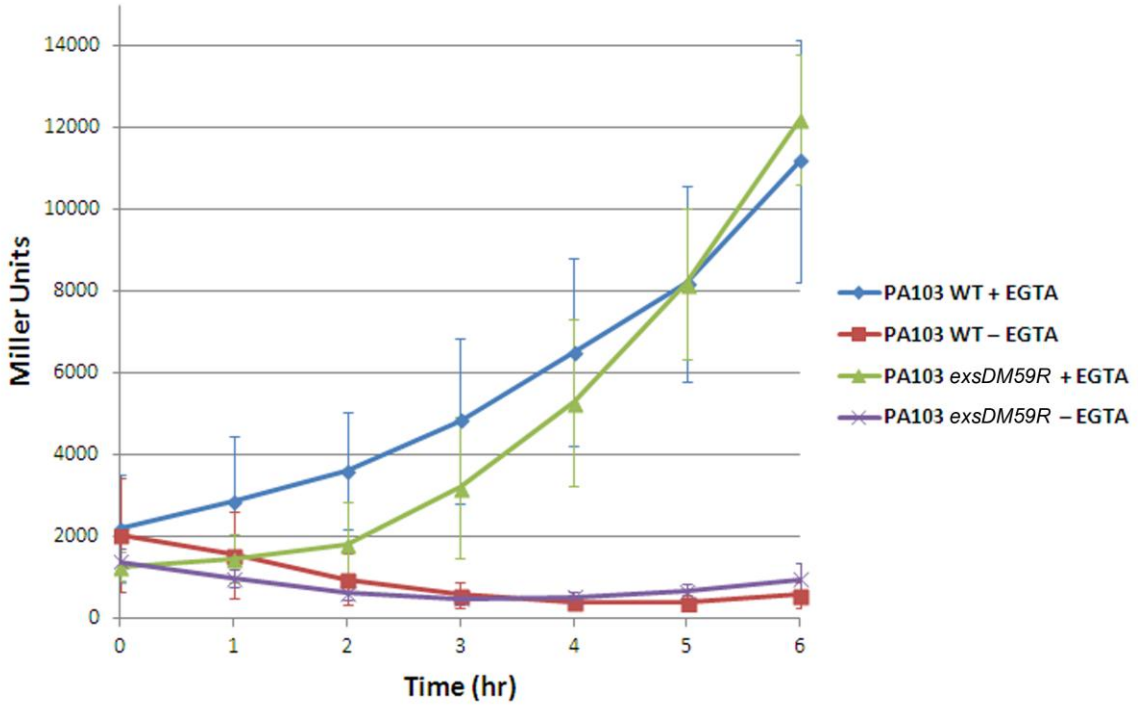


Fig. A.3. T3SS transcription activity of PA103 WT vs. PA103 *exsD*^{M59R}. β-galactosidase assay under inducing (+ EGTA) and non-inducing (– EGTA) conditions for type III secretion at 30 °C. Transcriptional activity was measured from the P_{exsD} promoter. This experiment was performed in quadruplicate.

REFERENCES

1. Haynes, W. C. & Rhodes, L. J. (1962) Comparative taxonomy of crystallogenic strains of *Pseudomonas aeruginosa* and *Pseudomonas chlororaphis*, *J Bacteriol.* **84**, 1080-4.
2. Propst, C. & Lubin, L. (1979) Light-mediated changes in pigmentation of *Pseudomonas aeruginosa* cultures, *Journal of General Microbiology.* **113**, 261-6.
3. Elrod, R. P. & Braun, A. C. (1942) *Pseudomonas aeruginosa*: Its role as a plant pathogen, *J Bacteriol.* **44**, 633-45.
4. Finkelstein, R., Raz, R., Stein, H., Reiss, D., Peleg, H., Merzbach, D. & Sobel, J. D. (1989) Bone and joint infections due to *Pseudomonas aeruginosa*: clinical aspects and treatment, *Israel Journal of Medical Sciences.* **25**, 123-6.
5. Hopkins, R. S., Abbott, D. O. & Wallace, L. E. (1981) Follicular dermatitis outbreak caused by *Pseudomonas aeruginosa* associated with a motel's indoor swimming pool, *Public Health Reports.* **96**, 246-9.
6. Stephenson, J. R., Heard, S. R., Richards, M. A. & Tabaqchali, S. (1985) Gastrointestinal colonization and septicaemia with *Pseudomonas aeruginosa* due to contaminated thymol mouthwash in immunocompromised patients, *The Journal of Hospital Infection.* **6**, 369-78.
7. Phillips, I. (1967) *Pseudomonas aeruginosa* respiratory tract infections in patients receiving mechanical ventilation, *The Journal of hygiene.* **65**, 229-35.
8. Church, D., Elsayed, S., Reid, O., Winston, B. & Lindsay, R. (2006) Burn wound infections, *Clinical Microbiology Reviews.* **19**, 403-34.
9. Mittal, R., Aggarwal, S., Sharma, S., Chhibber, S. & Harjai, K. (2009) Urinary tract infections caused by *Pseudomonas aeruginosa*: a minireview, *Journal of Infection and Public Health.* **2**, 101-11.
10. Carruthers, M. M. & Kanokvechayant, R. (1973) *Pseudomonas aeruginosa* endocarditis. Report of a case, with review of the literature, *The American Journal of Medicine.* **55**, 811-8.
11. Parrott, P. L., Terry, P. M., Whitworth, E. N., Frawley, L. W., Coble, R. S., Wachsmuth, I. K. & McGowan, J. E., Jr. (1982) *Pseudomonas aeruginosa* peritonitis associated with contaminated poloxamer-iodine solution, *Lancet.* **2**, 683-5.
12. Knight, V., Hardy, R. C. & Negrin, J., Jr. (1952) Meningitis due to *Pseudomonas aeruginosa*; intrathecal treatment with streptokinase and streptodornase and intramuscular and intrathecal treatment with neomycin, *Journal of the American Medical Association.* **149**, 1395-7.
13. Richardson, M. E. (1957) *Pseudomonas aeruginosa* septicemia, *The Journal of the American Osteopathic Association.* **57**, 135-7.
14. Shanson, D. C. (1990) Septicaemia in patients with AIDS, *Transactions of the Royal Society of Tropical Medicine and Hygiene.* **84 Suppl 1**, 14-6.
15. Rolston, K. V. & Bodey, G. P. (1992) *Pseudomonas aeruginosa* infection in cancer patients, *Cancer Investigation.* **10**, 43-59.

