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Mechanism of transcriptional activation by Pseudomonas aeruginosa ExsA

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MECHANISM OF TRANSCRIPTIONAL ACTIVATION BY *PSEUDOMONAS AERUGINOSA* EXSA

by

Christopher Anthony Vakulskas

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
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Thesis Supervisor: Associate Professor Timothy L. Yahr

ABSTRACT

ExsA is an AraC-family transcriptional regulator that controls expression of T3SS genes in *P. aeruginosa*. ExsA binds to DNA at T3SS promoters and activates transcription. In the work presented here I examine the stoichiometry, ligand-interaction properties, and transcriptional activation mechanism of ExsA. I determined that ExsA is largely monomeric in solution. ExsA binds T3SS promoter DNA with high affinity resulting in two ExsA-DNA complexes. Whereas the lower molecular weight complex represents a single molecule of ExsA bound to DNA, the higher molecular weight complex represents two molecules of ExsA bound to adjacent sites at T3SS promoters. I next analyzed the mechanism by which ExsD negatively effects ExsA function. Chromatin Immuno-Precipitation Assays (ChIP) demonstrate that ExsD inhibits the DNA-binding activity of ExsA *in vivo*. Finally, I characterized the mechanism of transcriptional activation by ExsA. ExsA-dependent promoters contain regions that resemble consensus σ^{70} -35 and -10 recognition hexamers. The spacing between these regions, however, is increased 4-5 bp compared to the σ^{70} consensus. Nevertheless, I demonstrate that T3SS promoters are dependent on σ^{70}-RNA polymerase (RNAP). Using the abortive initiation assay I discovered that ExsA recruits RNA polymerase to the P_{exsC} and P_{exsD} promoters. Potassium permanganate footprints indicate that following recruitment, RNAP facilitates unwinding of DNA at the -10 hexamer of T3SS promoters. Transcriptional activators generally recruit RNAP by contacting the α or σ^{70} subunits (or both). I have found that ExsA recruits RNAP to the P_{exsC} and P_{exsD} promoters by contacting region 4.2 of σ^{70}. Although I have established the role of the -10 hexamer, the function of a near-consensus, putative -35 remains puzzling. *in vitro* transcription assays on templates with mutations in the P_{exsC} -35 hexamer reveal that this region is dispensable for ExsA-independent transcription. These data may suggest the putative -35 hexamer is really just an ExsA binding site. Consistent with this hypothesis, I provide

evidence that suggests an extended -10 element at P_{exsC} may function to compensate for the lack of a -35 hexamer.

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To: Michael and Lynette Vakulskas who made all of this possible

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ExsA is an AraC-family transcriptional regulator that controls expression of T3SS genes in *P. aeruginosa*. ExsA binds to DNA at T3SS promoters and activates transcription. In the work presented here I examine the stoichiometry, ligand-interaction properties, and transcriptional activation mechanism of ExsA. I determined that ExsA is largely monomeric in solution. ExsA binds T3SS promoter DNA with high affinity resulting in two ExsA-DNA complexes. Whereas the lower molecular weight complex represents a single molecule of ExsA bound to DNA, the higher molecular weight complex represents two molecules of ExsA bound to adjacent sites at T3SS promoters. I next analyzed the mechanism by which ExsD negatively effects ExsA function. Chromatin Immuno-Precipitation Assays (ChIP) demonstrate that ExsD inhibits the DNA-binding activity of ExsA *in vivo*. Finally, I characterized the mechanism of transcriptional activation by ExsA. ExsA-dependent promoters contain regions that resemble consensus σ^{70} -35 and -10 recognition hexamers. The spacing between these regions, however, is increased 4-5 bp compared to the σ^{70} consensus. Nevertheless, I demonstrate that T3SS promoters are dependent on σ^{70}-RNA polymerase (RNAP). Using the abortive initiation assay I discovered that ExsA recruits RNA polymerase to the P_{exsC} and P_{exsD} promoters. Potassium permanganate footprints indicate that following recruitment, RNAP facilitates unwinding of DNA at the -10 hexamer of T3SS promoters. Transcriptional activators generally recruit RNAP by contacting the α or σ^{70} subunits (or both). I have found that ExsA recruits RNAP to the P_{exsC} and P_{exsD} promoters by contacting region 4.2 of σ^{70}. Although I have established the role of the -10 hexamer, the function of a near-consensus, putative -35 remains puzzling. *in vitro* transcription assays on templates with mutations in the P_{exsC} -35 hexamer reveal that this region is dispensable for ExsA-independent transcription. These data may suggest the putative -35 hexamer is really just an ExsA binding site. Consistent with this hypothesis, I provide

evidence that suggests an extended -10 element at P_{exsC} may function to compensate for the lack of a -35 hexamer.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF ABBREVIATIONS

T3SS	Type III secretion system
RNAP	RNA Polymerase
NTD	Amino-terminal domain
CTD	Carboxy-terminal domain
K_B	Equilibrium binding constant for RNA polymerase
k_2	Isomerization rate constant
K_{eq}	Apparent equilibrium constant
bp	Base pair
NT	Nucleotide
PCR	Polymerase chain reaction
EMSA	Electrophoretic mobility shift assay
R_f	Relative mobility
K_r	Retardation coefficient
τ	Lag time to open complex formation
ChIP	Chromatin immunoprecipitation
RACE	Rapid amplification of cDNA ends

CHAPTER I

INTRODUCTION

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative opportunistic bacterial pathogen found commonly in soil and other moist surfaces worldwide (Mena and Gerba 2009; Noura et al. 2009). *P. aeruginosa* has few nutrient requirements and can thrive under environmental stress (Cowell et al. 1999). *P. aeruginosa* is able to infect a wide variety of organisms including mammals, fish, invertebrates, and lower-order eukaryotes (Pukatzki et al. 2002; Rahme et al. 2000). Exposure to *P. aeruginosa* can cause a number of clinical manifestations including but not limited to pneumonia, skin/soft tissue infection, burn-wound complications, gastrointestinal disorders, endocarditis, meningitis, and urinary-tract infections (Veesenmeyer et al. 2009). *P. aeruginosa* is an emerging pathogen of clinical relevance due to the increased incidence of drug-resistant isolates from infected patients and is a frequently-isolated source of nosocomial pneumonia (Hocquet et al. 2007; Richards et al. 2000). Although community acquired infection has been reported, most newly acquired infections are thought to originate from environmental sources (usually nosocomial) infecting immunocompromised individuals with several comorbidities (Patel et al. 2002). Common nosocomial sources of the organism include ventilators, resuscitators, anaesthetic equipment, and catheters (Crouch Brewer et al. 1996; Fierer et al. 1967; Liedberg and Lundeberg 1989; Olds et al. 1972). Given its ability to persist in the hospital setting, it is not surprising that *P. aeruginosa* is the most common pathogen isolated from patients hospitalized longer than one week (Geffers et al. 2004). Although acute *P. aeruginosa* infections are often curable, a number of fulminant infections such as those from burn wounds or those that cause bacteremic pneumonia are associated with extremely high mortality rates (Kang et al. 2003). In addition to causing acute infections, *P. aeruginosa* frequently causes chronic infections in the lungs of cystic fibrosis patients (Lyczak et al. 2002). The most effective

treatment options for *P. aeruginosa* infections include aminoglycoside, quinolone, and cephalosporin antibiotics or combination therapy with multiple antiobiotics (Cunha 2002).

P. aeruginosa contains a number of virulence factors that contribute to acute and chronic infection. Several appendages are produced that mediate adherence including a type IV pilus and other non-pilus components such as neuraminidase, lipopolysaccharide, and flagella (Cacalano et al. 1992; Hahn 1997). In the absence of these adherent structures there is a dramatic decrease in the ability of *P. aeruginosa* to colonize host tissues (Kang et al. 1997; Sundin et al. 2002). The elastase proteins LasA and LasB are produced during *P. aeruginosa* infection, and these proteins enhance tissue destruction by increasing host-invasion (Cowell et al. 2003). A number of other extracellular factors like phospholipase and alginate are also important for the establishment of both acute and chronic infections (Berka et al. 1981; Pedersen et al. 1992). Finally, *P. aeruginosa* secretes a number of toxins that facilitate tissue destruction and phagocytic avoidance. For example, Exotoxin A is a secreted toxin that has a similar mechanism as diphtheria toxin and catalyzes the ribosyl transfer of ADP to protein synthesis elongation factor-2 (Pollack 1983; Pollack and Anderson 1978). Exotoxin-A is secreted in a multi-step process first involving transport to the periplasmic space via the *sec* pathway followed by outer membrane transport to the extracellular environment by the *xcp* system, collectively known as type II secretion (Sandkvist 2001). Lastly, a subset of toxins is transported across both membranes simultaneously via the type three secretion system (T3SS).

The Type III Secretion System (T3SS)

The T3SS consists of a macromolecular needle-like complex that facilitates the translocation of effector proteins from the bacterial to host-cell cytoplasm. The T3SS is functionally and structurally related to the well-studied flagellar secretion system and is found in many Gram-negative organisms that cause disease in plants and animals (Hauser

2009). The structural components of the T3SS can functionally be divided into regulators, the needle complex, the translocation apparatus, secreted effectors, and chaperones. Much of what is known about the *P. aeruginosa* needle complex is derived from the well-characterized needle complex in *Yersinia sp.* The needle complex consists of a multi-ring base and a needle-like filament. In *Yersinia sp.* an ATPase motor (YscN) powers the secretion system by unfolding effector proteins, placing them in a secretion-competent state (Blaylock et al. 2006). The YscJ and YscC proteins anchor the system to the inner and outer membranes, respectively (Marlovits et al. 2004; Woestyn et al. 1994). The needle filament consists of repeating subunits of the YscF protein, which serves as a conduit for protein secretion and may additionally serve as a sensor for host-cell contact (Pastor et al. 2005). Toxins and effectors are secreted through the needle complex and are translocated through the host-cell membrane via the YopB-YopD pore-forming complex and LcrV (Cornelis 2002a; Cornelis 2002b). The *P. aeruginosa* homologues PopB, PopD, and PcrV are secreted through the T3SS channel; PopB and PopD are sufficient for translocation pore formation of lipid vesicles *in vitro* (Schoehn et al. 2003). PcrV is thought to work in much the same fashion as the homologous protein in *Yersinia sp.* (LcrV) which forms a scaffold attached to the tip of the T3SS needle and functions as a chaperone for assembly of the YopBD complex (Mota 2006; Mueller et al. 2005).

The T3SS translocates four known effector toxins ExoS, ExoT, ExoU, and ExoY (Engel and Balachandran 2009). Most bacteria encoding a T3SS are known to secrete a large number of effectors yet *P. aeruginosa* is only known to secrete four toxins and surprisingly no clinical isolate typically encodes all four effectors (Feltman et al. 2001; Fleiszig et al. 1997). ExoS and ExoT are bifunctional cytotoxins with 76% amino acid similarity (Engel and Balachandran 2009). Both toxins contain ADP ribosyltransferase and GTPase-activating domains resulting in diverse effects in the host organism (Engel and Balachandran 2009). The GTPase-activating domain of ExoS/ExoT targets the eukaryotic factors Rac, Rho, and CDC42 (Goehring et al. 1999; Kazmierczak and Engel

2002). Constitutive inactivation of Rho causes alterations in cell morphology, inhibition of phagocytosis, and perturbed cell migration (Lerm et al. 2000) . The ADP-ribosyltransferase activity of both toxins differ in substrate specificity yet both require a eukaryotic 14-3-3-ligand cofactor (Liu et al. 1997; Ottmann et al. 2007). ExoS appears to have broad substrate specificity affecting host cytoskeletal structure, DNA synthesis, adherent properties, and cell suicide mechanisms (Barbieri and Sun 2004). Although few ADP-ribosylation targets for ExoS have been identified, the eukaryotic factors RAS and ERM are validated targets (Jansson et al. 2006; Maresso et al. 2007). RAS is a GTPase important for proliferation and cell-survival whereas ERM factors are important for actin-related processes (Barbieri and Sun 2004). In contrast, the ADP-ribosyltransferase activity of ExoT has narrow substrate specificity affecting a limited number of proteins (Barbieri and Sun 2004). Of particular importance are the effects of ExoT on the eukaryotic CRK I and CRK II adaptor proteins (Sun and Barbieri 2003). ExoT ADP-ribosyltransferase activity disrupts cell-cell signaling and arrests cell division (Kazmierczak and Engel 2002; Shafikhani and Engel 2006). ExoU is a potent phospholipase that causes rapid cell-death via its phospholipase-A activity (Phillips et al. 2003; Sato et al. 2003). Phospholipase A activity causes rapid hydrolysis of phospholipids with broad substrate specificity resulting in a rapid loss of host cell membrane integrity and necrosis (Tamura et al. 2004). Much like ExoS and ExoT, ExoU requires a eukaryotic cofactor, superoxide dismutase (SOD1). SOD1 was identified through fractionation of eukaryotic cell extracts and interestingly its ExoU-stimulatory activity seems to be independent of SOD1 enzymatic activity (Sato et al. 2006). Additionally, yeast extracts lacking SOD1 still exhibit ExoU-stimulatory activity suggesting additional cofactors may be present (Sato et al. 2006). ExoU targets phagocytes and epithelial barriers promoting both persistence and dissemination of *P. aeruginosa* (Hauser 2009). Finally, ExoY is an adenylate cyclase similar to CyaA from *Bordetella pertussis* and edema factor (EF) from *Bacillus anthracis* (Yahr et al. 1998).

ExoY is unique among known adenylate cyclase toxins in that it is not secreted by a type I or type II mechanism and delivery is limited to cells at the site of colonization (Yahr et al. 1998). ExoY causes a dramatic increase in the intracellular levels of cAMP, which results in an inhibition of bacterial uptake and causes cytoskeletal disruptions (Ichikawa et al. 2005; Yahr et al. 1998). A study has shown that although most *P. aeruginosa* strains carry the *exoY* gene few of them actually express it (Yahr et al. 1998). The overall contribution of ExoY to the virulence and dissemination of *P. aeruginosa* remains unclear.

The secretion of toxins by the T3SS pathway results in dramatic effects on host cell morphology and immune system function, yet effector-independent contributions are just now being realized. It has been hypothesized that the translocation pore alone is sufficient to cause host-cell death due to an increase in membrane permeability and/or an effect on activation of the immune response (Dacheux et al. 2001; Goure et al. 2004; Lee et al. 2005; Shafikhani et al. 2008). This hypothesis has never been thoroughly tested and a more likely alternative explanation is that the T3SS induces an immune response by the translocation of flagellin. The translocation of flagellin has been observed in a number of different organisms that express a T3SS (Badea et al. 2009; Sun et al. 2007). The translocation of flagellin activates caspase 1 through ice protease activating factor (Miao et al. 2008). This phenomenon has been observed in *Salmonella enterica* and has also been shown for the T4SS in *Legionella sp.* resulting in activation of the inflammasome and eventual pyroptosis (Franchi et al. 2006; Ren et al. 2006). Whether translocation of flagellin or other unknown T3SS-associated effectors contributes to *P. aeruginosa* pathogenesis remains unknown. The notion that host cells have evolved an innate, protective response to the formation of the bacterial translocation system is an intriguing possibility and worthy of further study.

Regulation of T3SS Gene Expression

T3SS gene expression is regulated at the transcriptional and post-transcriptional levels (Yahr and Wolfgang 2006). Induction of T3SS gene expression occurs via host cell contact and can be artificially stimulated by the sequestration of calcium from the growth medium (Frank 1997; Vallis et al. 1999). Mechanistically, it is unclear what host/pathogen-specific factors are involved in cell contact-mediated induction and how low calcium induces secretion. The T3SS is an energetically expensive process and is tightly regulated to produce a rapid response only when host cells are present (Hauser 2009). The T3SS is unique in that transcription is coupled to the state of the secretory apparatus by a cascade of regulatory proteins reminiscent of a partner switching mechanism (Figure 1).

At the heart of the T3SS regulatory cascade is ExsA, an AraC-family transcriptional activator. ExsA specifically binds T3SS promoters and activates transcription of T3SS gene expression (Brutinel et al. 2008; Hovey and Frank 1995). Under non-inducing conditions the activity of ExsA is negatively affected by the formation of a 1:1 stoichiometric complex with ExsD (ExsA-ExsD) (McCaw et al. 2002; Thibault et al. 2009). Concomitantly, the small regulatory protein ExsE remains cytosolic and bound to its chaperone ExsC forming a 2:1 stoichiometric complex ($ExsC_2$-ExsE) (Rietsch et al. 2005; Urbanowski et al. 2005; Zheng et al. 2007). Upon host cell contact or sequestration of calcium from the growth media, ExsE is translocated into host cells or secreted, respectively (Urbanowski et al. 2007). In the absence of ExsE, ExsC forms a 1:1 stoichiometric complex with ExsD ($ExsC_2$-$ExsD_2$) (Lykken et al. 2006; Zheng et al. 2007). Formation of the $ExsC_2$-$ExsD_2$ complex is energetically favored over formation of the ExsA-ExsD complex and thus frees ExsA to activate transcription (Brutinel et al. 2009a). This system is thought to be governed solely by protein affinity and in keeping with this hypothesis, the dissociation constant (K_d) for the $ExsC_2$-ExsE complex (1 nM) is lower than that of the $ExsC_2$-$ExsD_2$ complex (18 nM) suggesting that

ExsC preferentially binds ExsE (Zheng et al. 2007). Although the insolubility of free ExsD has hampered biochemical studies, we predict the dissociation constant of the ExsA-ExsD complex to be greater than 18 nM. This regulatory cascade is depicted in Figure 1 and is a summation of the following affinity hierarchy: ExsE > ExsC > ExsD > ExsA.