16. Gransden, W. R., Leibovici, L., Eykyn, S. J., Pitlik, S. D., Samra, Z., Konisberger, H., Drucker, M. & Phillips, I. (1995) Risk factors and a clinical index for diagnosis of *Pseudomonas aeruginosa* bacteremia, *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases.* **1**, 119-123.
17. Hashimoto, M., Hirouchi, S., Kosugi, J., Kohara, W. & Nakane, M. (1971) Morphological studies on fimbriae of *Pseudomonas aeruginosa* and *Aeromonas hydrophila* with special reference to their biological functions, *The Bulletin of Tokyo Medical and Dental University.* **18**, 105-22.
18. Doggett, R. G., Harrison, G. M. & Wallis, E. S. (1964) Comparison of some properties of *Pseudomonas aeruginosa* isolated from infections in persons with and without cystic fibrosis, *J Bacteriol.* **87**, 427-31.
19. Linker, A. & Evans, L. R. (1984) Isolation and characterization of an alginase from mucoid strains of *Pseudomonas aeruginosa*, *J Bacteriol.* **159**, 958-64.
20. Cryz, S. J. & Iglewski, B. H. (1980) Production of alkaline protease by *Pseudomonas aeruginosa*, *Journal of Clinical Microbiology.* **12**, 131-3.
21. Morihara, K., Tsuzuki, H., Oka, T., Inoue, H. & Ebata, M. (1965) *Pseudomonas aeruginosa* elastase. Isolation, crystallization, and preliminary characterization, *The Journal of Biological Chemistry.* **240**, 3295-304.
22. Berka, R. M., Gray, G. L. & Vasil, M. L. (1981) Studies of phospholipase C (heat labile hemolysin) in *Pseudomonas aeruginosa*, *Infection and Immunity.* **34**, 1071-4.
23. Liu, P. V. (1966) The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. II. Effects of lecithinase and protease, *The Journal of Infectious Diseases.* **116**, 112-6.
24. Scharmann, W. (1976) Cytotoxic effects of leukocidin from *Pseudomonas aeruginosa* on polymorphonuclear leukocytes from cattle, *Infection and Immunity.* **13**, 836-43.
25. Wilson, R., Pitt, T., Taylor, G., Watson, D., MacDermot, J., Sykes, D., Roberts, D. & Cole, P. (1987) Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia in vitro, *The Journal of Clinical Investigation.* **79**, 221-9.
26. Miles, A. A. & Khimji, P. L. (1975) Enterobacterial chelators of iron: their occurrence, detection, and relation to pathogenicity, *Journal of Medical Microbiology.* **8**, 477-90.
27. Naoi, M., Egami, F., Hamamura, N. & Homma, J. Y. (1958) [The toxic lipopolysaccharides of *Pseudomonas aeruginosa*], *Biochemische Zeitschrift.* **330**, 421-7.
28. Liu, P. V., Yoshii, S. & Hsieh, H. (1973) Exotoxins of *Pseudomonas aeruginosa*. II. Concentration, purification, and characterization of exotoxin A, *The Journal of Infectious Diseases.* **128**, 514-9.
29. Iglewski, B. H., Sadoff, J., Bjorn, M. J. & Maxwell, E. S. (1978) *Pseudomonas aeruginosa* exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A, *Proceedings of the National Academy of Sciences of the United States of America.* **75**, 3211-5.

30. Cowell, B. A., Chen, D. Y., Frank, D. W., Vallis, A. J. & Fleiszig, S. M. (2000) ExoT of cytotoxic *Pseudomonas aeruginosa* prevents uptake by corneal epithelial cells, *Infection and Immunity.* **68**, 403-6.
31. Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J. P., Fleiszig, S. M., Wu, C., Mende-Mueller, L. & Frank, D. W. (1997) ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury, *Molecular Microbiology.* **25**, 547-57.
32. Yahr, T. L., Vallis, A. J., Hancock, M. K., Barbieri, J. T. & Frank, D. W. (1998) ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system, *Proceedings of the National Academy of Sciences of the United States of America.* **95**, 13899-904.
33. Jean, D., Rezaiguia-Delclaux, S., Delacourt, C., Leclercq, R., Lafuma, C., Brun Buisson, C., Harf, A. & Delclaux, C. (1998) Protective effect of endotoxin instillation on subsequent bacteria-induced acute lung injury in rats, *American Journal of Respiratory and Critical Care Medicine.* **158**, 1702-8.
34. Meluleni, G. J., Grout, M., Evans, D. J. & Pier, G. B. (1995) Mucoid *Pseudomonas aeruginosa* growing in a biofilm *in vitro* are killed by opsonic antibodies to the mucoid exopolysaccharide capsule but not by antibodies produced during chronic lung infection in cystic fibrosis patients, *Journal of Immunology.* **155**, 2029-38.
35. Koch, C. & Hoiby, N. (1993) Pathogenesis of cystic fibrosis, *Lancet.* **341**, 1065-9.
36. Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S. & Lory, S. (2004) A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*, *Developmental Cell.* **7**, 745-54.
37. Burns, J. L., Gibson, R. L., McNamara, S., Yim, D., Emerson, J., Rosenfeld, M., Hiatt, P., McCoy, K., Castile, R., Smith, A. L. & Ramsey, B. W. (2001) Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis, *The Journal of Infectious Diseases.* **183**, 444-52.
38. Richards, M. J., Edwards, J. R., Culver, D. H. & Gaynes, R. P. (1999) Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System, *Critical Care Medicine.* **27**, 887-92.
39. Reuter, S., Sigge, A., Wiedeck, H. & Trautmann, M. (2002) Analysis of transmission pathways of *Pseudomonas aeruginosa* between patients and tap water outlets, *Critical care medicine.* **30**, 2222-8.
40. Grieco, M. H. (1972) *Pseudomonas* arthritis and osteomyelitis, *The Journal of Bone and Joint Surgery American Volume.* **54**, 1693-704.
41. Crouch Brewer, S., Wunderink, R. G., Jones, C. B. & Leeper, K. V., Jr. (1996) Ventilator-associated pneumonia due to *Pseudomonas aeruginosa*, *Chest.* **109**, 1019-29.
42. Garau, J. & Gomez, L. (2003) *Pseudomonas aeruginosa* pneumonia, *Current Opinion in Infectious Diseases.* **16**, 135-43.
43. Ohmagari, N., Hanna, H., Graviss, L., Hackett, B., Perego, C., Gonzalez, V., Dvorak, T., Hogan, H., Hachem, R., Rolston, K. & Raad, I. (2005) Risk factors for infections with multidrug-resistant *Pseudomonas aeruginosa* in patients with cancer, *Cancer.* **104**, 205-12.