In addition to secretion-mediated transcriptional regulation of T3SS gene expression, a host of unrelated factors are known to influence T3SS gene expression (Figure 2). Intracellular levels of cAMP modulate T3SS gene expression through the CRP-like transcriptional regulator Vfr (Wolfgang et al. 2003). Recent studies indicate that low Ca^{2+} conditions also influence cellular cAMP levels (Rietsch and Mekalanos 2006). cAMP levels are known to allosterically modulate Vfr activity and DNA microarray studies have implicated Vfr as a global regulator of type IV pili, type II secretion, and T3SS gene expression (Wolfgang et al. 2003). ExsA overexpression can complement mutants unable to produce Vfr and cAMP, suggesting these regulatory components act on a common pathway (Wolfgang et al. 2003). Conversely, Vfr overexpression and exogenous cAMP are unable to complement a mutant lacking ExsA, suggesting that the Vfr/cAMP effect occurs at or above the level of ExsA (Wolfgang et al. 2003). It is attractive to posit that Vfr may act synergistically with ExsA to directly regulate T3SS gene expression given the propensity for AraC-family members to interact with CRP-like proteins (Gallegos et al. 1997).

Other factors such as osmolarity, DNA damage, copper stress, and metabolic stress influence T3SS gene expression. Increased osmolarity, caused by either high NaCl or elevated sucrose concentrations, is known to modulate T3SS gene expression (Rietsch and Mekalanos 2006). Levels of cAMP are also elevated in response to osmotic stress, suggesting a possible link with the Vfr/cAMP pathway (Rietsch and Mekalanos 2006). The SOS DNA damage stress response is known to induce PtrB, a protein that negatively affects T3SS gene expression by an unknown mechanism (Wu and Jin 2005). PtrA is a

regulator that is induced by copper stress and is thought to negatively regulate T3SS gene expression during burn-wound infections (Ha et al. 2004). Recent studies suggest PtrA directly binds ExsA to negatively regulate its activity in *P. aeruginosa* strain PAK (Ha et al. 2004) yet similar studies in PA103 yield different results suggesting strain-specific effects (Vakulskas and Yahr, unpublished). Finally, disruptions in intracellular metabolite levels are known to influence T3SS gene expression. Mutations in pyruvate dehydrogenases, glucose transporters, and overproduction of efflux pumps all negatively affect T3SS gene expression through unknown mechanisms (Linares et al. 2005; Rietsch and Mekalanos 2006; Wolfgang et al. 2003).

In general, T3SS gene expression appears to be positively regulated in acute infections and negatively regulated in chronic infections. Recent data suggest that negative regulation of T3SS gene expression in a chronic infection is linked to alginate production, biofilm formation, and quorum sensing (Yahr and Wolfgang 2006). Alginate is an exopolysaccharide that increases the ability of *P. aeruginosa* to persist in the lungs of cystic fibrosis (CF) patients (Bendiak and Ratjen 2009; Martin et al. 1993). Alginate overproduction in CF patients occurs primarily because of mutations in *mucA,* the anti-sigma factor negatively regulating alginate production (Martin et al. 1993). Analysis of clinical isolates from CF-lungs suggests that the switch to alginate overproduction (*mucA* mutation) is observed simultaneously with a loss in T3SS gene expression (Wu et al. 2004). The short term, reversible loss of T3SS gene expression is thought to occur as a result of an increase in AlgU/AlgR production (Yahr and Wolfgang 2006). AlgU and AlgR are regulators (repressed by MucA) that negatively affect T3SS gene expression by an unknown mechanism (Yahr and Wolfgang 2006). The long term loss of T3SS gene expression observed in clinical isolates from CF patients, however, may be due to the rapid accumulation of mutations in factors positively regulating T3SS gene expression (*exsA*, *vfr*, etc.) (Wu et al. 2004).

T3SS gene expression is also linked to the expression of genes required for biofilm formation. Biofilm formation is regulated by the RetS and LadS sensor proteins (Ventre et al. 2006). Mutations in *retS* have reduced T3SS-dependent cytotoxicity and increased biofilm formation while *ladS* mutants have the opposite effect (Ventre et al. 2006). RetS and LadS are two-component regulators thought to be associated with sensing carbohydrates that mediate activity through the GacAS/*rsmZ* pathway (Heurlier et al. 2004; Ventre et al. 2006). The small, regulatory RNA rsmZ sequesters the CsrA-like RNA-binding protein RsmA (Ventre et al. 2006). Recent evidence suggests that RsmA post-transcriptionally regulates ExsA expression (Urbanowski and Yahr, unpublished). The current model predicts that RetS directly represses transcription of biofilm genes and *rsmZ* expression, thereby allowing free RsmA to enhance T3SS gene expression by stabilizing the *exsA* mRNA. The available data also suggest that RetS may function through the Vfr/cAMP pathway; as Vfr overexpression can complement a *retS* deletion restoring T3SS gene expression (Mulcahy et al. 2006). Finally, T3SS gene expression has been linked to quorum sensing as an inactive Rhl system shows increased T3SS gene expression (Bleves et al. 2005). The details of this interaction have yet to be determined but are worthy of further study.

ExsA and the AraC-family Transcriptional Regulators

ExsA is a member of the extremely diverse AraC family of transcriptional regulators. AraC homologues are found in both Gram-positive and Gram-negative organisms. These regulators can function to activate, repress, or bifunctionally modulate transcription (Gallegos et al. 1997). The hallmark feature of AraC family regulators is a conserved DNA-binding region comprised of approximately 100 amino acids (Egan 2002). This domain contains 1 or 2 helix-turn-helix DNA-binding motifs, which are usually sufficient to bind DNA and activate transcription (Egan 2002). AraC family regulators also typically have a non-conserved region containing either a ligand-

interaction or multimerization domain (or both). DNA-binding activity is usually affected by the presence of bound ligand. The ligands in these cases can be small sugars/metabolites, redox components, organic compounds, or proteins (Egan 2002; Plano 2004). For example, AraC regulates transcription of arabinose metabolism genes in response to the presence or absence of the simple sugar arabinose (Schleif 2003). An AraC dimer binds arabinose, inducing a conformational change in the complex that increases its affinity for DNA half-sites located at the *araI* locus (Schleif 2003). In this conformation, the arabinose-bound AraC dimer activates transcription by recruiting RNA polymerase (RNAP) and facilitating open complex formation (Zhang et al. 1996). In the absence of arabinose, the AraC dimer binds distal half sites; one located at the $araI_1$ locus and the other 210 base pairs upstream at the $araO_2$ locus (Schleif 2003). In this conformation the AraC dimer forms a repressive DNA loop that prevents access to promoters by RNAP (Schleif 2003). This is an example of not only how an AraC-family member is modulated by its bound ligand, but also of how it can both activate and repress transcription.

ExsA contains the conserved AraC-family DNA-binding region located in its carboxy terminus as well as an amino-terminal domain containing ExsD binding determinants and a self-interaction domain (Brutinel et al. 2009b). Electrophoretic mobility shift assays demonstrate that ExsA specifically binds T3SS promoters with high affinity (K_{eq} = 1-5 nM) (Brutinel et al. 2008; Hovey and Frank 1995). Two complexes of different mobility are observed at all tested promoters, with the most mobile shift product termed complex 1, and the least mobile shift complex termed complex 2 (Brutinel et al. 2008). ExsA protects similar regions of the P_{exsC}, P_{exsD}, and P_{exoT} promoters from DNAse I cleavage. The area of protection starts approximately 8 nucleotides downstream of the presumptive -35-like RNAP recognition hexamer and continues upstream as far as 30 nucleotides (Brutinel et al. 2008; Hovey and Frank 1995). This protection range is much greater than that of several well-characterized monomeric AraC-family regulators, which

suggests multiple ExsA molecules occupy T3SS promoters (Egan 2002). Point mutations were made in the P_{exoT} promoter and ExsA binding was assayed by EMSA to more finely map the ExsA binding site. The results of these experiments suggest that the downstream, -35-like region as well as the upstream, adenine-rich region of the P_{exoT} promoter (Figure 3) represent adjacent ExsA binding sites called sites 1 and 2, respectively (Brutinel et al. 2008; Brutinel et al. 2009b). In contrast to most AraC-family proteins, the ExsA binding site appears to completely overlap a presumptive -35 sequence (Brutinel et al. 2008). Mechanistically it is unclear how ExsA activates transcription, and what role a near-consensus -35 RNAP recognition hexamer plays in both activator-dependent and activator-independent transcription.

It is also interesting to note that ExsA bends DNA at the P_{exoT}, P_{exsD}, and P_{exsC} promoters and that bending is dependent on the carboxy-terminal domain (Brutinel et al. 2008; Brutinel et al. 2009b). The most dramatic example of ExsA-dependent DNA bending is seen at the P_{exsC} promoter where the DNA in complex 1 bends 37° and the DNA in complex 2 bends 78° (Brutinel et al. 2008). DNA bending by transcriptional activators has been shown to play a role in the formation of open complexes during transcription initiation and it is tempting to speculate a similar role for ExsA (Kerppola and Curran 1997).

The Bacterial σ^{70}-RNA Polymerase and the Kinetics of Transcription Initiation

The DNA-dependent σ^{70}-RNAP from *P. aeruginosa* is very similar to that of *E. coli*, containing nearly identical subunits and recognizing similar promoter consensus sequences (TTGACA/TATAAT) (Dominquez-Cuevas 2004). The RNAP complex is made up of five different subunits. The RNAP β subunit houses the catalytic machinery facilitating RNA chain initiation and elongation (Gross et al. 1996). The β' subunit is poorly understood but appears to have a role in non-specific DNA binding; this occurs

independent of the σ factor (Gross et al. 1996). At the heart of the complex is the α subunit which forms a dimer as part of the RNAP holoenzyme, with each α monomer containing two independent functional domains joined by a flexible linker (Gross et al. 1996). The α subunit amino terminus serves as a scaffold for RNAP core assembly by independently binding the β and β' subunits (Igarashi et al. 1991). In rare cases the α subunit amino terminus has been implicated as a target for transcriptional activators yet this domain does not seem to be critical for AraC-family protein-RNAP contacts (Egan et al. 2000). The α subunit carboxy terminal domain binds DNA regions known as UP elements which consist of AT tracts located upstream of the -35 hexamer (Gaal et al. 1996). In addition to having DNA-binding determinants, the α carboxy-terminal domain serves as a target for a number of transcriptional activators, including several AraC family members (Egan 2002). The α, β, and β' subunits make up what is collectively known as the RNAP core enzyme; this complex, when assembled on DNA, is fully capable of RNA chain elongation yet is incapable of specifically recognizing promoter sequences (Burgess et al. 1969).

The σ^{70} subunit, the most well-studied RNAP component, contains four independent regions of discrete function (Paget and Helmann 2003). The first σ^{70} region prevents non-specific association of σ^{70} with DNA in the absence of core RNAP (Paget and Helmann 2003). Curiously this region is only present in the primary σ factors (*rpoD*, *rpoS*, etc.) suggesting it may be critical for maximal expression of housekeeping genes. The second region contains DNA binding sequences important for recognizing the -10 or Pribnow recognition hexamer (Severinova et al. 1996). The third region contains sequences important for recognition of extended -10 promoters and is thought to have a role in transcription initiation (Paget and Helmann 2003). The fourth and final region of σ^{70} contains DNA binding elements that recognize the -35 hexamer and is important for the action of several transcriptional activators (Paget and Helmann 2003).

An additional RNAP subunit ω has been identified but its role in transcription remains unclear. It has been reported that *E. coli* mutants lacking the ω subunit have no obvious phenotype and grow normally in rich media (Mathew and Chatterji 2006; Mathew et al. 2006). Recent *in vitro* data suggests that this subunit may be important for refolding of denatured RNAP enzyme (Mathew et al. 2006). Additionally, it has been shown that the ω subunit can function as a target for a synthetic transcriptional activator; suggesting it theoretically could be an authentic target for activator function (Dove and Hochschild 1998).

RNAP is a complex enzyme that has evolved to recognize a diverse set of promoter architectures and functions in concert with hundreds of different transcriptional regulators. The initiation of RNA synthesis is also complex and is generally simplified by the identification of rate-limiting steps. The initiation of RNA synthesis requires template DNA, RNAP holoenzyme (α_2,β,β',σ^{70}), and the first few nucleotides of a transcript (Hsu 2009). The first step in initiation is closed complex formation and simply represents the initial recognition and binding of RNAP to promoter DNA (Hsu 2009). The rate of closed complex formation is limited by the ratio of RNAP to template DNA, as well as how efficiently RNAP can recognize the promoter. This efficiency is usually determined by the specific sequence of -35/-10 recognition hexamers as well as the presence or absence of additional promoter factors like UP elements or extended -10 sequences (Hsu 2009). The second step is isomerization to the open complex. The hallmark of the open complex is a region of single-stranded DNA (ssDNA) starting at the -10 hexamer and extending beyond the +1 site of transcription initiation. This step allows the β subunit of polymerase access to the template strand to initiate RNA chain synthesis and is easily detected by the presence of ssDNA-modifying agents such as potassium permanganate (Davis et al. 2007). The final step in initiation, promoter clearance, represents the transition to an elongation and is poorly understood. To date

transcriptional activators have been identified that affect each step in transcription initiation (Hsu 2009).

Research Proposal and Rationale

The AraC family of transcriptional regulators has been the subject of intense study for the past fifty years. Although the DNA-binding activity of a number of these regulators has been studied intensively, very little is known about how they activate transcription. AraC, the prototypical family member, has been shown to recruit RNAP to the promoter and affects isomerization of the resulting closed complex to an open complex (Zhang et al. 1996). Beyond that, only a few studies have hinted at the mechanism of transcriptional activation for other AraC-like proteins. I have developed a simple scheme to purify *P. aeruginosa* core RNAP and to reconstitute RNAP holoenzyme allowing the study of the regulation of ExsA-dependent transcription *in vitro*. In the second chapter of this manuscript I characterize the stoichiometry of ExsA both in solution and bound to T3SS promoters. Additionally, I show inhibition of ExsA DNA-binding activity by ExsD *in vivo.* In the third chapter I have used genetic and biochemical techniques to understand how ExsA interacts with ExsD, and how it interacts with RNAP to activate transcription. In the fourth and final chapter I determined which subunits of RNAP interact with ExsA and I provide convincing evidence that ExsA compensates for the lack of a -35 hexamer at T3SS promoters. I also provide a mechanism by which ExsA-independent transcription occurs in the absence of a -35 hexamer. Studying how ExsA interacts with RNAP and the mechanism of activation may aid in developing therapeutics designed to target both the T3SS as well as the bacterial RNAP. The following information supports this claim: (i) a novel therapeutic has already been developed specifically targeting ExsA (Bowser et al. 2007); (ii) the drug Virstatin has been developed to inhibit dimerization of the AraC-like protein

ToxT (Shakhnovich et al. 2007); and (iii) bacterial RNAP has been and will undoubtedly continue to be the target of novel therapeutics for decades (Chopra 2007).

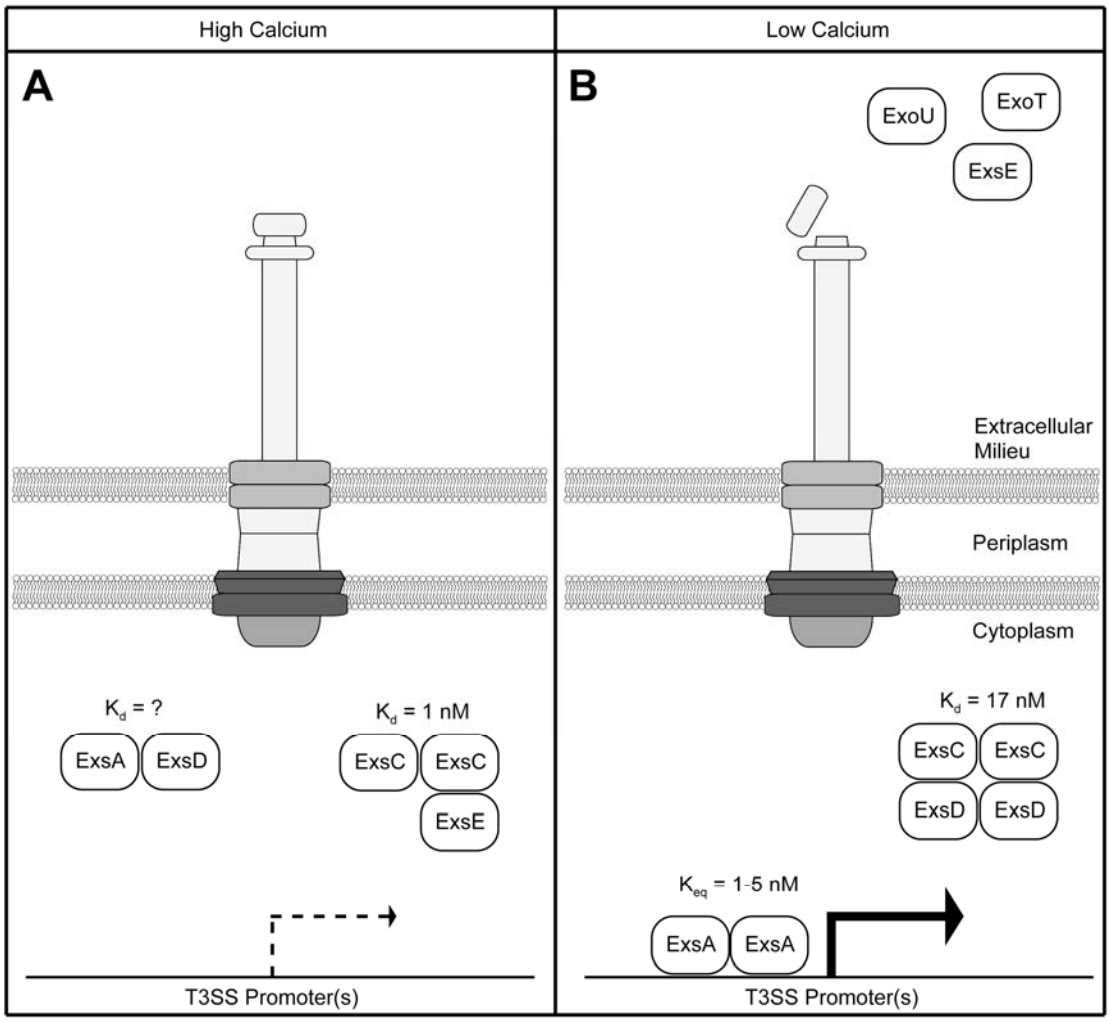

Figure 1. Schematic illustration of the *P. aeruginosa* T3SS regulatory system. T3SS-induction is accomplished by the presence of calcium-replete conditions (as depicted) or host-cell contact. (A) In an induced cell, ExsA-dependent gene expression is maximal and secretion or translocation of ExoU, ExoT, and ExsE takes place. The secretion or translocation of ExsE results in formation of the $ExsC_2$-$ExsD_2$ complex, which prevents ExsD from disrupting ExsA self-association and DNA-binding activities. (B) In the absence of induction, ExsE remains cytosolic and bound to ExsC. The $ExsC_2$-ExsE complex liberates ExsD which then is available to bind ExsA. The resulting ExsA-ExsD complex is incapable of binding DNA or associating with free ExsA rendering the complex transcription-incompetent.