44. Aloush, V., Navon-Venezia, S., Seigman-Igra, Y., Cabili, S. & Carmeli, Y. (2006) Multidrug-resistant *Pseudomonas aeruginosa*: risk factors and clinical impact, *Antimicrobial Agents and Chemotherapy.* **50**, 43-8.
45. Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P. & Edmond, M. B. (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study, *Clinical infectious Diseases: An Official Publication of the Infectious Diseases Society of America.* **39**, 309-17.
46. Arruda, E. A., Marinho, I. S., Boulos, M., Sinto, S. I., Caiaffa, H. H., Mendes, C. M., Oplustil, C. P., Sader, H., Levy, C. E. & Levin, A. S. (1999) Nosocomial infections caused by multiresistant *Pseudomonas aeruginosa*, *Infection Control and Hospital Epidemiology: The Official Journal of the Society of Hospital Epidemiologists of America.* **20**, 620-3.
47. Obritsch, M. D., Fish, D. N., MacLaren, R. & Jung, R. (2005) Nosocomial infections due to multidrug-resistant *Pseudomonas aeruginosa*: epidemiology and treatment options, *Pharmacotherapy.* **25**, 1353-64.
48. Bryan, L. E., Kwan, S. & Godfrey, A. J. (1984) Resistance of *Pseudomonas aeruginosa* mutants with altered control of chromosomal beta-lactamase to piperacillin, ceftazidime, and cefsulodin, *Antimicrobial Agents and Chemotherapy.* **25**, 382-4.
49. Reese, L. (1965) Nalidixic acid (Neggram) in the treatment of urinary infections, *Canadian Medical Association Journal.* **92**, 394-7.
50. Gershenfeld, L. & Guarini, J. R. (1951) The development of resistance by *Pseudomonas aeruginosa* to neomycin, streptomycin and chloromycetin, *American Journal of Pharmacy and the Sciences Supporting Public Health.* **123**, 182-93.
51. Angus, B. L., Carey, A. M., Caron, D. A., Kropinski, A. M. & Hancock, R. E. (1982) Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant, *Antimicrobial Agents and Chemotherapy.* **21**, 299-309.
52. Yoshimura, F. & Nikaido, H. (1982) Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes, *J Bacteriol.* **152**, 636-42.
53. Lebek, G. (1963) [The transmission of multiple resistance to antibiotics and chemotherapeutic agents in their significance for hospitalism with multiple resistant Gram-negative intestinal bacteria], *Zentralblatt fur bakteriologie, parasitenkunde, infektionskrankheiten und hygiene 1 abt medizinisch-hygienische bakteriologie, Virusforschung und Parasitologie Originale.* **191**, 387-95.
54. Hooper, D. C. & Wolfson, J. S. (1989) Bacterial resistance to the quinolone antimicrobial agents, *The American Journal of Medicine.* **87**, 17S-23S.
55. Pagani, L., Landini, P., Luzzaro, F., Debiaggi, M. & Romero, E. (1990) Emergence of cross-resistance to imipenem and other beta-lactam antibiotics in *Pseudomonas aeruginosa* during therapy, *Microbiologica.* **13**, 43-53.
56. Levy, S. B. (1992) Active efflux mechanisms for antimicrobial resistance, *Antimicrobial Agents and Chemotherapy.* **36**, 695-703.

57. Traub, W. H. (1970) Susceptibility of *Pseudomonas aeruginosa* to gentamicin sulfate *in vitro*: lack of correlation between disc diffusion and broth dilution sensitivity data, *Applied Microbiology.* **20**, 98-102.
58. Chadwick, P. (1969) Effect of carbenicillin on *Pseudomonas aeruginosa*, *Canadian Medical Association Journal.* **101**, 74-80.
59. Frithz-Lindsten, E., Du, Y., Rosqvist, R. & Forsberg, A. (1997) Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments, *Molecular Microbiology.* **25**, 1125-39.
60. Coburn, B., Sekirov, I. & Finlay, B. B. (2007) Type III secretion systems and disease, *Clinical Microbiology Reviews.* **20**, 535-49.
61. Troisfontaines, P. & Cornelis, G. R. (2005) Type III secretion: more systems than you think, *Physiology.* **20**, 326-39.
62. Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iijima, Y., Najima, M., Nakano, M., Yamashita, A., Kubota, Y., Kimura, S., Yasunaga, T., Honda, T., Shinagawa, H., Hattori, M. & Iida, T. (2003) Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*, *Lancet.* **361**, 743-9.
63. Garrity-Ryan, L., Kazmierczak, B., Kowal, R., Comolli, J., Hauser, A. & Engel, J. N. (2000) The arginine finger domain of ExoT contributes to actin cytoskeleton disruption and inhibition of internalization of *Pseudomonas aeruginosa* by epithelial cells and macrophages, *Infection and Immunity.* **68**, 7100-13.
64. Rocha, C. L., Coburn, J., Rucks, E. A. & Olson, J. C. (2003) Characterization of *Pseudomonas aeruginosa* exoenzyme S as a bifunctional enzyme in J774A.1 macrophages, *Infection and Immunity.* **71**, 5296-305.
65. Pederson, K. J. & Barbieri, J. T. (1998) Intracellular expression of the ADP ribosyltransferase domain of *Pseudomonas* exoenzyme S is cytotoxic to eukaryotic cells, *Molecular Microbiology.* **30**, 751-9.
66. Fraylick, J. E., La Rocque, J. R., Vincent, T. S. & Olson, J. C. (2001) Independent and coordinate effects of ADP-ribosyltransferase and GTPase-activating activities of exoenzyme S on HT-29 epithelial cell function, *Infection and Immunity.* **69**, 5318-28.
67. Lee, V. T., Smith, R. S., Tummler, B. & Lory, S. (2005) Activities of *Pseudomonas aeruginosa* effectors secreted by the Type III secretion system *in vitro* and during infection, *Infection and Immunity.* **73**, 1695-705.
68. Vance, R. E., Rietsch, A. & Mekalanos, J. J. (2005) Role of the type III secreted exoenzymes S, T, and Y in systemic spread of *Pseudomonas aeruginosa* PAO1 *in vivo*, *Infection and Immunity.* **73**, 1706-13.
69. Shaver, C. M. & Hauser, A. R. (2004) Relative contributions of *Pseudomonas aeruginosa* ExoU, ExoS, and ExoT to virulence in the lung, *Infection and Immunity.* **72**, 6969-77.
70. Zaborina, O., Kohler, J. E., Wang, Y., Bethel, C., Shevchenko, O., Wu, L., Turner, J. R. & Alverdy, J. C. (2006) Identification of multi-drug resistant *Pseudomonas aeruginosa* clinical isolates that are highly disruptive to the intestinal epithelial barrier, *Annals of Clinical Microbiology and Antimicrobials.* **5**, 14.

71. Diaz, M. H., Shaver, C. M., King, J. D., Musunuri, S., Kazzaz, J. A. & Hauser, A. R. (2008) *Pseudomonas aeruginosa* induces localized immunosuppression during pneumonia, *Infection and Immunity.* **76**, 4414-21.
72. Kurahashi, K., Kajikawa, O., Sawa, T., Ohara, M., Gropper, M. A., Frank, D. W., Martin, T. R. & Wiener-Kronish, J. P. (1999) Pathogenesis of septic shock in *Pseudomonas aeruginosa* pneumonia, *The Journal of Clinical Investigation.* **104**, 743-50.
73. Cowell, B. A., Evans, D. J. & Fleiszig, S. M. (2005) Actin cytoskeleton disruption by ExoY and its effects on *Pseudomonas aeruginosa* invasion, *FEMS Microbiology Letters.* **250**, 71-6.
74. Sayner, S. L., Frank, D. W., King, J., Chen, H., VandeWaa, J. & Stevens, T. (2004) Paradoxical cAMP-induced lung endothelial hyperpermeability revealed by *Pseudomonas aeruginosa* ExoY, *Circulation Research.* **95**, 196-203.
75. Yahr, T. L., Goranson, J. & Frank, D. W. (1996) Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway, *Molecular Microbiology.* **22**, 991-1003.
76. Feltman, H., Schulert, G., Khan, S., Jain, M., Peterson, L. & Hauser, A. R. (2001) Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*, *Microbiology.* **147**, 2659-69.
77. Fleiszig, S. M., Wiener-Kronish, J. P., Miyazaki, H., Vallas, V., Mostov, K. E., Kanada, D., Sawa, T., Yen, T. S. & Frank, D. W. (1997) *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S, *Infection and Immunity.* **65**, 579-86.
78. Schulert, G. S., Feltman, H., Rabin, S. D., Martin, C. G., Battle, S. E., Rello, J. & Hauser, A. R. (2003) Secretion of the toxin ExoU is a marker for highly virulent *Pseudomonas aeruginosa* isolates obtained from patients with hospital-acquired pneumonia, *The Journal of Infectious Diseases.* **188**, 1695-706.
79. Goehring, U. M., Schmidt, G., Pederson, K. J., Aktories, K. & Barbieri, J. T. (1999) The N-terminal domain of *Pseudomonas aeruginosa* exoenzyme S is a GTPase-activating protein for Rho GTPases, *The Journal of Biological Chemistry.* **274**, 36369-72.
80. Pederson, K. J., Vallis, A. J., Aktories, K., Frank, D. W. & Barbieri, J. T. (1999) The amino-terminal domain of *Pseudomonas aeruginosa* ExoS disrupts actin filaments via small-molecular-weight GTP-binding proteins, *Molecular Microbiology.* **32**, 393-401.
81. Riese, M. J., Goehring, U. M., Ehrmantraut, M. E., Moss, J., Barbieri, J. T., Aktories, K. & Schmidt, G. (2002) Auto-ADP-ribosylation of *Pseudomonas aeruginosa* ExoS, *The Journal of Biological Chemistry.* **277**, 12082-8.
82. Henriksson, M. L., Francis, M. S., Peden, A., Aili, M., Stefansson, K., Palmer, R., Aitken, A. & Hallberg, B. (2002) A nonphosphorylated 14-3-3 binding motif on exoenzyme S that is functional *in vivo*, *European Journal of Biochemistry / FEBS.* **269**, 4921-9.
83. Fu, H., Coburn, J. & Collier, R. J. (1993) The eukaryotic host factor that activates exoenzyme S of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family, *Proceedings of the National Academy of Sciences of the United States of America.* **90**, 2320-4.

84. Coburn, J., Kane, A. V., Feig, L. & Gill, D. M. (1991) *Pseudomonas aeruginosa* exoenzyme S requires a eukaryotic protein for ADP-ribosyltransferase activity, *The Journal of Biological Chemistry.* **266**, 6438-46.
85. Hauser, A. R. (2009) The type III secretion system of *Pseudomonas aeruginosa*: infection by injection, *Nature reviews Microbiology.* **7**, 654-65.
86. Barbieri, A. M., Sha, Q., Bette-Bobillo, P., Stahl, P. D. & Vidal, M. (2001) ADP ribosylation of Rab5 by ExoS of *Pseudomonas aeruginosa* affects endocytosis, *Infection and Immunity.* **69**, 5329-34.
87. Krall, R., Schmidt, G., Aktories, K. & Barbieri, J. T. (2000) *Pseudomonas aeruginosa* ExoT is a Rho GTPase-activating protein, *Infection and Immunity.* **68**, 6066-8.
88. Kazmierczak, B. I. & Engel, J. N. (2002) *Pseudomonas aeruginosa* ExoT acts *in vivo* as a GTPase-activating protein for RhoA, Rac1, and Cdc42, *Infection and Immunity.* **70**, 2198-205.
89. Liu, S., Yahr, T. L., Frank, D. W. & Barbieri, J. T. (1997) Biochemical relationships between the 53-kilodalton (Exo53) and 49-kilodalton (ExoS) forms of exoenzyme S of *Pseudomonas aeruginosa*, *J Bacteriol.* **179**, 1609-13.
90. Shafikhani, S. H. & Engel, J. (2006) *Pseudomonas aeruginosa* type III-secreted toxin ExoT inhibits host-cell division by targeting cytokinesis at multiple steps, *Proceedings of the National Academy of Sciences of the United States of America.* **103**, 15605-10.
91. Deng, Q., Sun, J. & Barbieri, J. T. (2005) Uncoupling Crk signal transduction by *Pseudomonas* exoenzyme T, *The Journal of Biological Chemistry.* **280**, 35953-60.
92. Garrity-Ryan, L., Shafikhani, S., Balachandran, P., Nguyen, L., Oza, J., Jakobsen, T., Sargent, J., Fang, X., Cordwell, S., Matthay, M. A. & Engel, J. N. (2004) The ADP ribosyltransferase domain of *Pseudomonas aeruginosa* ExoT contributes to its biological activities, *Infection and Immunity.* **72**, 546-58.
93. Geiser, T. K., Kazmierczak, B. I., Garrity-Ryan, L. K., Matthay, M. A. & Engel, J. N. (2001) *Pseudomonas aeruginosa* ExoT inhibits *in vitro* lung epithelial wound repair, *Cellular Microbiology.* **3**, 223-36.
94. Shafikhani, S. H., Morales, C. & Engel, J. (2008) The *Pseudomonas aeruginosa* type III secreted toxin ExoT is necessary and sufficient to induce apoptosis in epithelial cells, *Cellular Microbiology.* **10**, 994-1007.
95. Sato, H., Frank, D. W., Hillard, C. J., Feix, J. B., Pankhaniya, R. R., Moriyama, K., Finck-Barbancon, V., Buchaklian, A., Lei, M., Long, R. M., Wiener-Kronish, J. & Sawa, T. (2003) The mechanism of action of the *Pseudomonas aeruginosa*-encoded type III cytotoxin, ExoU, *The EMBO Journal.* **22**, 2959-69.
96. Phillips, R. M., Six, D. A., Dennis, E. A. & Ghosh, P. (2003) *In vivo* phospholipase activity of the *Pseudomonas aeruginosa* cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors, *The Journal of Biological Chemistry.* **278**, 41326-32.
97. Tamura, M., Ajayi, T., Allmond, L. R., Moriyama, K., Wiener-Kronish, J. P. & Sawa, T. (2004) Lysophospholipase A activity of *Pseudomonas aeruginosa* type III secretory toxin ExoU, *Biochemical and Biophysical Research Communications.* **316**, 323-31.