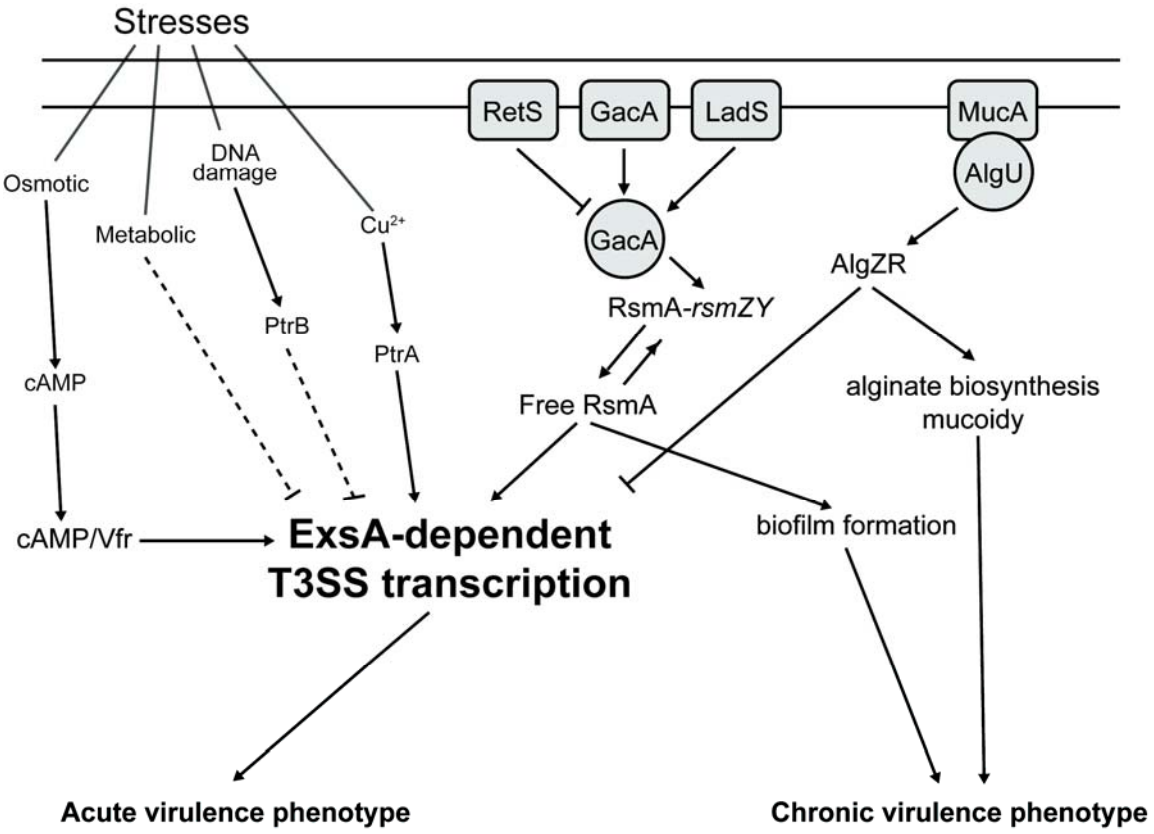

FIGURE 2. Environmental signals and regulatory systems that influence T3SS gene expression. The solid lines indicate regulatory circuits that have been demonstrated experimentally whereas the dashed lines represent hypothetical regulatory circuits. See text for details.

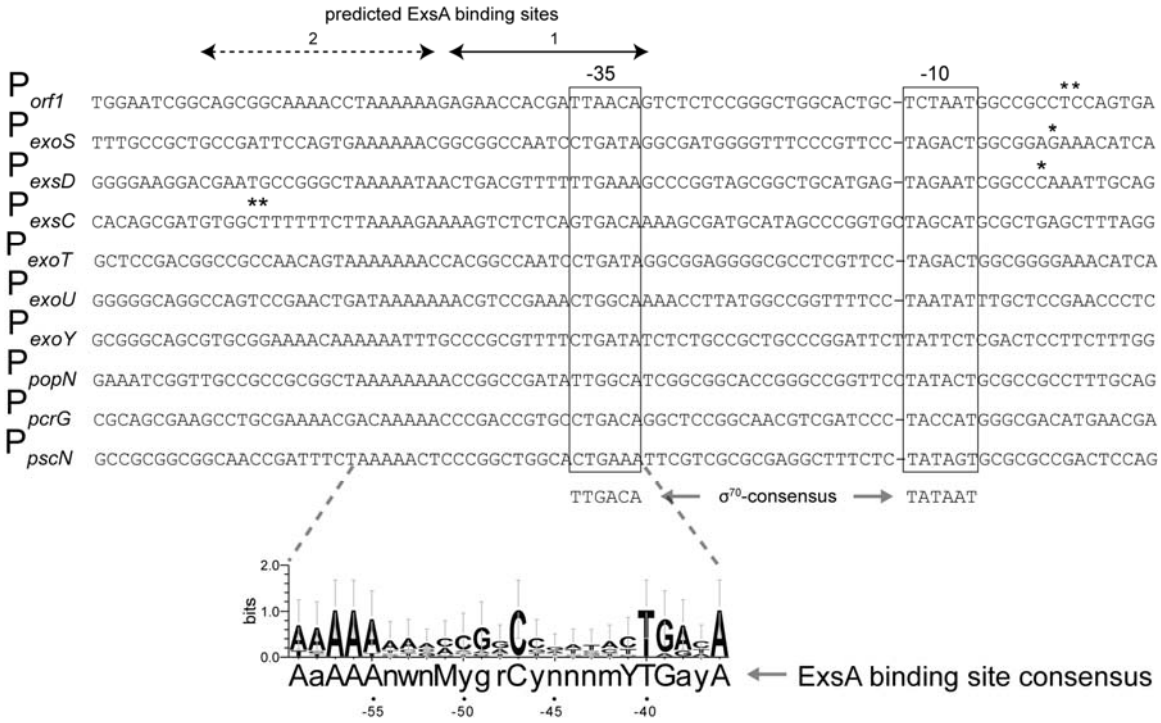

FIGURE 3. CLUSTALW alignments of ExsA-dependent promoters. The predicted ExsA DNA-binding sites, mapped transcription start sites (asterisks), and putative -35/-10 hexamers (boxed) are indicated. A WebLogo illustration of the consensus ExsA DNA-binding site is indicated. The letter height represents the frequency at which each base occurs at the position in the alignment. Nucleotides present > 90% or > 80% but less than 90% in the CLUSTALW alignment are indicated in the consensus sequence by capital and lowercase letters, respectively. W = A or T, M = A or C, Y = C or T, R = A or G, K = G or T, and n = any nucleotide.

CHAPTER II

THE STOICHIOMETRY OF EXSA AND IN VIVO INHIBITION OF DNA-BINDING ACTIVITY BY EXSD

Introduction

The AraC-family of transcriptional activators is diverse and consists of members that function as monomers, dimers, and higher order oligomers (Egan 2002). Deciphering the stoichiometry of AraC-like regulators has been somewhat elusive given the tendency for these proteins to aggregate and become insoluble (Schleif 2003). The prototypical member of the family, AraC, binds DNA as a dimer and has been studied in solution by isolating its amino terminal domain, which renders the protein highly soluble (Hendrickson and Schleif 1985; Weldon et al. 2007). Experiments with this truncated derivative reveal that AraC exists as a dimer in solution with or without arabinose (LaRonde-LeBlanc and Wolberger 2000). Other AraC-family members like MarA consist solely of the ~100 amino acid DNA-binding domain and function as monomers (Rhee et al. 1998). Determining the stoichiometry of ExsA when in solution will allow for a better understanding of how ExsA interacts with its known ligands and with DNA (monomer assembly, dimer recognition, etc.). Revealing the stoichiometry of ExsA when bound to T3SS promoters will also aid in determining both the RNAP interaction mechanism (class I or class II) and the precise nature of the ExsA consensus binding site(s).

A previous study of ExsA was complicated by the addition of a large solubility tag making the interpretation of experimental results difficult (Hovey and Frank 1995). Our laboratory has generated an amino-terminal histidine-tagged ExsA derivative that is very amenable to *in vitro* study when purified in the presence of the non-ionic detergent, Tween-20 (Brutinel et al. 2008). Using highly pure, decahistidine-tagged ExsA, it has been shown that ExsA most likely binds DNA with either 1 or 2 molecules per single binding site (Brutinel et al. 2008). Data supporting this hypothesis include

electrophoretic mobility shift assays demonstrating two ExsA-DNA shift complexes of distinct mobility and the corresponding DNAse I protection experiments showing a protection region suggestive of two adjacent ExsA proteins (Brutinel et al. 2008). The data also indicate the presence of 2 adjacent ExsA binding sites at the P_{exoT} promoter. EMSA experiments utilizing truncated P_{exoT} promoter derivatives indicate that deletions in the upstream A-rich region (site 2) only affect formation of shift complex 2 (Brutinel et al. 2008). Deletions of the downstream -35-like region (site 1), however, ablate the formation of both shift complexes 1 and 2. These data suggest that two binding sites are present and that occupation of site 1 by ExsA is required for occupation of site 2. Interestingly, whereas mutations in site 2 only affect formation of EMSA shift complex 2, mutations in either binding site drastically affect ExsA-dependent transcription (Brutinel et al. 2008), indicating that occupation of both binding sites is required for ExsA-dependent activation. Although the available data suggest 2 ExsA binding sites exist at T3SS promoters, it is unclear whether ExsA binds DNA as a monomer, multiple monomers, or as an oligomer.

ExsD negatively regulates ExsA activity through a direct binding interaction (McCaw et al. 2002). Recent *in vitro* data indicates that ExsD does so by forming a 1:1 stoichiometric complex (ExsA-ExsD), which perturbs the DNA-binding activity of ExsA (Brutinel et al. 2009a; Thibault et al. 2009). It has also been shown that ExsD may function by antagonizing both the DNA-binding activity and the self-association properties of ExsA (Brutinel et al. 2009a). ExsD is insoluble in solution and therefore *in vitro* studies have relied on testing the DNA binding activity of purified ExsA-ExsD complex (Brutinel et al. 2009b; Thibault et al. 2009). The ExsA-ExsD complex can be purified to homogeneity and is highly soluble (Brutinel et al. 2009a; Thibault et al. 2009). Because of the presence of competing hypotheses for ExsD function (inhibition of ExsA self-assocation and ExsD repression) it remains to be seen whether the inhibition of ExsA

DNA-binding activity by ExsD is physiologically relevant *in vivo* (Bernhards et al. 2009; Brutinel et al. 2009a).

In this chapter I determine the stoichiometry of ExsA in solution and when bound to DNA at three different T3SS promoters. I employed glycerol gradient ultracentrifugation to determine the stoichiometry of free $ExsA_{His}$ relative to protein standards of known molecular weight. Furthermore, I utilized a previously-established EMSA-based method (Orchard and May 1993) to determine the molecular weight of complexes 1 and 2 at the P_{exoT}, P_{exsC}, and P_{exsD} promoters. I used the chromatin immunopreciptiation assay (ChIP) to test whether ExsD inhibits ExsA DNA-binding activity *in vivo.* This represents the first complete stoichiometric characterization of a full-length AraC-family protein *in vitro* and the first direct *in vivo* evidence demonstrating modulation of the DNA-binding activity of an AraC-like protein by its bound ligand.

Materials and Methods

Glycerol gradient centrifugation

The relative sedimentation of ExsA was compared with non-denatured protein standards of known molecular weight (BSA monomer [66 kDa]; carbonic anhydrase [29 kDa]; and lactalbumin [14.2 kDa], Sigma) in a glycerol gradient. Purified $ExsA_{His}$ (2 µg) was loaded onto a 5–20% linear glycerol gradient in 4 ml of ExsA DNA-binding buffer (10 mM Tris [pH 7.5], 100 mM KCl, 1 mM EDTA, 5% glycerol and 100 µg ml^{-1} bovine serum albumin) supplemented with 0.5% Tween-20. This gradient alongside an identical gradient containing 5 µg each of protein standard was centrifuged (247,000 g) for 27 h at 4°C in a Beckman SW60ti rotor. Fractions (200 µl) were collected and subjected to SDS-PAGE followed by silver staining or anti-ExsA immunoblot analyses.

Stoichiometry of the $ExsA_{His}$–promoter probe complexes

Molecular weight determination of ExsA–DNA complexes was performed as described (Orchard and May, 1993). Annealed oligonucleotide probes (50 bp) were end-labelled with [γ-^{32}P]-ATP for use in DNA binding reactions. EMSAs were performed as follows. Reactions (19 ul) containing end-labelled specific and non-specific probes (0.25 nM each), 25 ng ml^{-1} poly (2′-deoxyinosinic-2′-deoxycytidylic acid) (Sigma, St Louis, MO), and 10 µl of 2X binding buffer (20 mM Tris [pH 7.5], 200 mM KCl, 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol and 200 µg ml^{-1} bovine serum albumin) were incubated for 5 min at 25°C. $ExsA_{His}$ was added to the indicated concentrations in a final reaction volume of 20 µl and incubated at 25°C for 15 min. ExsA–DNA complexes were immediately subjected to electrophoresis in 4.5%, 5%, 6%, 7%, 8%, 9% and 10% polyacrylamide (0.25X TBE) gels alongside non-denatured protein standards (β-amylase [200 kDa], BSA dimer [132 kDa] and monomer [66 kDa], carbonic anhydrase [29 kDa] and α-lactalbumin [14 kDa]). Protein standards were detected by Coomassie blue staining and ExsA–DNA complexes were visualized by autoradiography. Imaging was performed using an FLA-7000 phosphorimager (Fujifilm) and MultiGauge v3.0 software (Fujifilm) for data analyses. The migration distance of each DNA–protein complex and protein standard was divided by the migration distance of the bromophenol blue dye yielding a relative mobility (R_f). The logarithm of each R_f was plotted as a function of acrylamide concentration and the slope of each best-fit calibration curve (denoted K_r for retardation coefficient) was plotted against the molecular weight to yield a standard curve. The molecular weight of each ExsA–DNA complex was estimated from the standard curve and the reported values are an average of three independent experiments.

Chromatin immunoprecipitation assay (ChIP)

P. aeruginosa strains were grown with vigorous aeration in tryptic soy broth (TSB) supplemented with 100 mM monosodium glutamate and 1% glycerol to an OD_{600} of 0.6 at 30°C. Protein-DNA complexes were crosslinked by the addition of

formaldehyde (final concentration of 1%) and cultures were shaken at 150 rpm for 20 min at 30°C. Reactions were quenched by adding 5 ml 1.0 M glycine [pH 8.0]. The cells were harvested by centrifugation (2,000 x g for 10 min at 4°C), washed 2 times with ice cold PBS, and suspended in 500 µl lysis buffer (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 20% sucrose, 10 mM EDTA) containing protease inhibitor cocktail (complete mini, EDTA-free protease inhibitor cocktail, Roche, Indianapolis, IN) and 2 mg ml^{-1} lysozyme. After 30 min on ice, 500 µl of 2x IP buffer (100 mM Tris-HCl [pH 7.0], 300 mM NaCl, 2% Triton X-100, 40 mM EDTA) was added to the reaction and incubated at 37°C for 10 min, followed by 2 min on ice. Samples were sonicated and unbroken cells were sedimented by centrifugation (16,000 x g for 5 min at 4°C). The supernatant was passed through a 0.22 µM filter and subjected to immunoprecipitation. Anti-ExsA polyclonal antibody (2 µg) was incubated with the samples for 4 hours at 4°C followed by the addition of Protein A magnetic beads (25 µl, New England Biolabs) for 45 min at 4°C. Beads were washed 3 times with 1X IP Buffer and 2 times with TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Beads were suspended in 100 µl TE buffer and the formaldehyde crosslinks were reversed by incubation for 8 hrs at 65°C. The eluate (5 µl) was used in PCR reactions with primers that amplify ~200 bp region of the P_{exsD}, P_{exsC}, and P_{fleQ} promoters (5'- ATACGAATTCTTCCAGCCAGTCCTATTTCA and 5'-GACAGGTACCCCTGCTCCATTCTCTGCCTTG for P_{exsD}, 5'-TGATGAATTCGCCTCCTAAAGCTCAG and 5' -ATACGAATTCTTCCAGCCAGTCCTATTTCAC for P_{exsC}, and 5' -TTAGGTACCCACCAGATGTTCGGATAAGT and 5'-TTAGAATTCCGAATGGGTCTCGCTCGACC for P_{fleQ}). The resulting PCR products were visualized on an agarose gel stained with ethidium bromide.