98. Sato, H., Feix, J. B. & Frank, D. W. (2006) Identification of superoxide dismutase as a cofactor for the *Pseudomonas* type III toxin, ExoU, *Biochemistry.* **45**, 10368-75.
99. Stirling, F. R., Cuzick, A., Kelly, S. M., Oxley, D. & Evans, T. J. (2006) Eukaryotic localization, activation and ubiquitinylation of a bacterial type III secreted toxin, *Cellular Microbiology.* **8**, 1294-309.
100. Rabin, S. D., Veesenmeyer, J. L., Bieging, K. T. & Hauser, A. R. (2006) A C terminal domain targets the *Pseudomonas aeruginosa* cytotoxin ExoU to the plasma membrane of host cells, *Infection and Immunity.* **74**, 2552-61.
101. Hauser, A. R., Kang, P. J. & Engel, J. N. (1998) PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence, *Molecular Microbiology.* **27**, 807-18.
102. Vallis, A. J., Finck-Barbancon, V., Yahr, T. L. & Frank, D. W. (1999) Biological effects of *Pseudomonas aeruginosa* type III-secreted proteins on CHO cells, *Infection and Immunity.* **67**, 2040-4.
103. Yahr, T. L., Hovey, A. K., Kulich, S. M. & Frank, D. W. (1995) Transcriptional analysis of the *Pseudomonas aeruginosa* exoenzyme S structural gene, *J Bacteriol.* **177**, 1169-78.
104. Shen, D. K., Quenee, L., Bonnet, M., Kuhn, L., Derouazi, M., Lamotte, D., Toussaint, B. & Polack, B. (2008) Orf1/SpcS chaperones ExoS for type three secretion by *Pseudomonas aeruginosa*, *Biomedical and Environmental Sciences: BES.* **21**, 103-9.
105. Finck-Barbancon, V., Yahr, T. L. & Frank, D. W. (1998) Identification and characterization of SpcU, a chaperone required for efficient secretion of the ExoU cytotoxin, *J Bacteriol.* **180**, 6224-31.
106. Schoehn, G., Di Guilmi, A. M., Lemaire, D., Attree, I., Weissenhorn, W. & Dessen, A. (2003) Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in *Pseudomonas*, *The EMBO Journal.* **22**, 4957-67.
107. Quinaud, M., Chabert, J., Faudry, E., Neumann, E., Lemaire, D., Pastor, A., Elsen, S., Dessen, A. & Attree, I. (2005) The PscE-PscF-PscG complex controls type III secretion needle biogenesis in *Pseudomonas aeruginosa*, *The Journal of Biological Chemistry.* **280**, 36293-300.
108. Urbanowski, M. L., Lykken, G. L. & Yahr, T. L. (2005) A secreted regulatory protein couples transcription to the secretory activity of the *Pseudomonas aeruginosa* type III secretion system, *Proceedings of the National Academy of Sciences of the United States of America.* **102**, 9930-5.
109. Rietsch, A., Vallet-Gely, I., Dove, S. L. & Mekalanos, J. J. (2005) ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*, *Proceedings of the National Academy of Sciences of the United States of America.* **102**, 8006-11.
110. Woestyn, S., Allaoui, A., Wattiau, P. & Cornelis, G. R. (1994) YscN, the putative energizer of the *Yersinia* Yop secretion machinery, *J Bacteriol.* **176**, 1561-9.
111. Blaylock, B., Riordan, K. E., Missiakas, D. M. & Schneewind, O. (2006) Characterization of the *Yersinia enterocolitica* type III secretion ATPase YscN and its regulator, YscL, *J Bacteriol.* **188**, 3525-34.

112. Koster, M., Bitter, W., de Cock, H., Allaoui, A., Cornelis, G. R. & Tommassen, J. (1997) The outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex, *Molecular Microbiology.* **26**, 789-97.
113. Burghout, P., Beckers, F., de Wit, E., van Boxtel, R., Cornelis, G. R., Tommassen, J. & Koster, M. (2004) Role of the pilot protein YscW in the biogenesis of the YscC secretin in *Yersinia enterocolitica*, *J Bacteriol.* **186**, 5366-75.
114. Journet, L., Agrain, C., Broz, P. & Cornelis, G. R. (2003) The needle length of bacterial injectisomes is determined by a molecular ruler, *Science.* **302**, 1757-60.
115. Burns, R. E., McDaniel-Craig, A. & Sukhan, A. (2008) Site-directed mutagenesis of the *Pseudomonas aeruginosa* type III secretion system protein PscJ reveals an essential role for surface-localized residues in needle complex function, *Microbial Pathogenesis.* **45**, 225-30.
116. Goure, J., Pastor, A., Faudry, E., Chabert, J., Dessen, A. & Attree, I. (2004) The V antigen of *Pseudomonas aeruginosa* is required for assembly of the functional PopB/PopD translocation pore in host cell membranes, *Infection and Immunity.* **72**, 4741-50.
117. Mota, L. J. (2006) Type III secretion gets an LcrV tip, *Trends in Microbiology.* **14**, 197-200.
118. Sawa, T., Yahr, T. L., Ohara, M., Kurahashi, K., Gropper, M. A., Wiener-Kronish, J. P. & Frank, D. W. (1999) Active and passive immunization with the *Pseudomonas* V antigen protects against type III intoxication and lung injury, *Nature Medicine.* **5**, 392-8.
119. Holder, I. A., Neely, A. N. & Frank, D. W. (2001) PcrV immunization enhances survival of burned *Pseudomonas aeruginosa*-infected mice, *Infection and Immunity.* **69**, 5908-10.
120. Hovey, A. K. & Frank, D. W. (1995) Analyses of the DNA-binding and transcriptional activation properties of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* exoenzyme S regulon, *J Bacteriol.* **177**, 4427-36.
121. Fuchs, E. L., Brutinel, E. D., Klem, E. R., Fehr, A. R., Yahr, T. L. & Wolfgang, M. C. (2010) *In vitro* and *in vivo* characterization of the *Pseudomonas aeruginosa* cyclic AMP (cAMP) phosphodiesterase CpdA, required for cAMP homeostasis and virulence factor regulation, *J Bacteriol.* **192**, 2779-90.
122. Dong, Y. H., Zhang, X. F. & Zhang, L. H. (2013) The global regulator Crc plays a multifaceted role in modulation of type III secretion system in *Pseudomonas aeruginosa*, *MicrobiologyOpen.* **2**, 161-72.
123. Smith, R. S., Wolfgang, M. C. & Lory, S. (2004) An adenylate cyclase-controlled signaling network regulates *Pseudomonas aeruginosa* virulence in a mouse model of acute pneumonia, *Infection and Immunity.* **72**, 1677-84.
124. Whitchurch, C. B., Beatson, S. A., Comolli, J. C., Jakobsen, T., Sargent, J. L., Bertrand, J. J., West, J., Klausen, M., Waite, L. L., Kang, P. J., Tolker-Nielsen, T., Mattick, J. S. & Engel, J. N. (2005) *Pseudomonas aeruginosa* fimL regulates multiple virulence functions by intersecting with Vfr-modulated pathways, *Molecular Microbiology.* **55**, 1357-78.