Results

Oligomeric state of purified $ExsA_{His}$ and stoichiometry of $ExsA_{His}$-promoter probe complexes

To examine the oligomeric state of ExsA in solution I employed glycerol gradient centrifugation. Purified $ExsA_{His}$ (300 nM) was applied to a linear 5–20% glycerol gradient and centrifuged for 27 h. Fractions were then collected, subjected to SDS-PAGE, and stained with silver or immunoblotted for $ExsA_{His}$. Molecular weight standards consisting of BSA (66 kDa), carbonic anhydrase (29 kDa) and α-lactalbumin (14 kDa) were analyzed in the same manner and used to generate a standard curve. The majority of $ExsA_{His}$ applied to the gradient co-migrated with the 29 kDa marker indicating that $ExsA_{His}$ (which has a predicted molecular mass of 34 kDa) is monomeric in solution (Fig. 4A). We also noted a minor $ExsA_{His}$ peak (representing < 5% of the material applied to the gradient and not visible in Fig. 4A) that co-migrated with the 66 kDa marker. Although it is possible that $ExsA_{His}$ is dimeric but dissociates to monomers within the glycerol gradient, the fact that $ExsA_{His}$ sediments as a tightly defined monomeric peak rather than being spread between the 66 kDa and 29 kDa markers argues against this possibility. Furthermore, I estimate the $ExsA_{His}$ concentration within the monomeric peak to be ~60 nM when accounting for dilution. This concentration is well within the range required for $ExsA_{His}$ DNA-binding activity (~5 nM) (Brutinel et al. 2008). These data suggest that purified $ExsA_{His}$ is monomeric under conditions permissive for DNA binding.

To determine the number of $ExsA_{His}$ molecules bound to shift complexes 1 and 2 we utilized the method of Orchard and May (1993). This method requires the use of relatively small EMSA probes (50 bp). Using probes of this size we find that $ExsA_{His}$ specifically shifts the P_{exsC}, P_{exsD} and P_{exoT} promoters and again yields two distinct shift products (Fig. 4B). These data are similar to our EMSA findings using 200 bp probes (Brutinel et al. 2008) with the notable exception that the mobility of shift product 2 for

the 50 bp P_{exsC} promoter probe is now similar to the mobility of shift product 2 for the P_{exsD} and P_{exoT} promoter probes (Fig. 4B). It was previously determined that ExsA binding imparts a significant bend of the P_{exsC} promoter. Therefore the most likely explanations for the altered mobility observed with the 50 bp P_{exsC} probe are that $ExsA_{His}$ does not elicit bending of the 50 bp P_{exsC} promoter probe or that the effect of bending the 50 bp promoter probe is not detectable by gel electrophoresis.

The molecular weight of shift complexes 1 and 2 was determined by comparing the $ExsA_{His}$–promoter probe complexes to a standard curve generated from a panel of non-denatured proteins of known molecular weight (Fig. 4C). By this method the calculated molecular weight of shift product 1 for the P_{exsC}, P_{exsD} and P_{exoT} promoter probes is 70, 75 $\pm$ 2 and 74 $\pm$ 1 kDa respectively. Subtracting the molecular weight of the 50 bp probe (~33 kDa) yields molecular weights of 37, 42, and 41 kDa, respectively. These values are consistent with shift product 1 resulting from one molecule of $ExsA_{His}$ bound to the promoter probes. The calculated molecular weights for P_{exsC}, P_{exsD}, and P_{exoT} shift products 2 are 65 $\pm$ 1, 68 $\pm$ 1 and 68 $\pm$ 1 kDa, respectively, after subtracting the molecular weight of the 50 bp probe, and is consistent with two molecules of bound $ExsA_{His}$ resulting in shift product 2.

The data presented thus far are consistent with $ExsA_{His}$ functioning as a monomer. A trivial explanation for that finding, however, is that the Tween-20 required to maintain $ExsA_{His}$ in a soluble state dissociates dimeric $ExsA_{His}$ into monomers. To address this possibility whole-cell extracts from *E. coli* expressing $ExsA_{His}$ were prepared in the absence or presence of 0.5 % Tween-20, and then used immediately in an EMSA. The $ExsA_{His}$ extracts generated shift products 1 and 2 irrespective of the absence or presence of Tween-20 for each of the promoter probes tested (Brutinel et al. 2008). These combined data demonstrate that $ExsA_{His}$ most likely binds DNA as a monomer.

ExsD inhibits the DNA-binding activity of ExsA *in vivo*

Electrophoretic mobility shift assays demonstrate that the ExsA-ExsD complex does not possess DNA-binding activity *in vitro*. Furthermore, Dr. Evan Brutinel in my lab has determined that ExsA liberated from the ExsA-ExsD complex by the presence of ExsC protein binds DNA *in vitro* (Brutinel et al. 2009b). To determine whether ExsD also inhibits the DNA binding activity of ExsA *in vivo*, I performed a chromatin immunoprecipitation (ChIP) assay. The ideal comparison would be between cells grown under high Ca^{2+} conditions where ExsD inhibits ExsA-dependent transcription versus cells grown under inducing conditions (low Ca^{2+}) where ExsC inhibits the negative regulatory activity of ExsD and ExsA is bound to promoter DNA. The fact that ExsA levels increase 3-4 fold under inducing conditions, however, would make it difficult to determine whether an increase in DNA-binding activity reflected increased ExsA expression or a lack of inhibition by ExsD. For this reason I designed a system in which ExsA expression remains constant and then assessed whether ExsD influences the fraction of ExsA bound to DNA. To this end Δ*exsA* and Δ*exsA*Δ*exsD* (Δ*exsAD*) mutants carrying a chromosomally integrated ExsA-dependent transcriptional reporter ($P_{exsD\text{-}lacZ}$) were transformed with either a vector control (pJN105) or a plasmid constitutively expressing low levels of ExsA (pExsA). The resulting strains were grown under non-inducing conditions for T3SS gene expression and assayed for β-galactosidase activity. As expected, the activity of the $P_{exsD\text{-}lacZ}$ reporter was low in the absence of *exsA* (Fig. 5A, pJN105). Introduction of pExsA into the Δ*exsA* mutant resulted in only a modest increase in $P_{exsD\text{-}lacZ}$ reporter activity (~2-fold) due to the negative regulatory function of ExsD. In contrast, $P_{exsD\text{-}lacZ}$ reporter activity increased dramatically in the Δ*exsAD* mutant transformed with pExsA. Immunoblots of whole cell lysates confirmed that the steady-state levels of ExsA expression were similar in both the Δ*exsA* and Δ*exsAD* strains transformed with pExsA (Fig. 5B). This finding suggests that the ability of ExsA to activate transcription differs in these two backgrounds.

To determine if the difference in expression of the $P_{exsD\text{-}lacZ}$ reporter correlated with changes in the DNA-binding activity of ExsA, cells from log-phase cultures were treated with formaldehyde to crosslink ExsA to chromosomal DNA. The cellular DNA was then sonicated to generate 500–1000 bp fragments and subjected to immunoprecipitation with polyclonal ExsA antibody. Following reversal of the formaldehyde crosslinks by heat treatment, the cellular DNA co-precipitating with ExsA was used as template in a PCR with primers that amplify the ExsA-dependent P_{exsD} or P_{exsC} promoters, and the ExsA-independent P_{fleQ} promoter as a negative control. Strong PCR products representing the P_{exsD} or P_{exsC} promoters were seen in samples isolated from cells expressing ExsA in the absence of ExsD (Fig. 5C, lane 6), and the amount of those products was significantly reduced in samples isolated from strains expressing ExsD (lane 5). PCR products were absent in reactions lacking ExsA (Fig. 5C, lanes 3-4), ExsA antibody (lane 2), or when using primers to P_{fleQ} (lanes 3-6). Although the PCR method of detection used in these experiments is semi-quantitative at best, two pieces of data indicate that the difference in P_{exsD} promoter signal in the absence and presence of ExsD is greater than shown: First the subtraction of 2 PCR cycles greatly affects signal from the *exsA* mutant whereas signal from the *exsAD* double mutant is largely unaffected suggesting that the levels of template are greater than visibly appreciated (Fig. 5). Second, increasing the concentration of ExsA antibody had no apparent effect on PCR signal from the *exsA* mutant; however, it had a dramatic effect on signal from the *exsAD* double mutant suggesting that the α-ExsA antibody was limiting (data not shown).

Discussion

In the present study I have determined the stoichiometry of ExsA in solution and when bound to DNA in complexes 1 and 2. My data demonstrate that shift product 1 represents one molecule of $ExsA_{His}$ bound to the promoter and is consistent with my observation that purified $ExsA_{His}$ is monomeric under conditions known to support DNA-

binding (Fig. 4A). My data further indicate that shift product 2 represents two molecules of $ExsA_{His}$ bound to the promoter. Although I cannot fully resolve whether the two molecules of $ExsA_{His}$ are bound to a single site or two adjacent sites on the promoter, I favor the latter possibility based on the following data: First, the minimal $ExsA_{His}$ binding region on the P_{exoT} promoter (27 bp) derived from mapping studies is larger than the monomeric binding sites (17–21 bp) of several well-characterized AraC proteins like MarA and SoxS (Brutinel et al. 2008; Li and Demple 1994; Martin et al. 1996). Second, the extended size of the DNAse I footprints (~45 bp) of ExsA bound to several T3SS promoters is also larger than one would expect for a single binding site (Brutinel et al. 2008). Third, whereas mutations in the adenine-rich region of the P_{exoT} promoter disrupt formation of complex 2 but have little effect on complex 1 formation, mutations introduced near the -35 site impair formation of both complex 1 and 2 (Brutinel et al. 2008). Mutagenesis studies indicate that ExsA must be bound to both binding sites on the P_{exoT} promoter in order to activate transcription (Brutinel et al. 2008). Based on these data I propose a monomer assembly pathway in which the monomer bound at site 1 facilitates binding of a second ExsA monomer to the upstream site 2. Although the Hill plot data from an earlier study does not support cooperative binding interactions at the P_{exoT} and P_{exsD} promoters (Brutinel et al. 2008; Brutinel et al. 2009b), the following lines of evidence suggest cooperative interactions occur at these promoters: (i) the afore-mentioned promoter mapping and mutagenesis studies indicating ExsA bound to site 1 is required for binding to site 2; (ii) DNAse I footprints show no evidence of ExsA preferentially binding sites 1 or 2, suggesting occupation of site 1 causes immediate occupation of site 2; and (iii) occupation of site 1 by ExsA cooperatively enhances occupation of site 2 at the P_{exsC} promoter (Brutinel et al. 2008; Brutinel et al. 2009b).

In addition to characterizing the stoichiometry of ExsA I provide the first evidence that ExsD inhibits ExsA DNA-binding activity *in vivo*. ExsD is only one of two described anti-activators that targets a member of the AraC family of transcriptional

activators, the other being OspD1 from *Shigella flexneri* (Parsot et al. 2005). Recent studies in my lab indicate that ExsD may also function by disrupting ExsA self-association (Brutinel et al. 2009a). Although it has been shown that ExsD binds to the amino-terminal domain of ExsA and that the amino terminal domain is required for the self-association and cooperative binding properties, it is unclear whether this phenomenon is physiologically relevant (Brutinel et al. 2009b). I favor a model in which the primary function of ExsD is to antagonize the DNA-binding activity of ExsA. Data in support of this hypothesis include the observation that ExsD antagonizes ExsA shift complex 1 suggesting it can do so independent of preventing self-association. Future studies may elucidate whether ExsD functions by one or more of these mechanisms.

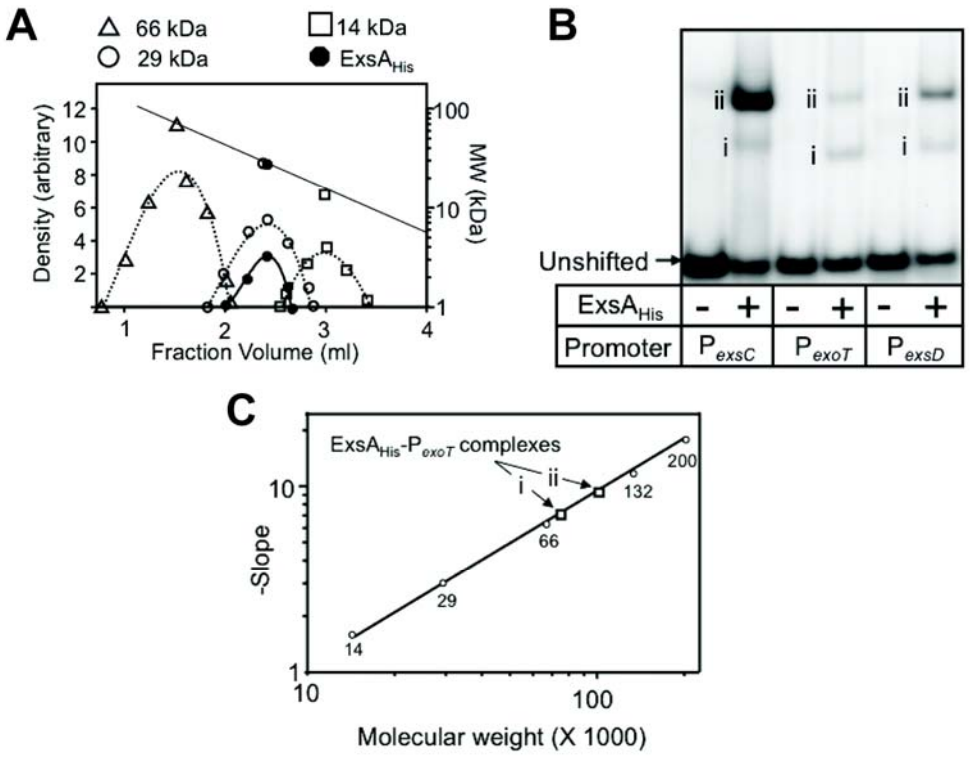

FIGURE 4. Stoichiometry of ExsA. A**.** Purified $ExsA_{His}$ is monomeric in solution. Molecular weight standards (66 kDa, 29 kDa, and 14 kDa] and $ExsA_{His}$ were applied to a 4 ml 5–20% glycerol gradient and centrifuged for 27 h. Fractions (200 µl, indicated on the x-axis) were subjected to SDS-PAGE and proteins were detected by silver staining and quantified by densitometry (arbitrary units, indicated on the left y-axis) to determine the elution peak. A standard curve was generated by plotting the elution peak of each standard (x-axis) against its molecular weight (MW) (indicated on the right y-axis). The empirically determined molecular weight of $ExsA_{His}$ (27.9 kDa) based on the standard curve is comparable to the molecular weight (34.1 kDa) predicted from the amino acid sequence. B. EMSA using 50 bp probes containing the ExsA binding site from the P_{exsC}, P_{exoT} and P_{exsD} promoters. Shift products 1 and 2 are indicated. C. Representative experiment demonstrating the method for calculating the number of $ExsA_{His}$ molecules bound to P_{exoT} shift products 1 and 2. A standard curve (Ferguson Plot) was generated by plotting the gradient (-Slope, on the y-axis) of each calibration curve against the molecular weight of protein standards (14, 29, 66, 132 and 200 kDa, on the x-axis). The molecular weights of $ExsA_{His}$–DNA complexes 1 (74.3 kDa) and 2 (100.8 kDa) were determined from the standard curve as indicated and the molecular weight of the DNA probe (33 kDa) was then subtracted to yield the molecular weight of the protein bound to shift products 1 (41.3 kDa) and 2 (67.8 kDa).

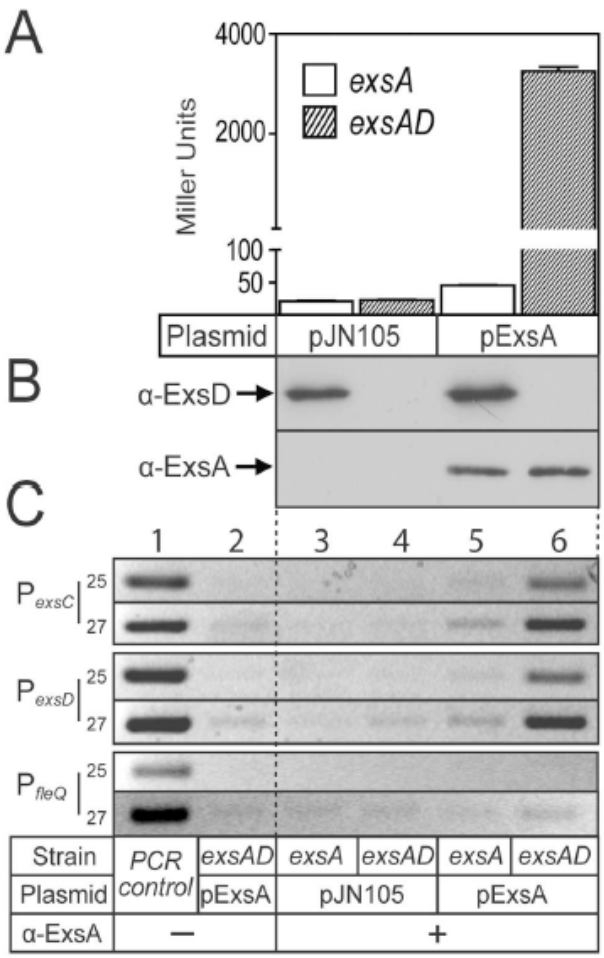

FIGURE 5. ExsD inhibits the DNA-binding activity of ExsA *in vivo*. (A - B) An *exsA* mutant or an *exsA*,*exsD* double mutant carrying the $P_{exsD\text{-}lacZ}$ reporter was transformed with a vector control (pJN105) or an expression plasmid (p2UY95, labeled pExsA in the Figure) that constitutively expresses low levels of ExsA. The resulting strains were grown under non-inducing conditions for T3SS gene expression and assayed for β-galactosidase activity (A) or protein expression levels (B) by performing immunoblots of whole cells lysates using the indicated antibodies. The reported values are the average of three independent experiments and error bars indicate the standard error of the mean. (C) Chromatin immunoprecipitation (ChIP) assays performed in the presence or absence of ExsD. Cells were treated with formaldehyde to crosslink ExsA to the DNA and processed for ChIP assays using polyclonal anti-ExsA antibody. The immunoprecipitate was then used in a PCR reaction with primers designed to amplify 200 bp regions of the P_{exsC}, P_{exsD}, and P_{fleQ} promoters. The PCR reactions were programmed to run for 25 or 27 extension cycles as indicated. The resulting PCR products were separated on an agarose gel and stained with ethidium bromide. *P. aeruginosa* chromosomal DNA was used as a positive control (lane 1) for the PCR and reactions lacking antibody served as a negative control (lane 2) for chromosomal contamination.

CHAPTER III

MECHANISM OF TRANSCRIPTIONAL ACTIVATION BY PSEUDOMONAS AERUGINOSA EXSA

Introduction

The primary regulator of T3SS gene expression in *Pseudomonas aeruginosa* is ExsA (Frank and Iglewski 1991; Yahr and Frank 1994; Yahr et al. 1995). ExsA controls T3SS gene expression by directly binding to and activating transcription from all T3SS promoters (Brutinel et al. 2008; Hovey and Frank 1995). Whereas the DNA-binding properties of ExsA have been characterized through genetic and biochemical studies (Brutinel et al. 2008; Brutinel et al. 2009b; Hovey and Frank 1995), the mechanism of transcriptional activation is not known. Transcriptional start sites for several ExsA-dependent promoters have been mapped by primer extension (Yahr and Frank 1994; Yahr and Wolfgang 2006). The transcriptional start sites for the P_{exsD}, P_{exoS}, and P_{orf1} promoters are favorably positioned downstream from near-consensus -10 (TATAAT) and -35 (TTGACA) recognition hexamers typical of σ^{70}-dependent promoters in both *E. coli* and *P. aeruginosa* (Dominquez-Cuevas 2004; Hawley and McClure 1983). Atypical, however, is the apparent increase in spacing (21-22 bp) between the -10 and -35 elements of ExsA-dependent promoters when compared to the optimal spacing of 17 bp for typical σ^{70}-dependent promoters (Brutinel et al. 2008). Whether these near-consensus promoter sequences of ExsA-dependent promoters truly serve as recognition hexamers for RNAP-σ^{70} is not known.

Transcriptional activators generally function by recruiting RNAP to non-standard promoters and/or facilitating isomerization to an open complex. ExsA is an AraC-family transcriptional activator of which the known activation mechanisms include both the recruitment of RNAP and isomerization from a closed to an open complex (Gallegos et al. 1997). Despite containing DNA-binding determinants very similar to other AraC-like proteins, ExsA binds promoters that appear to more closely resemble that activated by

Spo0A sporulation activator from *Bacillus sp.* Spo0A binds sites that completely overlap the -35 sequence and activates transcription from promoters containing near-consensus recognition hexamers but suboptimal promoter spacing (Seredick and Spiegelman 2004; Strauch et al. 1990). Recent data has suggested that Spo0A promotes formation of a second closed complex by repositioning prebound RNAP (Kumar and Moran 2008). Whether ExsA functions by a similar mechanism is not known.

In this chapter I characterize the mechanism of transcriptional activation by ExsA. Genetic data demonstrate that the putative-10 and -35 regions of T3SS promoters are poor RNAP recognition hexamers and that ExsA does not simply overcome a promoter spacing limitation. Biochemical data demonstrate that ExsA primarily facilitates transcription originating ~10 nucleotides downstream of -10 hexamers by the recruitment of RNAP and only modestly affects isomerization to an open complex.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are summarized in Table 1. *Escherichia coli* strains were maintained on L-agar plates containing the following antibiotics as necessary (gentamicin [15 μg ml^{-1}], ampicillin [50 or 100 μg ml^{-1}], tetracycline [10 μg ml^{-1}], kanamycin [50 μg ml^{-1}], spectinomycin [50 μg ml^{-1}]). *P. aeruginosa* strains were maintained on Vogel Bonner minimal medium (Vogel and Bonner 1956) with antibiotics as indicated (gentamicin [100 μg ml^{-1}], carbenicillin [300 μg ml^{-1}], tetracycline [50 μg ml^{-1}]). To assay for T3SS gene expression *P. aeruginosa* strains were grown with vigorous aeration at 30°C in TSB supplemented with 100 mM monosodium glutamate, 1% glycerol, and 2 mM EGTA as indicated. β-galactosidase assays were performed as previously described and the reported values are the average of at least three independent experiments (Dasgupta et al. 2004).

Plasmid construction and promoter mutagenesis

The primer sequences used to generate PCR products and the vectors into which each product was cloned are provided in Tables 2 and 3, respectively. The P_{tacI} constructs were generated by cloning annealed complementary oligonucleotides with KpnI/HindIII ends into mini-CTX-lacZ. Point mutations in the P_{tacI} promoter were introduced by Quikchange site-directed mutagenesis (Stratagene). To limit β-galactosidase toxicity, *E. coli* subcloning strains were transformed with the $LacI^q$-overexpressing plasmid pMS421 (Grana et al. 1988).

Purification of ExsA and RNAP

$ExsA_{His}$ was purified by metal-affinity chromatography and shown to possess DNA binding activity by EMSA as previously described (Brutinel et al. 2008). RNAP was purified from *P. aeruginosa* strain AK1012 (lacking expression of LPS O-antigen) as previously described (Burgess and Jendrisak 1975) with the following modifications. Overnight cultures were diluted to an OD_{600} of 0.1 into 5 L TSB and grown with shaking at 37˚C. At an OD_{600} of 1.0, cells were harvested by centrifugation (10 min, 6000 x g, 4˚C), washed with 500 ml of 0.85% NaCl, collected by centrifugation, and resuspended in 60 mls purification buffer (20 mM Tris-HCl [pH 8.0], 0.05 mM EDTA, 1.7 mM PMSF, 0.3 mM DTT, and 5% glycerol) containing 0.1 M NaCl. Cells were lysed via passage through a French pressure cell and unbroken cells were removed by centrifugation (30 min, 35,000 x g, 4˚C). Poly-ethyleneimine ([0.5% w/v final concentration] Sigma) was added to the soluble fraction and incubated at 4°C for 30 min. with constant stirring. The precipitate was collected by centrifugation (30 min, 35,000 x g, 4˚C) and washed with purification buffer containing 0.25 M NaCl using a Dounce-homogenizer. Following centrifugation RNAP was extracted with purification buffer containing 0.8 M NaCl. Insoluble material was removed by centrifugation and solid ammonium sulfate (30% final concentration) was added to the soluble fraction and

incubated at 4°C for 2 hours with constant stirring. Insoluble material was removed by centrifugation and ammonium sulfate (60% final concentration) was added to the soluble fraction and allowed to precipitate as above. The precipitate was collected by centrifugation, homogenized in 11 ml of purification buffer containing 0.1 M NaCl, and dialyzed for 18 hours at 4°C against 2 L purification buffer containing 0.1 M NaCl. Prior to heparin column chromatography the material was subjected to ultracentrifugation (100,000 x g for 30 min at 4°C) to ensure solubility. Soluble material was loaded onto a 5 ml heparin column, developed with a linear elution gradient (0.1 to 1 M NaCl), and peak fractions (based on polymerase activity) were pooled. The heparin column was repeated a second time followed by a final purification using superdex-300 gel filtration chromatography. Purified polymerase was stored at -20°C in purification buffer containing 0.1 M NaCl and 50% glycerol. The specific activity of RNAP was determined as described previously (Allan and Kropinski 1987). One unit of RNAP activity is defined as the amount of enzyme required to incorporate 1 pMol of UMP into acid-precipitable material in 20 min. Protein samples were denatured in SDS-PAGE sample buffer and electrophoresed on 15% acrylamide denaturing gels. Gels were analyzed by Coomassie blue and silver-staining methods.

Transcription templates

Supercoiled plasmid templates containing the P_{exsD} promoter fused to the *rpoC* terminator were generated and purified as described previously (Brutinel et al. 2009b). Supercoiled minicircle templates were created by cloning PCR-generated P_{exsC} and P_{exsD} promoters (nucleotides -238 to +192 relative to the transcriptional start site) as SacI/KpnI fragments into pSA508 (Choy and Adhya 1993). The resulting plasmids add 29 additional bases upstream of the *rpoC* transcriptional terminator to generate 221 base transcripts from each promoter. Minicircle purification was as described previously (Choy and Adhya 1993) with the following modifications: T3SS promoter-containing

pSA508 derivatives were introduced by transformation into *E. coli* strain SA1751 [λint+ xis439 cI857 (cro-chlA)ΔH1]. Transformants were grown in 800 mls of LB containing ampicillin (50 μg ml^{-1}) to an OD_{600} of 0.8 at 30°C, heat-shocked at 42° for 15 min, returned to 30°C in an ice-water bath, and grown at 30°C for an additional 30 min. Cells were harvested by centrifugation and plasmid DNA was isolated with the Fast-Ion Plasmid Maxi kit (IBI Scientific, Peosta, IA). Transcription templates were subjected to agarose gel electrophoresis and visualized by methylene blue staining. Supercoiled DNA was excised, gel purified, and analytical samples were examined by agarose gel electrophoresis to confirm that the preparations were largely free of nicked template.

in vitro transcription assays

Single-round transcription assays (20 μL final volume) were performed by incubating $ExsA_{His}$ (35 nM) with supercoiled transcription templates (2 nM) at 25°C in 1x transcription buffer (40 nM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 0.01% Tween-20, and 1 mM DTT) containing the initiating nucleotides ATP or GTP (0.75 mM) for the P_{exsD} and P_{exsC} promoters, respectively. After 10 min 25 nM *E. coli* RNAP holoenzyme (Epicentre, Madison, WI) or *P. aeruginosa* RNAP holoenzyme was added and open complexes were allowed to form for 5 min at 30°C. Elongation was allowed to proceed by the addition of the remaining unlabeled nucleotides (0.75 mM each including 5 μCi [α^{32}P-CTP]) in 1x transcription buffer containing heparin (50 μg ml^{-1} final concentration). Reactions were stopped after 10 min at 30°C by the addition of stop buffer (20 μl, [98% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol]). Samples were heated to 95°C for 5 min. and electrophoresed immediately on 5% denaturing urea polyacrylamide gels.

Transcription start site identification

RNA was isolated from mid-log phase (OD_{600} 0.8) bacterial cells using RNAprotect reagent and an RNA mini isolation kit (Qiagen, Valencia, CA). Purified

mRNA (200 ng) and gene-specific, antisense primers (positioned ~500 bases downstream of the translational start sites) were used in reverse transcription reactions to generate cDNA for the P_{exsD} and P_{exsC} promoters with the SuperScript III First-Strand Synthesis System (Invitrogen). Reverse transcriptase reactions were allowed to proceed at 55°C for 30 min. and terminated by incubation at 70°C for 15 min. cDNA was purified using the MinElute PCR purification kit (Qiagen). The 5' ends of the resulting cDNAs were identified using the PCR-based method of 5' RACE (Scotto-Lavino et al. 2006); ten independent clones for each promoter were sequenced to confirm the start site.

Abortive initiation assays

The steady-state properties of the abortive initiation assay have been described previously (McClure 1980). Abortive initiation assays were performed with P_{exsD} or P_{exsC} supercoiled minicircle templates (2 nM) in the presence and absence of $ExsA_{His}$ (35 nM) in 1x transcription buffer. The substrates for the abortive initiation reactions were as follows: for the P_{exsD} promoter, 1 mM ATP, 1 mM UTP, and 0.33 μCi [α-^{32}P] UTP to form pppApApApUpU and pppApApUpU; for the P_{exsC} promoter, 1 mM GTP, 1 mM CTP, 1 mM UTP, and 0.33 μCi [α-^{32}P] UTP to form pppGpCpUpUpU and pppCpUpUpU. Reactions including $ExsA_{His}$ were incubated in 1x transcription buffer with template DNA for 10 min at 25°C prior to nucleotide/RNAP addition. To measure the lag time to open complex formation (τ) two separate reactions were performed for each of the seven RNAP concentrations tested (25, 28.6, 33.3, 40, 50, 66.7, 100 nM). The first set of reactions contained template, $ExsA_{His}$ (35 nM as indicated), and substrate nucleotides in 1x transcription buffer and was initiated by the addition of RNAP. The second set of reactions contained template, $ExsA_{His}$ (35 nM), and RNAP in 1x transcription buffer and were preincubated for 60 min at 30°C. Transcription was initiated by the addition of nucleotides. Both sets of reactions were allowed to proceed at 30°C and samples were taken at various time points (1 – 120 min), terminated with stop

buffer, and electrophoresed on denaturing 25% polyacrylamide gels. Gels were subjected to phosphorimaging and densitometry. The rate of abortive synthesis was calculated for reactions initiated with nucleotides by linear regression analysis (least-squares). Curves plotted for reactions initiated by RNAP addition were analyzed by drawing a line through the curve but parallel to the reaction initiated by nucleotide addition. τ_{obs} was also obtained for these curves by linear regression analysis by solely using values 3 times greater than the initial estimate for τ_{obs} as described previously (McClure 1980) yielding results comparable to the first method. GraphPad Prism (GraphPad Software, Inc) was used to plot abortive initiation data and evaluate τ_{obs}.

Potassium permanganate footprinting

Supercoiled minicircles carrying P_{exsC} or P_{exsD} were used as templates for the potassium permanganate footprinting reactions (Zhang et al. 1996). Reactions containing $ExsA_{His}$ were incubated for 10 min at 25°C to allow DNA-binding in 1x potassium permanganate reaction buffer (40 mM Tris-HCl pH [7.5], 25 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Tween 20, and 100 ng ml^{-1} BSA). RNAP was added to the indicated concentrations and open complex formation was allowed to proceed for 3 min at 25°C. Potassium permanganate (Sigma-Aldrich, St. Louis, MO) was immediately added (10 mM final concentration) and allowed to modify DNA for 1 min at 25°C. Reactions were stopped with termination buffer (0.5 M Potassium Acetate [pH 7.0], 1.5 M 2-mercaptoethanol, 5 mM EDTA) and purified with a PCR column purification kit (IBI Scientific) and eluted into 30 µl elution buffer (10 mM Tris-HCl [pH 8.5]). Modification by potassium permanganate was detected by primer extension. Primers (50 pmol) were end-labeled with 50 µCi [γ-^{32}P]-ATP (Perkin Elmer) and 10 U polynucleotide kinase (New England Biolabs, Ipswich, MA) as instructed by the manufacturer. Primer extension reactions (10 µl) were performed on the potassium permanganate-modified plasmids with 1 mM dNTPs, 0.5 µl end-labeled primer, 1.25 units sequencing grade Taq

DNA polymerase (Promega, Madison, WI) in 1x sequencing buffer (50 mM Tris-HCl [pH 9.0], 2 mM $MgCl_2$) under the following conditions: 1 cycle of 2 min at 94°C, 30 cycles of 0.5 min at 94°C, 0.5 min at 55°C, and 1 min at 72°C. Stop buffer (10 µl) was immediately added to each reaction to terminate DNA synthesis. Dideoxy sequencing reactions for A and T were generated using the same thermocycling program and the following reaction components: 5 fmol P_{exsC}/P_{exsD} minicircle, 0.5 pmol labeled primer, 1.25 units sequencing grade Taq DNA polymerase, 500 µM termination nucleotide (ddATP or ddTTP), and 20 µM elongation nucleotides (dCTP, 7-deaza-dGTP, dATP, dTTP). Sequencing reactions were terminated with an equal volume of stop buffer and run alongside primer extension reactions on denaturing 6% polyacrylamide gels. Gels were dried and visualized by phosphorimaging and analysis performed with MultiGauge v3.0 software (Fujifilm).