125. Bange, G., Kummerer, N., Engel, C., Bozkurt, G., Wild, K. & Sinning, I. (2010) FlhA provides the adaptor for coordinated delivery of late flagella building blocks to the type III secretion system, *Proceedings of the National Academy of Sciences of the United States of America.* **107**, 11295-300.
126. Shen, D. K., Filopon, D., Chaker, H., Boullanger, S., Derouazi, M., Polack, B. & Toussaint, B. (2008) High-cell-density regulation of the *Pseudomonas aeruginosa* type III secretion system: implications for tryptophan catabolites, *Microbiology.* **154**, 2195-208.
127. Anderson, G. G., Yahr, T. L., Lovewell, R. R. & O'Toole, G. A. (2010) The *Pseudomonas aeruginosa* magnesium transporter MgtE inhibits transcription of the type III secretion system, *Infection and Immunity.* **78**, 1239-49.
128. Wu, W., Badrane, H., Arora, S., Baker, H. V. & Jin, S. (2004) MucA-mediated coordination of type III secretion and alginate synthesis in *Pseudomonas aeruginosa*, *J Bacteriol.* **186**, 7575-85.
129. Van Alst, N. E., Wellington, M., Clark, V. L., Haidaris, C. G. & Iglewski, B. H. (2009) Nitrite reductase NirS is required for type III secretion system expression and virulence in the human monocyte cell line THP-1 by *Pseudomonas aeruginosa*, *Infection and Immunity.* **77**, 4446-54.
130. Shen, D. K., Filopon, D., Kuhn, L., Polack, B. & Toussaint, B. (2006) PsrA is a positive transcriptional regulator of the type III secretion system in *Pseudomonas aeruginosa*, *Infection and Immunity.* **74**, 1121-9.
131. Wu, W. & Jin, S. (2005) PtrB of *Pseudomonas aeruginosa* suppresses the type III secretion system under the stress of DNA damage, *J Bacteriol.* **187**, 6058-68.
132. Jin, Y., Yang, H., Qiao, M. & Jin, S. (2011) MexT regulates the type III secretion system through MexS and PtrC in *Pseudomonas aeruginosa*, *J Bacteriol.* **193**, 399-410.
133. Hogardt, M., Roeder, M., Schreff, A. M., Eberl, L. & Heesemann, J. (2004) Expression of *Pseudomonas aeruginosa exoS* is controlled by quorum sensing and RpoS, *Microbiology.* **150**, 843-51.
134. Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T., Camara, M., Haas, D. & Williams, P. (2001) The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*, *J Bacteriol.* **183**, 6676-83.
135. Mulcahy, H., O'Callaghan, J., O'Grady, E. P., Adams, C. & O'Gara, F. (2006) The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human airway epithelial cells by positively regulating the type III secretion system, *Infection and Immunity.* **74**, 3012-5.
136. Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S. & Filloux, A. (2006) Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes, *Proceedings of the National Academy of Sciences of the United States of America.* **103**, 171-6.
137. Zolfaghar, I., Angus, A. A., Kang, P. J., To, A., Evans, D. J. & Fleiszig, S. M. (2005) Mutation of *retS*, encoding a putative hybrid two-component regulatory protein in *Pseudomonas aeruginosa*, attenuates multiple virulence mechanisms, *Microbes and Infection / Institut Pasteur.* **7**, 1305-16.

138. O'Callaghan, J., Reen, F. J., Adams, C., Casey, P. G., Gahan, C. G. & O'Gara, F. (2012) A novel host-responsive sensor mediates virulence and type III secretion during *Pseudomonas aeruginosa*-host cell interactions, *Microbiology.* **158**, 1057-70.
139. Wolfgang, M. C., Lee, V. T., Gilmore, M. E. & Lory, S. (2003) Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway, *Developmental Cell.* **4**, 253-63.
140. Brencic, A., McFarland, K. A., McManus, H. R., Castang, S., Mogno, I., Dove, S. L. & Lory, S. (2009) The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs, *Molecular Microbiology.* **73**, 434-45.
141. Vallis, A. J., Yahr, T. L., Barbieri, J. T. & Frank, D. W. (1999) Regulation of ExoS production and secretion by *Pseudomonas aeruginosa* in response to tissue culture conditions, *Infection and Immunity.* **67**, 914-20.
142. Rietsch, A. & Mekalanos, J. J. (2006) Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*, *Molecular Microbiology.* **59**, 807-20.
143. Rietsch, A., Wolfgang, M. C. & Mekalanos, J. J. (2004) Effect of metabolic imbalance on expression of type III secretion genes in *Pseudomonas aeruginosa*, *Infection and Immunity.* **72**, 1383-90.
144. Zhou, L., Wang, J. & Zhang, L. H. (2007) Modulation of bacterial type III secretion system by a spermidine transporter dependent signaling pathway, *PloS One.* **2**, e1291.
145. Bleves, S., Soscia, C., Nogueira-Orlandi, P., Lazdunski, A. & Filloux, A. (2005) Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1, *J Bacteriol.* **187**, 3898-902.
146. Singh, G., Wu, B., Baek, M. S., Camargo, A., Nguyen, A., Slusher, N. A., Srinivasan, R., Wiener-Kronish, J. P. & Lynch, S. V. (2010) Secretion of *Pseudomonas aeruginosa* type III cytotoxins is dependent on *Pseudomonas* quinolone signal concentration, *Microbial Pathogenesis.* **49**, 196-203.
147. Thibault, J., Faudry, E., Ebel, C., Attree, I. & Elsen, S. (2009) Anti-activator ExsD forms a 1:1 complex with ExsA to inhibit transcription of type III secretion operons, *The Journal of Biological Chemistry.* **284**, 15762-70.
148. Dasgupta, N., Lykken, G. L., Wolfgang, M. C. & Yahr, T. L. (2004) A novel anti anti-activator mechanism regulates expression of the *Pseudomonas aeruginosa* type III secretion system, *Molecular Microbiology.* **53**, 297-308.
149. Vakulskas, C. A., Brady, K. M. & Yahr, T. L. (2009) Mechanism of Transcriptional Activation by *Pseudomonas aeruginosa* ExsA, *Journal of Bacteriology.* **191**, 6654-6664.
150. Gallegos, M. T., Schleif, R., Bairoch, A., Hofmann, K. & Ramos, J. L. (1997) Arac/XylS family of transcriptional regulators, *Microbiology and Molecular Biology Reviews : MMBR.* **61**, 393-410.
151. Egan, S. M. (2002) Growing repertoire of AraC/XylS activators, *J Bacteriol.* **184**, 5529-32.
152. Schleif, R. (2003) AraC protein: a love-hate relationship, *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology.* **25**, 274-82.

153. Martin, R. G. & Rosner, J. L. (2001) The AraC transcriptional activators, *Current Opinion in Microbiology.* **4**, 132-7.
154. Gralla, J. D. (1989) Bacterial gene regulation from distant DNA sites, *Cell.* **57**, 193-5.
155. Brutinel, E. D., Vakulskas, C. A. & Yahr, T. L. (2009) Functional domains of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* type III secretion system, *J Bacteriol.* **191**, 3811-21.
156. Rhee, S., Martin, R. G., Rosner, J. L. & Davies, D. R. (1998) A novel DNA-binding motif in MarA: the first structure for an AraC family transcriptional activator, *Proceedings of the National Academy of Sciences of the United States of America.* **95**, 10413-8.
157. Kwon, H. J., Bennik, M. H., Demple, B. & Ellenberger, T. (2000) Crystal structure of the *Escherichia coli* Rob transcription factor in complex with DNA, *Nature Structural Biology.* **7**, 424-30.
158. Kaniga, K., Bossio, J. C. & Galan, J. E. (1994) The *Salmonella typhimurium* invasion genes invF and invG encode homologues of the AraC and PulD family of proteins, *Molecular Microbiology.* **13**, 555-68.
159. Lambert de Rouvroit, C., Sluiters, C. & Cornelis, G. R. (1992) Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of *Yersinia enterocolitica*, *Molecular Microbiology.* **6**, 395-409.
160. Hoe, N. P., Minion, F. C. & Goguen, J. D. (1992) Temperature sensing in *Yersinia pestis*: regulation of *yopE* transcription by *lcrF*, *J Bacteriol.* **174**, 4275-86.
161. Hoe, N. P. & Goguen, J. D. (1993) Temperature sensing in *Yersinia pestis*: translation of the LcrF activator protein is thermally regulated, *J Bacteriol.* **175**, 7901-9.
162. Savelkoul, P. H., Willshaw, G. A., McConnell, M. M., Smith, H. R., Hamers, A. M., van der Zeijst, B. A. & Gaastra, W. (1990) Expression of CFA/I fimbriae is positively regulated, *Microbial Pathogenesis.* **8**, 91-9.
163. DiRita, V. J., Parsot, C., Jander, G. & Mekalanos, J. J. (1991) Regulatory cascade controls virulence in *Vibrio cholerae*, *Proceedings of the National Academy of Sciences of the United States of America.* **88**, 5403-7.
164. Zhou, X., Shah, D. H., Konkel, M. E. & Call, D. R. (2008) Type III secretion system 1 genes in *Vibrio parahaemolyticus* are positively regulated by ExsA and negatively regulated by ExsD, *Molecular Microbiology.* **69**, 747-64.
165. Zhou, X., Konkel, M. E. & Call, D. R. (2010) Regulation of type III secretion system 1 gene expression in *Vibrio parahaemolyticus* is dependent on interactions between ExsA, ExsC, and ExsD, *Virulence.* **1**, 260-72.
166. Kodama, T., Yamazaki, C., Park, K. S., Akeda, Y., Iida, T. & Honda, T. (2010) Transcription of *Vibrio parahaemolyticus* T3SS1 genes is regulated by a dual regulation system consisting of the ExsACDE regulatory cascade and H-NS, *FEMS Microbiology Letters.* **311**, 10-7.
167. Brutinel, E. D., Vakulskas, C. A., Brady, K. M. & Yahr, T. L. (2008) Characterization of ExsA and of ExsA-dependent promoters required for expression of the *Pseudomonas aeruginosa* type III secretion system, *Molecular Microbiology.* **68**, 657-71.

168. King, J. M., Brutinel, E. D., Marsden, A. E., Schubot, F. D. & Yahr, T. L. (2012) Orientation of *Pseudomonas aeruginosa* ExsA monomers bound to promoter DNA and base-specific contacts with the P_{exoT} promoter, *J Bacteriol.* **194**, 2573-85.
169. Brutinel, E. D., King, J. M., Marsden, A. E. & Yahr, T. L. (2012) The distal ExsA binding site in *Pseudomonas aeruginosa* type III secretion system promoters is the primary determinant for promoter-specific properties, *J Bacteriol.* **194**, 2564-72.
170. Vakulskas, C. A., Brutinel, E. D. & Yahr, T. L. (2010) ExsA recruits RNA polymerase to an extended -10 promoter by contacting region 4.2 of sigma-70, *J Bacteriol.* **192**, 3597-607.
171. McCaw, M. L., Lykken, G. L., Singh, P. K. & Yahr, T. L. (2002) ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon, *Molecular Microbiology.* **46**, 1123-33.
172. Brutinel, E. D., Vakulskas, C. A. & Yahr, T. L. (2010) ExsD inhibits expression of the *Pseudomonas aeruginosa* type III secretion system by disrupting ExsA self-association and DNA binding activity, *J Bacteriol.* **192**, 1479-86.
173. Bernhards, R. C., Jing, X., Vogelaar, N. J., Robinson, H. & Schubot, F. D. (2009) Structural evidence suggests that antiactivator ExsD from *Pseudomonas aeruginosa* is a DNA binding protein, *Protein Sci.* **18**, 503-13.
174. Khare, D., Ziegelin, G., Lanka, E. & Heinemann, U. (2004) Sequence-specific DNA binding determined by contacts outside the helix-turn-helix motif of the ParB homolog KorB, *Nature Structural & Molecular Biology.* **11**, 656-63.
175. Bingle, L. E., Macartney, D. P., Fantozzi, A., Manzoor, S. E. & Thomas, C. M. (2005) Flexibility in repression and cooperativity by KorB of broad host range IncP-1 plasmid RK2, *Journal of Molecular Biology.* **349**, 302-16.
176. Sosunova, E., Sosunov, V., Kozlov, M., Nikiforov, V., Goldfarb, A. & Mustaev, A. (2003) Donation of catalytic residues to RNA polymerase active center by transcription factor Gre, *Proceedings of the National Academy of Sciences of the United States of America.* **100**, 15469-74.
177. Lykken, G. L., Chen, G., Brutinel, E. D., Chen, L. & Yahr, T. L. (2006) Characterization of ExsC and ExsD self-association and heterocomplex formation, *J Bacteriol.* **188**, 6832-40.
178. Vogelaar, N. J., Jing, X., Robinson, H. H. & Schubot, F. D. (2010) Analysis of the crystal structure of the ExsC.ExsE complex reveals distinctive binding interactions of the *Pseudomonas aeruginosa* type III secretion chaperone ExsC with ExsE and ExsD, *Biochemistry.* **49**, 5870-9.
179. Zheng, Z., Chen, G., Joshi, S., Brutinel, E. D., Yahr, T. L. & Chen, L. (2007) Biochemical characterization of a regulatory cascade controlling transcription of the *Pseudomonas aeruginosa* type III secretion system, *The Journal of Biological Chemistry.* **282**, 6136-42.
180. Nallamsetty, S., Austin, B. P., Penrose, K. J. & Waugh, D. S. (2005) Gateway vectors for the production of combinatorially-tagged His_6-MBP fusion proteins in the cytoplasm and periplasm of *Escherichia coli*, *Protein Sci.* **14**, 2964-71.