Results

Near-consensus -35 and -10 sequences at T3SS promoters are poor RNA polymerase recognition hexamers

Each of the ExsA-dependent promoters used in this study (P_{exsC} and P_{exsD}) contain hexamers that match the consensus -10 and -35 regions of σ^{70}-dependent promoters at 4/6 or 5/6 of the nucleotide positions (Fig. 3). Nevertheless, it is not known whether the -10 and/or -35 regions are truly recognized by RNAP-σ^{70}. One reason for this uncertainty is that the -10 and -35 regions of ExsA-dependent promoters are separated by 21 or 22 bp whereas optimal spacing for σ^{70}-dependent promoters is 17 bp (Allan and Kropinski 1987; Harley and Reynolds 1987; Hawley and McClure 1983; Warne and deHaseth 1993). One interpretation of these data is that the increased spacing between the -10 and -35 regions prevents transcription in the absence of ExsA and that ExsA functions by overcoming the increased spacing. If this were true, I would predict that reducing the spacing of ExsA-dependent promoters to 17 bp would reconstitute a functional σ^{70}-

dependent promoter and eliminate the requirement for ExsA. To test this hypothesis, Keith Brady in our lab created a series of *lacZ* transcriptional reporters in which the spacing between the -10 and -35 regions of P_{exoT} and P_{exsD} promoters was decreased (16 bp) from the native spacing of 21 bp. Whereas expression of the native P_{exoT} and P_{exsD} reporters is ExsA-dependent, reporter derivatives in which the spacing was decreased to 16 bp were not utilized efficiently in either the absence or presence of ExsA. The lack of activity for the P_{exoT} and P_{exsD} reporters was somewhat surprising as I expected that reducing the spacing to the σ^{70}-dependent consensus of 16 bp might result in constitutive, ExsA-independent expression. To test the hypothesis that sequences outside the -35 and -10 regions might be affecting promoter activity, hybrid reporters were constructed by replacing the -10 and/or -35 regions of the $P_{tacI\text{-}lacZ}$ reporter with the corresponding elements from the P_{exsD} promoter (Fig. 6A). Whereas the native $P_{tacI\text{-}lacZ}$ reporter had significant activity in the absence of *exsA*, the hybrid $P_{tacI\text{-}lacZ}$ reporters containing the -10 and/or -35 regions from the P_{exsD} promoter demonstrated a 7-14-fold reduction in activity (Fig. 6B). These combined data indicate that even when properly spaced and in the appropriate context, the putative -10 and -35 hexamers of P_{exsD} are suboptimal for σ^{70}-dependent transcription.

ExsA activates transcription at T3SS promoters *in vitro*

To examine the mechanism of ExsA-dependent transcriptional activation, I developed a single-round in vitro transcription assay using $ExsA_{His}$ purified from *E. coli* (Fig. 7A, lane 1) and RNAP-σ^{70} holoenzyme purified from *P. aeruginosa* (lane 2) or RNAP-σ^{70} holoenzyme from *E. coli* (obtained commercially). The specific activity of RNAP-σ^{70} isolated from *P. aeruginosa* was 3-4 fold lower than that of *E. coli* RNAP-σ^{70} (data not shown). The initial transcription template consisted of supercoiled plasmid DNA carrying the P_{exsD} promoter fused to a strong transcriptional terminator ($rpoC_{ter}$). The plasmid template was pre-incubated with $ExsA_{His}$, RNAP-σ^{70}, and the initiating

nucleotide for transcription (ATP, as determined below) for 15 min. Ribonucleotides (including [α-^{32}P]-CTP) were then added in the presence of heparin (to prevent RNAP-σ^{70} from reinitiating) and transcripts were allowed to elongate for 10 min. RNAP-σ^{70} from either *E. coli* or *P. aeruginosa* generated the expected terminated transcript of 261 nucleotides in an ExsA-dependent manner (Fig. 7B). Since the *E. coli* RNAP holoenzyme used for these studies is σ^{70}-saturated, and the *P. aeruginosa* RNAP holoenzyme isolated from log phase cells is presumed to be largely σ^{70}-saturated (Fig. 7A, lane 2), I conclude that ExsA-dependent promoters are σ^{70}-dependent.

ExsA-independent transcription was not observed at high RNAP concentrations (200 nM) and at incubation times as long as 2 h (data not shown). Because the transcription templates used in these experiments are self-replicating, supercoiled plasmids (~5 kb in length), I hypothesized that strong, constitutive plasmid promoters might out-compete the P_{exsD} or P_{exsC} promoters for RNAP occupancy in the absence of ExsA. A similar result was previously observed for the *gal* promoters in *E. coli* and addressed by constructing small supercoiled plasmid templates called minicircles (Choy and Adhya 1993). To generate P_{exsC} and P_{exsD} minicircles the promoters were cloned upstream of the *rpoC* transcriptional terminator in the parental vector pSA508 (3.4 kb). The minicircles excise in vivo from the parental plasmid as supercoiled plasmids through a temperature-dependent recombination event. The resulting P_{exsC} and P_{exsD} minicircles (~0.83 kb) consist solely of the cloned promoters fragments (0.43 kb), the *rpoC* transcriptional terminator, and residual plasmid sequences. Similar to the findings presented in Fig. 7B, the larger parental P_{exsC} or P_{exsD} plasmids were permissive for ExsA-dependent transcription while ExsA-independent transcripts were undetectable (Fig. 7C). Minicircle templates derived from the parental plasmids, however, supported both ExsA-dependent and -independent transcription although the amount of terminated transcript in the absence of ExsA was significantly reduced for both the P_{exsC} or P_{exsD} minicircle templates. Detection of ExsA-independent transcription from the minicircle

templates is consistent with the possibility that strong promoters on the parental plasmid out-compete the P_{exsC} and P_{exsD} promoters for RNAP-σ^{70} occupancy and suggests that RNAP-σ^{70} binds to T3SS promoters poorly in the absence of ExsA.

Transcription from the P_{exsC} and P_{exsD} promoters initiates 8 to 9 NT downstream of near-consensus Pribnow boxes

Transcriptional start sites for several ExsA-dependent promoters were previously mapped by primer extension ((Yahr and Frank 1994; Yahr et al. 1995). As expected of σ^{70}-dependent promoters, the P_{exsD}, P_{exoS}, and P_{orf1} promoters initiate transcription 7-9 bp downstream of the -10 Pribnow box (Fig. 8). The P_{exsC} start site, however, mapped to two adjacent nucleotides located ~50 bases upstream of the putative -10 region. To resolve this apparent discrepancy 5' rapid amplification of cDNA ends (RACE) and abortive initiation assays were used to reexamine the transcriptional start sites for the P_{exsC} and P_{exsD} promoters.

For the RACE assays mRNA was isolated from wild-type PA103 and an *exsA* mutant grown under inducing (+EGTA) conditions for T3SS gene expression. The mRNA was reverse transcribed into cDNA using gene specific primers (*exsC* or *exsD*) and cloned into a plasmid vector for sequencing. At least 10 clones were sequenced for each promoter/RNA sample. Consistent with the previous primer extension data, the 5' end of the *exsD* mRNA mapped to nucleotides located 7-9 bases downstream of the -10 region in both wild-type and the *exsA* mutant (Fig. 8). The 5' end of the *exsC* mRNA, however, mapped to two distinct regions located 50 bp upstream and 8-9 bp downstream of the -10 region. Whereas the position of the former site is consistent with the previous primer extension data, the location of the latter site is nearly identical to the P_{exsD}, P_{exoS}, and P_{orf1} promoters with respect to the putative -10/-35 regions (Fig. 8). Subsequent studies (described below) indicate that the P_{exsC} promoter initiates transcription at the second site.

An inherent limitation of 5' RACE analysis is that the exact starting nucleotide cannot always be determined. To more precisely map the start sites, I analyzed abortive transcription products. Abortive RNA synthesis is thought to occur at all promoters and results from RNAP that initiates transcription but fails to clear the promoter and randomly aborts transcription generally producing transcripts less than 20 nt (Hsu 2009). By starving an *in vitro* transcription reaction for one or more nucleotides, and incorporating specific radiolabeled nucleotides, the length of the abortive transcripts can reveal the exact transcriptional start site. The abortive initiation assays for the P_{exsC} promoter contained GTP, CTP and radiolabeled UTP. Under these conditions the putative start site located 50 bp upstream of the -10 region would generate a 10-12 nt transcript before terminating at an adenine while the start site located 8-9 bp downstream of the -10 region would generate a 4-5 nt product. In the presence of $ExsA_{His}$ two abortive transcripts were generated (Fig. 9A, lane 4). The shorter product was nearly identical in size to the abortive transcript (4 nt, pppAAUU) from the well-characterized P_{I8UV5} promoter when provided only ATP and radiolabled UTP (data not shown). These data are consistent with the aborted P_{exsC} transcripts representing pppCUUU (4 nt) and pppGCUUU (5 nt) and indicate that P_{exsC} transcription initiates from the G and C nucleotides located 8 and 9 nucleotides downstream of the -10 region (Fig. 8). The fact that the same products were not detected when the transcription reactions lacked UTP further supports this conclusion (Fig. 9A, lanes 1-2). The pppCUUU and pppGCUUU abortive transcripts were also generated in the absence of $ExsA_{His}$ albeit to a much lesser extent and only after an extended incubation period (Fig. 9A, lanes 6). The failure to detect the 10-12 nt product from the site located 50 bp upstream of the -10 region suggests that this start site mapped by primer extension and RACE may be an artifact.

Abortive initiation assays for the P_{exsD} promoter that were limited to ATP and radiolabeled UTP generated two ExsA-dependent products (Fig. 9B, lanes 1-2; pppAAUU and pppAAAUU) the shorter of which is identical in size to the aborted

transcript generated by the P_{lacUV5} promoter (data not shown). This finding is consistent with transcription initiating at the adenine nucleotides located 7-8 bp downstream of the -10 region (Fig. 8). To confirm this finding the assays were repeated in the presence of ATP, UTP, and radiolabeled GTP where only the terminal nucleotide would be labeled. As expected the resulting abortive products (pppAAUUG and pppAAAUUG) were 1 nt longer (Fig. 9B, lanes 2 vs 4) and ExsA-dependent (lane 3 vs 4).

The ability to measure ExsA-independent abortive products can provide information regarding the mechanism of transcriptional activation by ExsA (discussed below). Unfortunately, extended incubation of RNAP-σ^{70} with the P_{exsD} promoter in the absence of ExsA resulted in the appearance of background bands which raised questions as to whether the abortive transcripts were truly arising from the P_{exsD} promoter. To determine whether the aborted products were indeed derived from P_{exsD}, a mutant promoter ($P_{exsD+GG}$) was generated in which two additional guanine nucleotides were added between nucleotides +6 and +7. When compared to the wild-type P_{exsD} promoter, the products generated from the $P_{exsD+GG}$ promoter (pppAAUUGGG and pppAAAUUGGG) were 2 bases greater in length (Fig. 9C, lane 2 vs 3) and, importantly, were clearly detected in absence of $ExsA_{His}$ if the incubation time was extended to 240 min.

ExsA promotes an early step in transcription initiation

The most common rate-limiting steps during transcriptional initiation are closed and open complex formation (Hsu 2009). Closed complexes result from the binding of RNAP to the promoter; transition of the closed complex to an open complex involves unwinding of the -10 region of the promoter to single stranded DNA (ssDNA). Open complex formation provides RNAP access to the template strand and is required for subsequent elongation of the transcript (Hsu 2009). The open complex can be detected with the DNA modification reagent potassium permanganate which preferentially

oxidizes pyrimidine bases in ssDNA (Akman et al. 1990). To determine whether ExsA is required for the initial steps in initiation of P_{exsC} and P_{exsD} transcription, the minicircles carrying these promoters were incubated with $ExsA_{His}$ and/or RNAP-σ^{70} and then subjected to potassium permanganate modification. Modified minicircles were then used as templates in primer extension reactions with DNA polymerase and radiolabeled primers. DNA polymerase terminates transcription at bases oxidized by potassium permanganate, and the resulting enrichment of the terminated fragments indicates the regions of ssDNA. Incubation of the P_{exsC} and P_{exsD} minicircles with both $ExsA_{His}$ and a low concentration of RNAP-σ^{70} (1.5 nM) resulted in strong permanganate modification of the -10 regions within each promoter (Fig. 10A-B). Weaker modification of the same regions could also be seen with RNAP-σ^{70} alone. These findings demonstrate that RNAP-σ^{70} can bind to the P_{exsD} and P_{exsC} promoters independent of ExsA and that ExsA facilitates transcriptional initiation by enhancing recruitment of RNAP-σ^{70} to the promoter and/or promoting isomerization to an open complex.

ExsA facilitates a rate-limiting step prior to open complex formation at the P_{exsC} and P_{exsD} promoters

Potassium permanganate footprints indicate that ExsA functions at the level of transcription initiation but cannot discern whether ExsA recruits RNAP and/or promotes isomerization to the open complex. The abortive initiation assay, however, can be used to estimate the isomerization rate constant and overall reaction rate for a given promoter by measuring the lag time to steady-state synthesis of abortive transcripts. To analyze the kinetics of transcription initiation at the P_{exsC} and P_{exsD} promoters, abortive transcripts were generated at varying concentrations of RNAP-σ^{70} in the absence or presence of saturating concentrations of $ExsA_{His}$ and the lag time (τ_{obs}) to open complex formation was recorded for each RNAP-σ^{70} concentration on a Tau (τ) plot. The resulting τ plots for both the P_{exsC} and P_{exsD} promoters shows an inverse linear relationship between the

lag time to open complex formation and RNAP-σ^{70} concentration (Fig. 11A-B). The overall reaction rates for the P_{exsC} and P_{exsD} promoters increased 13- and 11-fold in the presence of $ExsA_{His}$, respectively (Table 4). In both cases, the stimulatory effect of $ExsA_{His}$ resulted primarily from an increase in the equilibrium binding constant for RNAP-σ^{70} (5-8 fold) although there was also a modest increase in the isomerization rate constant (~2-fold). These data indicate that the primary mechanism by which ExsA stimulates transcription is through recruitment of RNAP-σ^{70} to the P_{exsC} and P_{exsD} promoters prior to open complex formation.

Discussion

In the present study I find that purified $ExsA_{His}$ and RNAP-σ^{70} isolated from either *P. aeruginosa* or *E. coli* is sufficient to activate transcription from T3SS promoters *in vitro*. This is consistent with previous studies demonstrating that T3SS genes are expressed maximally during exponential growth phase (Ha and Jin 2001; Hogardt et al. 2004; Shen et al. 2008) and the fact that the -10 and -35 regions of T3SS promoters are similar to the σ^{70} consensus sequences from *E. coli* (Grana et al. 1988; Hawley and McClure 1983) and *P. aeruginosa* (Dominquez-Cuevas 2004). In addition, potassium permanganate footprinting assays reveal RNAP-σ^{70}-dependent unwinding of the P_{exsD} and P_{exsC} -10 regions, and this was greatly enhanced in the presence of $ExsA_{His}$. Based on these data I conclude that ExsA primarily utilizes RNAP-σ^{70} to activate T3SS gene expression. Our findings, however, do not preclude the possibility that alternative sigma factors might also be involved in ExsA-dependent gene expression.

5' RACE was used to map the general region of the P_{exsC} and P_{exsD} transcription start sites in vivo and abortive initiation products were used for more precise mapping in vitro. Although the P_{exsD} start site matched previously published primer extension data, two apparent start sites were observed for the P_{exsC} promoter. Whereas the first site is located upstream of the ExsA binding site, the second site is located downstream of the -

10 element (Fig. 8). I believe the latter site is the true P_{exsC} start site given its proximity to the ExsA-binding site and RNAP recognition elements, and its similarity to the positions of the P_{exsD}, P_{exoS}, and P_{orf1} start sites. The apparent upstream start site may result from transcriptional read-through from the upstream P_{pcrG} promoter. In this regard it is worth noting the poly(dA) and poly(dT) tracts located just downstream of the start site mapped by primer extension and 5' RACE (Fig. 8) and that both of those techniques rely upon reverse transcriptase (RT) extension from mRNA templates. RT is known to pause at sites of secondary structure and at poly(A) and poly(U) nucleotide runs (Harrison et al. 1998; Klarmann et al. 1993). I believe the apparent upstream start site represents pausing of RT that results from either the poly(A)/poly(U) sequence or secondary structure associated with a transcriptional terminator. In either case this finding suggests that transcriptional read-through from the upstream P_{pcrG} promoter, which is also ExsA-dependent, contributes to expression of the *exsCEBA* operon and could represent another point at which ExsA expression levels are regulated.

AraC activates transcription by enhancing the RNAP equilibrium binding and the open complex isomerization rate constants (Zhang et al. 1996). These activities have been attributed to protein-protein interactions between AraC and RNAP-σ^{70} (Martin and Rosner 2001). Given the similarities between ExsA and AraC with respect to the location of the activator binding site, I hypothesized that ExsA might activate transcription through similar mechanisms. Abortive initiation assays, however, indicate that $ExsA_{His}$ only marginally altered the isomerization rate constant but had a significant effect on the equilibrium binding constant (5-8 fold) at both the P_{exsC} and P_{exsD} promoters. These results indicate that ExsA primarily functions by enhancing recruitment of RNAP-σ^{70} to the promoter prior to open complex formation. The effect of $ExsA_{His}$ on transcriptional initiation *in vitro* (11-13 fold) is much lower than that observed *in vivo* (100-1000 fold) (McCaw et al. 2002). The most likely explanation for this discrepancy is that the level of ExsA-independent transcription seen *in vitro* is artificially elevated due

to the absence of competing promoters (Fig. 7D), a condition never seen *in vivo*. Other contributing factors include the inherent limitations of an *in vitro* assay, the use of minicircle transcription templates as a mimic for chromosomal DNA, and the absence of factors that may be required for maximal ExsA-dependent transcription. For example, ExsA may possess a co-activator as is seen for the AraC family members (VirF and MxiE) that regulate T3SS gene expression in *Salmonella typhimurium* and *Shigella flexneri*, respectively. Alternatively, some AraC-family members function together with catabolite activator protein (CAP) to regulate gene expression. It is interesting to note that the *P. aeruginosa* homolog of CAP (Vfr) is required for T3SS gene expression through an undetermined pathway (Wolfgang et al. 2003). Development of an *in vitro* transcription assay provides a means to test whether factors known to influence T3SS gene expression in *P. aeruginosa* do so by directly modulating ExsA-dependent activation.

ExsA-dependent promoters are unusual in that the putative -10 and -35 promoter elements are separated by 21 or 22 bp when compared to the 17 bp typical of σ^{70}-dependent promoters. The mechanism of Spo0A-dependent transcriptional activation in *Bacillus subtilis* closely parallels the ExsA situation where in both cases the -10 and -35 sites are separated by 21-22 bp and the activator binding sites overlap with and extend upstream of the -35 regions. These observations initially raised concerns as to whether the -35 region was an authentic determinant for binding of RNAP-σ^{A} (the equivalent of RNAP-σ^{70} in *B. subtilis*) to Spo0A-dependent promoters (York et al. 1992). The findings that reducing the spacing between the -10 and -35 regions of the *spoIIG* promoter to 17 bp results in Spo0A-independent transcription and that RNAP-σ^{A} footprints the -35 site independently of Spo0A (Bird et al. 1996), however, indicate that the -35 region is recognized by RNAP-σ^{A} and that Spo0A activates transcription by suppressing the -10 and -35 spacing constraint. The current model proposes that RNAP binds to the *spoIIG* promoter independently of Spo0A through low specificity interactions between RNAP-σ^{A}

and the -35 region. Spo0A binding then repositions RNAP-σ^A 4 bp downstream of the -35 region such that region 2 of σ^A can interact with the -10 region resulting in open complex formation (Kumar and Moran 2008).

While it is clear from permanganate footprinting experiments that the -10 regions of P_{exoT} and P_{exsD} isomerize to open complexes, the role of the putative -35 regions remains unclear. Permanganate footprints and abortive transcripts demonstrate that RNAP-σ^{70} is capable of binding to the P_{exoT} and P_{exsD} promoters in the absence of ExsA. In this regard ExsA-dependent promoters are similar to Spo0A-dependent promoters in that RNAP can bind independently of the activator. Binding of RNAP-σ^{70} to the P_{exoT} and P_{exsD} promoters, however, was only detected in the absence of competing promoters and may not reflect the *in vivo* situation. A notable difference between the ExsA and Spo0A systems is the effect of altered spacing between the -10 and -35. Based upon the Spo0A model I hypothesized that reducing the spacing between the -10 and -35 regions of ExsA-dependent promoters to 16 bp would result in ExsA-independent activity. Both the P_{exsD} and P_{exoT} promoters, however, lacked ExsA-independent expression at the optimal spacing of 16 bp (Vakulskas et al. 2009). This was particularly surprising for the P_{exsD} promoter because the -10 and -35 regions closely match the σ^{70} consensus. Even more striking was the finding that the -10 and -35 regions of P_{exsD} are poor substitutes for the corresponding elements of the P_{tacI} promoter (Fig. 6B). These findings suggest that the -10 and -35 regions of P_{exsD} function as poor recognition elements for RNAP-σ^{70} and support our conclusion that the primary role of ExsA is to facilitate recruitment of RNAP-σ^{70} to the promoter. Future experiments will focus on characterization of the ExsA-RNAP-σ^{70} interaction, the regions of σ^{70} that interact with T3SS promoters in both the presence and absence of ExsA, and whether the -35 region contributes to the binding of RNAP-σ^{70}.

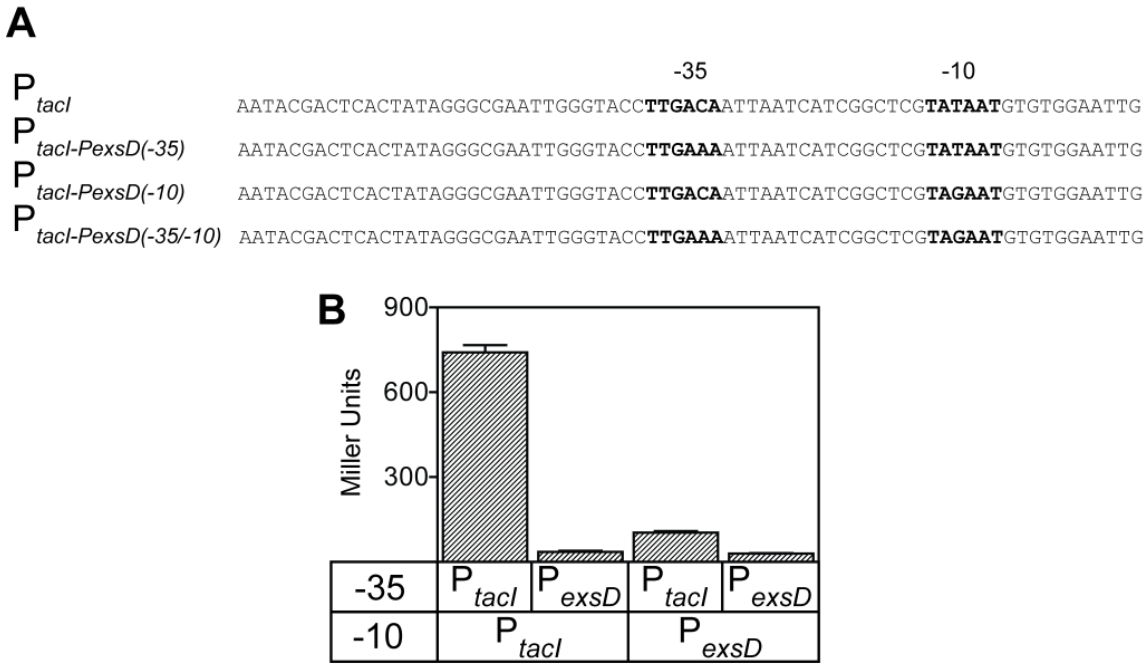

FIGURE 6. The role of the -10 and -35 regions in ExsA-independent expression. (A) Diagram of the native P_{exsD}, P_{tacI}, and hybrid promoters derived from both P_{exsD} and P_{tacI}. The P_{tacI}-$P_{exsD(-35)}$-lacZ, P_{tacI}-$P_{exsD(-10)}$-lacZ, and P_{tacI}-$P_{exsD(-35/-10)}$-lacZ reporters were constructed by replacing the -10 and/or -35 regions of P_{tacI} with the corresponding regions of P_{exsD}. The putative -10 and -35 regions are indicated in bold typeface. (B) Wild-type PA103 and *exsA*::Ω carrying the indicated reporters were cultured under T3SS inducing conditions and assayed for β-galactosidase activity. The reported values represent the average of three independent experiments and error bars indicate the standard error of the mean.

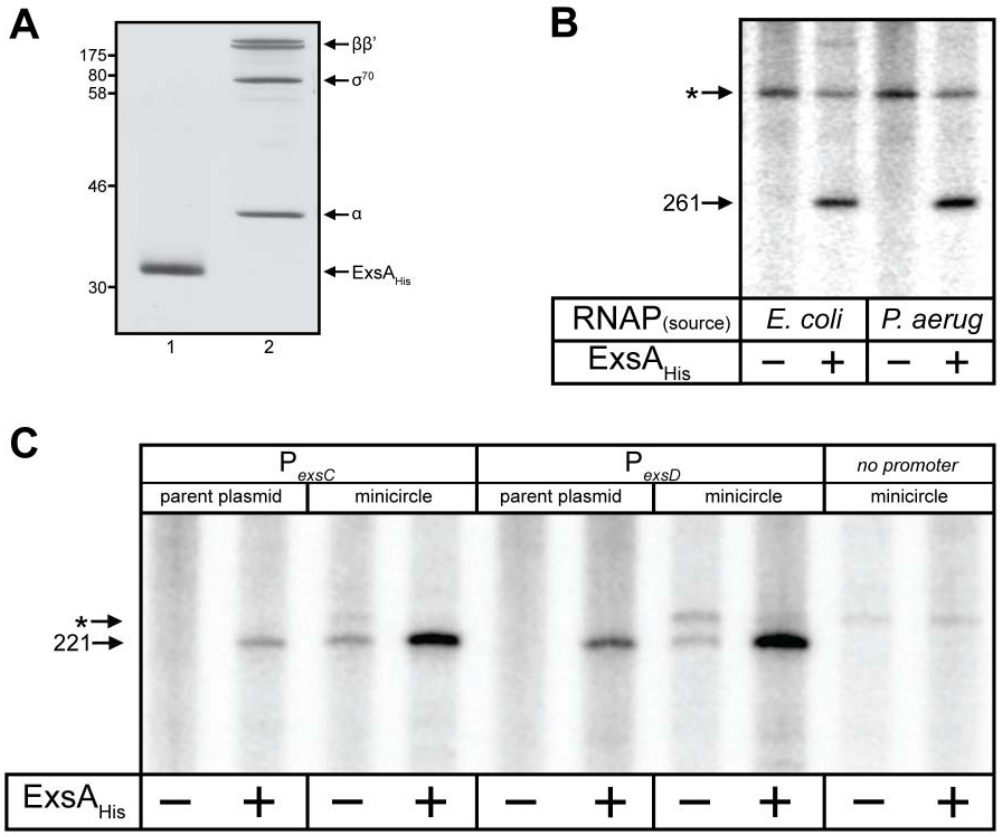

FIGURE 7. Purified $ExsA_{His}$ activates transcription *in vitro*. (A) Silver-stained gel of $ExsA_{His}$ purified from *E. coli* (lane 1) and σ^{70}-RNAP purified from *P. aeruginosa* (lane 2). Molecular mass standards (in kDa) are indicated on the left. (B) Single-round *in vitro* transcription assays. $ExsA_{His}$ (35 nM) was incubated with 2 nM supercoiled P_{exsD} promoter template (pOM90-P_{exsD}) at 25˚C in the presence of rATP. After 10 min RNAP from *E. coli* or *P. aeruginosa* (25 nM of each, normalized to the specific activity of *E. coli* RNAP) was added and the reaction was incubated for 5 min at 30°C. Heparin and the remaining ribonucleotides (including 5 μCi [α^{32}P-CTP]) were immediately added and the reaction was incubated for 10 min at 30°C. Reactions were terminated, and the resulting products were electrophoresed on a 5% polyacrylamide-urea gel, and subjected to phosphorimaging. The ExsA-dependent terminated transcript (261 nt) from the P_{exsD} promoter is indicated. A transcript originating from an undetermined plasmid promoter is indicated with an asterisk. (C) Single-round *in vitro* transcription assays were performed as above using the parental pSA508-P_{exsC}/pSA508-P_{exsD} plasmid templates or minicircles derived from the parental plasmids. The terminated transcript (221 nt) from these templates is indicated. A transcript originating from a weak plasmid promoter is indicated with an asterisk.

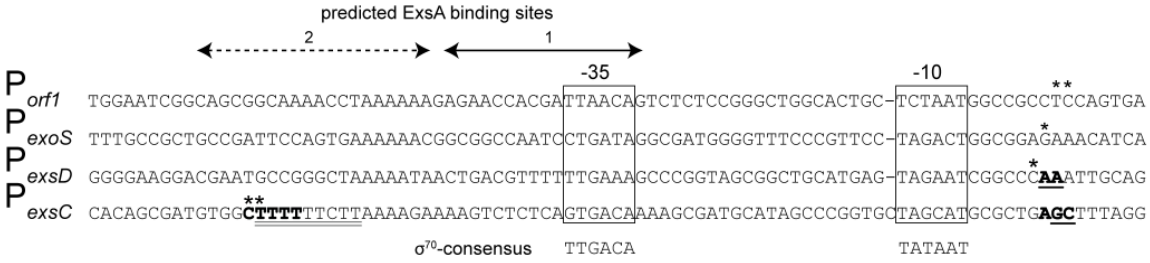

FIGURE 8. Diagram of the P_{orf1}, P_{exoS}, P_{exsD}, and P_{exsC} promoters. The putative -10 and -35 regions are boxed. Transcription start sites previously mapped by primer extension (53, 54) are indicated with an asterisk. The start sites mapped in this study by RACE analysis and by abortive initiation assays are in bold typeface or underlined, respectively. A putative transcriptional terminator from a transcript located upstream of the P_{exsC} promoter is indicated by the double underline.

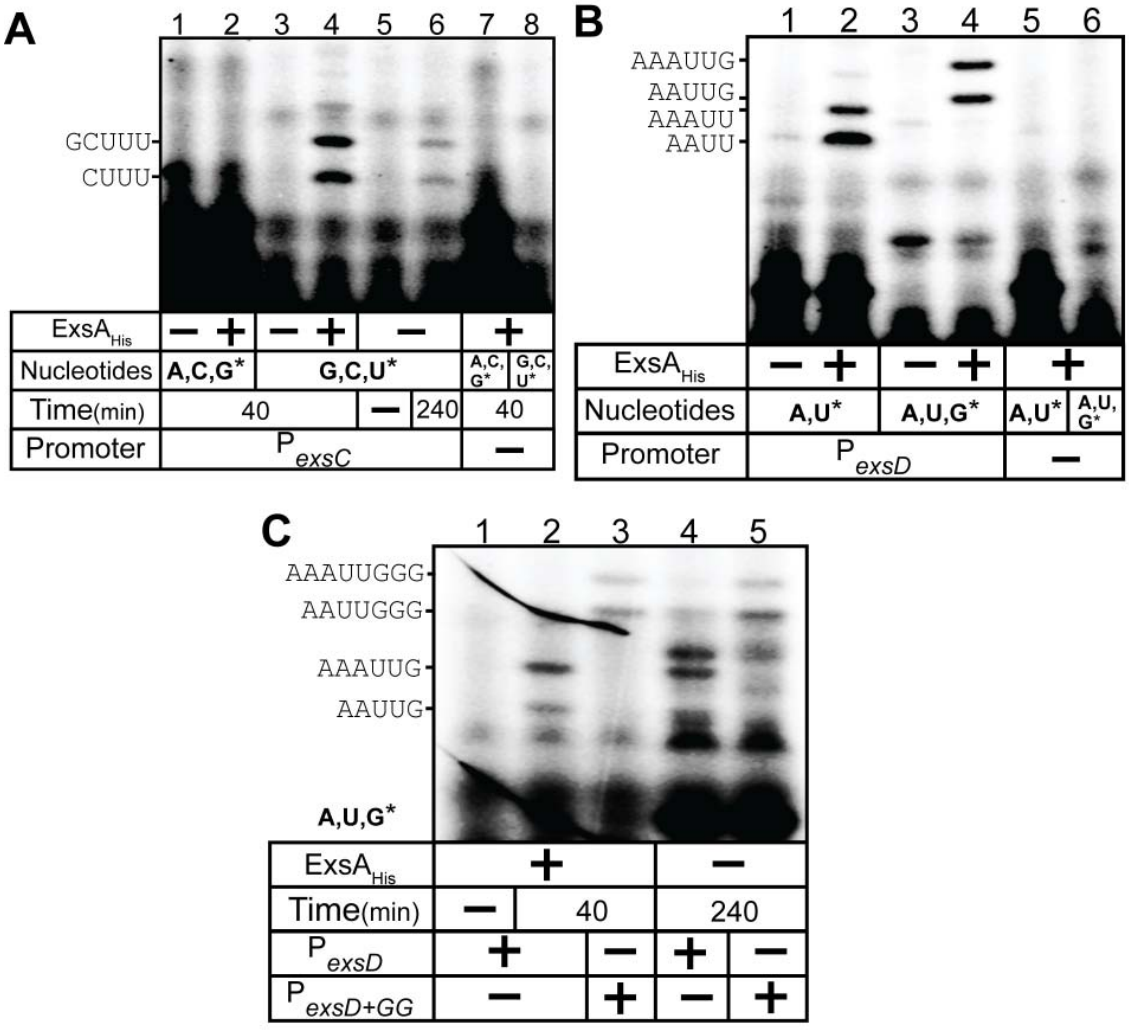

FIGURE 9. Abortive initiation assays for the P_{exsC} and P_{exsD} promoters. Reactions using the P_{exsC} (A) or P_{exsD} (B-C) minicircle templates were allowed to proceed for 40 min or 240 min, as indicated, with RNAP and substrate nucleotide sets (asterisk indicates labeled nucleotide) in the absence or presence of $ExsA_{His}$. Reactions were terminated and the products were electrophoresed through a 25% denaturing polyacrylamide gel and visualized by phosphorimaging. Control reactions using a supercoiled minicircle template lacking T3SS promoters were performed with each substrate nucleotide set in the presence of $ExsA_{His}$. (C) Abortive initiation assays with the P_{exsD} and modified $P_{exsD+GG}$ promoters in the absence or presence of $ExsA_{His}$. Abortive initiation reactions were allowed to proceed for 240 min in the presence of RNAP, unlabeled ATP/UTP, and labeled GTP. Control reactions in the presence of $ExsA_{His}$ were incubated for 40 min.