181. Yahr, T. L. & Frank, D. W. (1994) Transcriptional organization of the trans regulatory locus which controls exoenzyme S synthesis in *Pseudomonas aeruginosa*, *J Bacteriol.* **176**, 3832-38.
182. Timmes, A., Rodgers, M. & Schleif, R. (2004) Biochemical and physiological properties of the DNA binding domain of AraC protein, *Journal of Molecular Biology.* **340**, 731-8.
183. Kapust, R. B., Tozser, J., Fox, J. D., Anderson, D. E., Cherry, S., Copeland, T. D. & Waugh, D. S. (2001) Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency, *Protein Eng.* **14**, 993-1000.
184. Allan, B. & Kropinski, A. M. (1987) DNA-dependent RNA polymerase from *Pseudomonas aeruginosa*, *Biochem Cell Biol.* **65**, 776-82.
185. Finney, A. H., Blick, R. J., Murakami, K., Ishihama, A. & Stevens, A. M. (2002) Role of the C-terminal domain of the alpha subunit of RNA polymerase in LuxR-dependent transcriptional activation of the lux operon during quorum sensing, *J Bacteriol.* **184**, 4520-8.
186. Depuydt, P., Benoit, D., Vogelaers, D., Claeys, G., Verschraegen, G., Vandewoude, K., Decruyenaere, J. & Blot, S. (2006) Outcome in bacteremia associated with nosocomial pneumonia and the impact of pathogen prediction by tracheal surveillance cultures, *Intensive Care Med.* **32**, 1773-81.
187. Gaynes, R. & Edwards, J. R. (2005) Overview of nosocomial infections caused by Gram-negative bacilli, *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America.* **41**, 848-54.
188. Johnson, L. E., D'Agata, E. M., Paterson, D. L., Clarke, L., Qureshi, Z. A., Potoski, B. A. & Peleg, A. Y. (2009) *Pseudomonas aeruginosa* bacteremia over a 10-year period: multidrug resistance and outcomes in transplant recipients, *Transpl Infect Dis.* **11**, 227-34.
189. Trautmann, M., Lepper, P. M. & Haller, M. (2005) 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.* **33**, S41-9.
190. Wine, J. J. (1999) The genesis of cystic fibrosis lung disease, *The Journal of Clinical Investigation.* **103**, 309-12.
191. Hauser, A. R., Cobb, E., Bodi, M., Mariscal, D., Valles, J., Engel, J. N. & Rello, J. (2002) Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*, *Critical Care Medicine.* **30**, 521-8.
192. Hsu, D. I., Okamoto, M. P., Murthy, R. & Wong-Beringer, A. (2005) Fluoroquinolone-resistant *Pseudomonas aeruginosa*: risk factors for acquisition and impact on outcomes, *J Antimicrob Chemother.* **55**, 535-41.
193. Roy-Burman, A., Savel, R. H., Racine, S., Swanson, B. L., Revadigar, N. S., Fujimoto, J., Sawa, T., Frank, D. W. & Wiener-Kronish, J. P. (2001) Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections, *The Journal of Infectious Diseases.* **183**, 1767-74.

194. Angus, A. A., Evans, D. J., Barbieri, J. T. & Fleiszig, S. M. (2010) The ADP ribosylation domain of *Pseudomonas aeruginosa* ExoS is required for membrane bleb niche formation and bacterial survival within epithelial cells, *Infection and Immunity.* **78**, 4500-10.
195. Dacheux, D., Toussaint, B., Richard, M., Brochier, G., Croize, J. & Attree, I. (2000) *Pseudomonas aeruginosa* cystic fibrosis isolates induce rapid, type III secretion-dependent, but ExoU-independent, oncosis of macrophages and polymorphonuclear neutrophils, *Infection and Immunity.* **68**, 2916-24.
196. Evans, D. J., Frank, D. W., Finck-Barbancon, V., Wu, C. & Fleiszig, S. M. (1998) *Pseudomonas aeruginosa* invasion and cytotoxicity are independent events, both of which involve protein tyrosine kinase activity, *Infection and Immunity.* **66**, 1453-9.
197. Maresso, A. W., Deng, Q., Pereckas, M. S., Wakim, B. T. & Barbieri, J. T. (2007) *Pseudomonas aeruginosa* ExoS ADP-ribosyltransferase inhibits ERM phosphorylation, *Cellular Microbiology.* **9**, 97-105.
198. Saliba, A. M., Filloux, A., Ball, G., Silva, A. S., Assis, M. C. & Plotkowski, M. C. (2002) Type III secretion-mediated killing of endothelial cells by *Pseudomonas aeruginosa*, *Microbial Pathogenesis.* **33**, 153-66.
199. Sato, H. & Frank, D. W. (2004) ExoU is a potent intracellular phospholipase, *Molecular Microbiology.* **53**, 1279-90.
200. Zolfaghar, I., Evans, D. J., Ronaghi, R. & Fleiszig, S. M. (2006) Type III secretion dependent modulation of innate immunity as one of multiple factors regulated by *Pseudomonas aeruginosa* RetS, *Infection and Immunity.* **74**, 3880-9.
201. Minamino, T. & Namba, K. (2008) Distinct roles of the FliI ATPase and proton motive force in bacterial flagellar protein export, *Nature.* **451**, 485-8.
202. Paul, K., Erhardt, M., Hirano, T., Blair, D. F. & Hughes, K. T. (2008) Energy source of flagellar type III secretion, *Nature.* **451**, 489-92.
203. Wurtzel, O., Yoder-Himes, D. R., Han, K., Dandekar, A. A., Edelheit, S., Greenberg, E. P., Sorek, R. & Lory, S. (2012) The single-nucleotide resolution transcriptome of *Pseudomonas aeruginosa* grown in body temperature, *PLoS Pathogens.* **8**, e1002945.
204. Dotsch, A., Eckweiler, D., Schniederjans, M., Zimmermann, A., Jensen, V., Scharfe, M., Geffers, R. & Haussler, S. (2012) The *Pseudomonas aeruginosa* transcriptome in planktonic cultures and static biofilms using RNA sequencing, *PloS One.* **7**, e31092.
205. Daines, D. A., Granger-Schnarr, M., Dimitrova, M. & Silver, R. P. (2002) Use of LexA-based system to identify protein-protein interactions *in vivo*, *Methods in Enzymology.* **358**, 153-61.
206. Bernhards, R. C., Marsden, A. E., Esher, S. K., Yahr, T. L. & Schubot, F. D. (2013) Self-trimerization of ExsD limits inhibition of the *Pseudomonas aeruginosa* transcriptional activator ExsA *in vitro*, *The FEBS Journal.* **280**, 1084-94.
207. Guilbault, C., Saeed, Z., Downey, G. P. & Radzioch, D. (2007) Cystic fibrosis mouse models, *American Journal of Respiratory Cell and Molecular Biology.* **36**, 1-7.