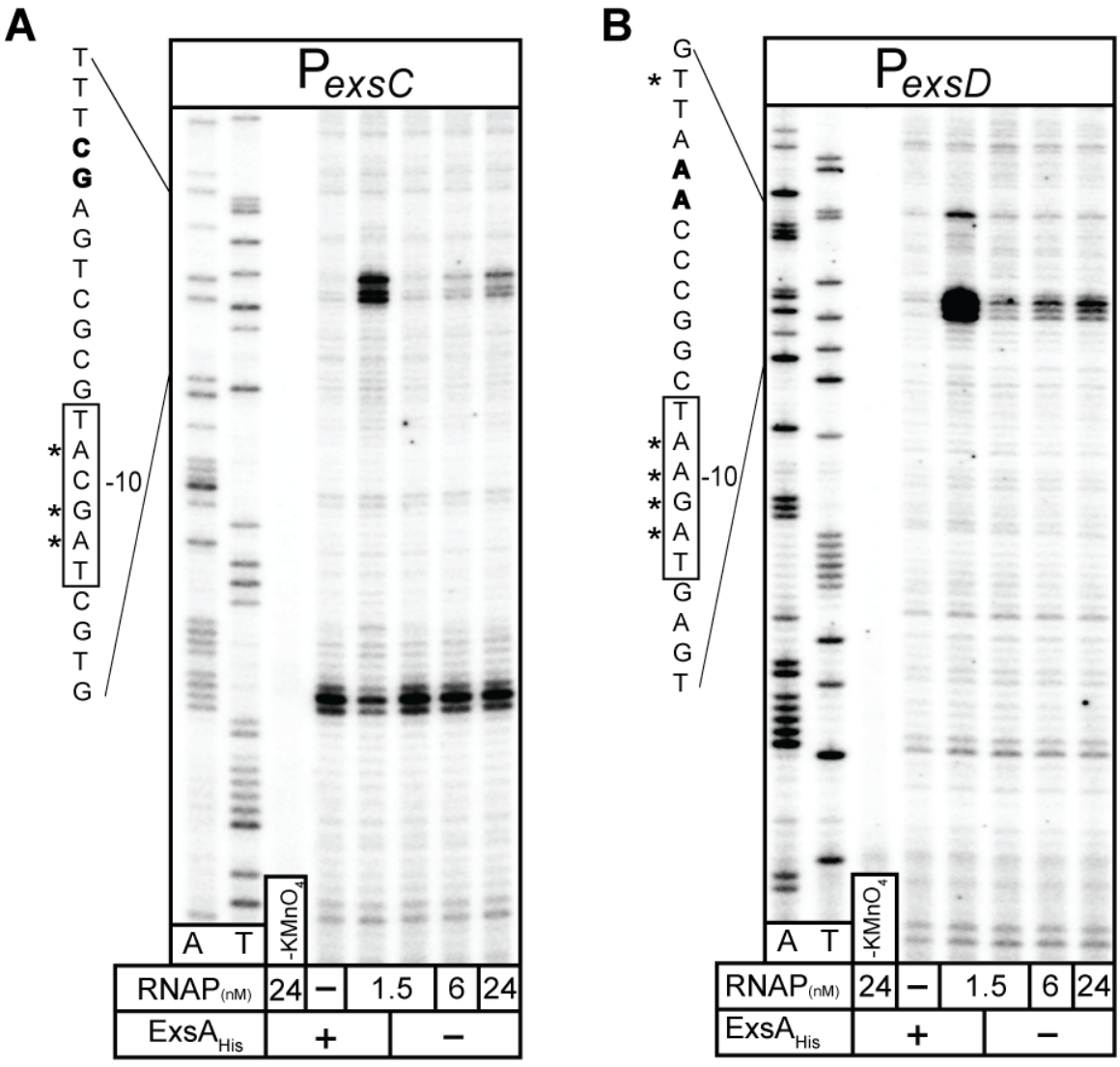

FIGURE 10. ExsA stimulates formation of open complexes as measured by potassium permanganate footprinting. (A-B) Supercoiled minicircles carrying the P_{exsC} (A) or P_{exsD} (B) promoters (1.6 nM) were incubated in the absence (-) or presence (+) of $ExsA_{His}$ (30 nM) for 10 min. RNAP was added to the indicated concentrations and incubated for 3 min. Reactions were then treated with potassium permanganate (except -$KMnO_4$ control) and the modified minicircles were used as templates in primer extension reactions with a radiolabeled coding strand primer. Primer extension products were subjected to denaturing electrophoresis and phosphorimaging. Dideoxy sequencing reactions for A and T are indicated. The diagrams to the left show the transcriptional start sites (bold typeface), the -10 regions (boxed), and the nucleotides modified by potassium permanganate (indicated with asterisks).

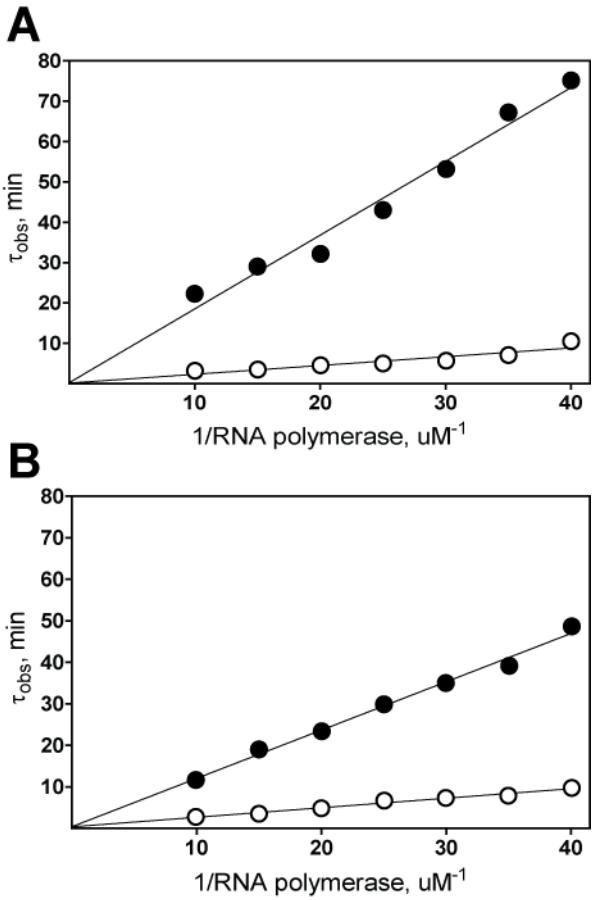

FIGURE 11. Kinetics of transcription initiation at the P_{exsC} and P_{exsD} promoters. (A-B) Tau plots for the P_{exsC} (A) and P_{exsD} (B) promoters in the presence (open circles) and absence (closed cirlces) of $ExsA_{His}$. Values for τobs were calculated from abortive initiation assays measuring synthesis of the products pppGCUUU (P_{exsC}) and pppAAAUU (P_{exsD}). Calculated values for τobs were plotted on the ordinate as a function of reciprocal RNAP concentration.

TABLE 1

Bacterial strains and plasmids used in Chapter III

Strain or plasmid	Relevant characteristics	Reference
P. aeruginosa strains		
PA103	wild-type parental strain	(Frank et al. 1994)
PA103 *exsA*::Ω	insertional mutant lacking ExsA	(Frank et al. 1994)
AK1012	Transcriptional activator, no T3SS expression defective LPS core; *O* antigen deficient for RNAP purification	(Jarrell and Kropinski 1977)
Escherichia coli strains		
DH5α	*recA* cloning strain	(Hanahan 1983)
SA1751	thermoinducible Int expression from the cryptic prophage for minicircle recombination	(Choy and Adhya 1993)
Plasmids		
pMS421	*LacI*^{q} expression plasmid for mini-CTX cloning	(Grana et al. 1988)
mini-CTX-*lacZ*	vector for single-copy integration of *lacZ* reporters onto the *P. aeruginosa* chromosomal *attB* site	(Hoang et al. 2000)
mini-CTX-P_{exoT}	transcriptional fusion of the P_{exoT} promoter to *lacZ*	(McCaw et al. 2002)
mini-CTX-P_{exoT} (17-24)	$P_{exoT\text{-}lacZ}$ fusions with -10/-35 spacing deletions and insertions	this study
mini-CTX-P_{exoT} (C)	$P_{exoT\text{-}lacZ}$ fusion with a portion of the -10/-35 spacer region changed to its complementary sequence	this study
mini-CTX-P_{exsD}	transcriptional fusion of the P_{exsD} promoter to *lacZ*	(McCaw et al. 2002)
mini-CTX-P_{exsD} (17A,B,C)	$P_{exsD\text{-}lacZ}$ fusion with 4 base deletions in the -10/-35 spacing nucleotides	this study
mini-CTX-P_{tacI}	transcriptional fusion of the constitutive P_{tacI} promoter to *lacZ*	this study
mini-CTX-$P_{exsD\text{-}tacI16}$	$P_{exsD\text{-}lacZ}$ fusion with -10/-35 spacing nucleotides from P_{tacI}	this study
mini-CTX-$P_{tacI\text{-}PexsD}$ (-35)	$P_{tacI\text{-}lacZ}$ fusion with the -35 sequence from P_{exsD}	this study
mini-CTX-$P_{tacI\text{-}PexsD}$ (-10)	$P_{tacI\text{-}lacZ}$ fusion with the -10 sequence from P_{exsD}	this study
mini-CTX-$P_{tacI\text{-}PexsD}$ (-35/-10)	$P_{tacI\text{-}lacZ}$ fusion with the -10 and -35 sequences from P_{exsD}	this study
pOM90-P_{exsD}	plasmid *in vitro* transcription template containing the P_{exsD} promoter	(Brutinel et al. 2009b)
pSA508	Parent vector for supercoiled minicircle yielding pMCP	(Choy and Adhya 1993)
pSA508-P_{exsD}	P_{exsD} template vector; yielding minicircle $pMCP_{exsD}$	this study
pSA508-P_{exsC}	P_{exsC} template vector; yielding minicircle $pMCP_{exsC}$	this study
pSA508-$P_{exsD+GG}$	$P_{exsD+GG}$ template vector; yielding minicircle $pMCP_{exsD+GG}$	this study

TABLE 2

Primers used in Chapter III

Primer ID	Primer Sequence (5'- 3')
36444516	ACGTTCTAGAAAGCTTGGCTGCACGCCGAGCCGC
37630269	ACGTGTCGACGAATTCGCAATTTGGGCCGATTCTACT
39456110	CTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTA
39456728	AGCTTAATTGTTATCCGCTCACAATTCCACACATTATACGAGCCGATGATTAATTGTCAAGGTAC
39647294	AGGGCGAATTGGGTACCTTGAAAATTAATCATCGGCTCGT
39647293	ACGAGCCGATGATTAATTTTCAAGGTACCCAATTCGCCCT
39647291	CGCTCACAATTCCACACATTCTACGAGCCGATGATTAATT
39647292	AATTAATCATCGGCTCGTAGAATGTGTGGAATTGTGAGCG
25444811	ACCGAATTCGGACTCACGATACAAACTGCTCGA
25444813	CGTGGAATTCATGCTCTTCGCGTTCAGTCC
36893485	ATAGCAGGTACCGATTCCGGACTCACGATACA
36893487	TATCATGAGCTCGATCAGCGAGCGGAGAATCCT
36893484	ATAGCAGGTACCCCACATCGGCCTCCAGCAAC
36893489	ATATCTGAGCTCAGCCAGAAGCAGAAGGTCGAG
38524736	GAGTAGAATCGGCCCAAATTGGGCAGGCTCTGACGAG
38524735	CTCGTCAGAGCCTGCCCAATTTGGGCCGATTCTACTC

TABLE 3

Construction of plasmids used in Chapter III

Figure	PCR Product	Primer Pair	Cloning Vector
Fig. 6	mCTX-PexsD	36444516-37630269	miniCTX-lacZ
Fig. 6	mCTX-PtacI	39456110-39456728	miniCTX-lacZ
Fig. 6	mCTX-PtacI-PexsD(-35)	39647294-39647293	miniCTX-PtacI-lacZ
Fig. 6	mCTX-PtacI-PexsD(-10)	39647291-39647292	miniCTX-PtacI-lacZ
Fig. 6	mCTX-PtacI-PexsD(-35/-10)	39647291-39647292	miniCTX-PtacI-PexsD(-35)-lacZ
Fig. 7B	pOM90-PexsD	25444811-25444813	pOM90
Fig. 7C, 9-11	pSA508-PexsD	36893485-36893487	pSA508
Fig. 7C, 9-11	pSA508-PexsC	36893484-36893489	pSA508
Fig. 9C	pSA508-PexsD+GG	38524736-38524735	pSA508-PexsD

TABLE 4

Kinetic parameters of the P_{exsC} and P_{exsD} promoters

Promoter	τ^a	$k_2{}^b$	$K_B{}^c$	k_2 x $K_B{}^d$
P_{exsC}	13.8	72	1.3	9.4
P_{exsC} + $ExsA_{His}$	8.1	123	10	123
P_{exsD}	52.7	18.9	3.4	6.4
P_{exsD} + $ExsA_{His}$	22.6	44	16.5	72.6

[a] lag time (sec) to open complex formation

[b] isomerization rate constant (sec^{-1} x 10^3)

[c] equilibrium binding constant for RNAP (M^{-1} x 10^{-5})

[d] overall reaction rate for open complex formation (M^{-1} sec^{-1} x 10^{-3})

CHAPTER IV

EXSA RECRUITS RNA POLYMERASE TO AN EXTENDED -10 PROMOTER BY CONTACTING REGION 4.2 OF SIGMA-70

Introduction

The central regulator of T3SS gene expression is ExsA (Frank and Iglewski 1991; Yahr and Frank 1994; Yahr et al. 1995). ExsA directly binds to 10 different promoters and activates transcription of the core genes required for assembly and function of the T3SS (Brutinel et al. 2008; Vakulskas et al. 2009). Work presented in chapter III demonstrated that ExsA is dependent on σ^{70} for transcriptional activation (Vakulskas et al. 2009). In addition, ExsA-dependent promoters contain apparent σ^{70}-RNAP hexamers that closely resemble the *P. aeruginosa* consensus sequences (TTGACA and TATAAT for the -35 and -10 sites, respectively) (Brutinel et al. 2008; Hovey and Frank 1995). I determined the placement of the -10 hexamers and transcription start sites for several ExsA-dependent promoters by 5' RACE mapping and potassium permanganate footprinting experiments (Vakulskas et al. 2009; Yahr and Frank 1994). These experiments indicated that σ^{70}-dependent transcription originates from the same start sites in the presence and absence of ExsA (Vakulskas et al. 2009). I established the role of the -10 hexamers and provided evidence that near-consensus -35 hexamers are not authentic determinants for the initial binding of RNAP to T3SS promoters (Vakulskas et al. 2009). Consistent with this hypothesis, by a kinetic analysis of abortive transcript production from the P_{exsC} and P_{exsD} promoters, I reveal that the primary function of ExsA is to recruit σ^{70}-RNAP to promoter DNA (Vakulskas et al. 2009).

AraC-family transcriptional regulators typically promote transcription through specific contacts with the α and σ^{70} subunits of RNAP (Martin and Rosner 2001). The transcriptional activation mechanisms facilitated by these proteins fall into one of two classes. Class I activation usually involves an activator DNA-binding site that is located ≥ 20 bp upstream of a -35 hexamer. Class I activation is mediated by specific contacts

between the activator protein and the carboxy-terminal domain of the RNAP alpha (α) subunit. Class II activation occurs when the activator-binding site is positioned in proximity to, or overlaps the -35 hexamer. In these cases, the activator protein contacts the σ^{70} subunit and the activator may additionally bind the α subunit. In this mechanism of activation, RNAP recruitment is thought to occur by interaction with the α subunit while isomerization from a closed to open complex is thought to occur through interactions with the σ^{70} subunit.

The σ^{70} RNAP subunit functions as a specificity factor during transcription initiation. There are four main regions of σ^{70}, each thought to possess a discrete function. Region 1 prevents non-specific association of σ^{70} with promoter DNA in the absence of core RNAP (Paget and Helmann 2003). Region 2 contains a DNA-binding domain important for interaction with the -10 hexamer (Paget and Helmann 2003). Although much less is known about the role of region 3, the available data suggests it binds extended -10 promoters and may play a role in the initiation of transcription (Paget and Helmann 2003). Region 4 can be subdivided into two distinct sections (4.1 and 4.2). Although the role of region 4.1 is poorly understood, region 4.2 contains a DNA-binding domain that recognizes the -35 hexamer. The carboxy-terminal end of region 4.2 also interacts with a diverse group of class II transcriptional activators. For example, the AraC-family regulators RhaR and RhaS, (involved in metabolism of the sugar rhamnose) contact several amino acids in region 4.2 of σ^{70} and this interaction is required for transcriptional activation (Wickstrum and Egan 2004).

In this chapter I test for an interaction between ExsA and the RNAP α and σ^{70} subunits. My data indicates that ExsA functions as a class II transcriptional activator at the P_{exsC} and P_{exsD} promoters and contacts several amino acids in region 4.2 of σ^{70}. I also provide evidence that the -35-like element of the P_{exsC} promoter is not an authentic RNAP recognition hexamer for ExsA-independent or -dependent transcription and I demonstrate

that ExsA-independent transcription at the P_{exsC} promoter requires an extended -10 promoter element.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are summarized in Table 1. *Escherichia coli* strains were maintained on LB-agar plates containing the following antibiotics/chemicals as necessary (gentamicin [15 μg ml^{-1}], ampicillin [50 or 100 μg ml^{-1}], tetracycline [10 μg ml^{-1}], kanamycin [50 μg ml^{-1}], indole-3-acrylic acid [0.5 mM*)*. *P. aeruginosa* strains were maintained on Vogel Bonner minimal medium (Vogel and Bonner 1956) with antibiotics as indicated (carbenicillin [300 μg ml^{-1}] and tetracycline [50 μg ml^{-1}]). For experiments where LuxR was utilized the co-factor 3oxo-hexanoyl-L-homoserine-lactone (Sigma Aldrich) was added (200 nM). To assay for ExsA-dependent gene expression in the presence of mutant and wild-type RNAP subunits, E.coli strains were inoculated from LB-agar plates grown overnight into 10 ml of LB to an OD_{600}-0.1 and grown with vigorous aeration at 30°C to an OD_{600}-0.6. β-galactosidase assays were performed as previously described (Vakulskas et al. 2009) and the reported values are the average of at least three independent experiments.

Plasmid construction and promoter mutagenesis

The $P_{exsC\text{-}lacZ}$, $P_{exsD\text{-}lacZ}$, and $P_{exoT\text{-}lacZ}$ transcriptional reporters were generated by PCR amplifying the promoters and cloning into the KpnI/EcoRI (P_{exsC}/P_{exsD}) or SalI/EcoRI (P_{exoT}) sites of the λ integration plasmid pAH125 (Haldimann and Wanner 2001). The $P_{luxI\text{-}lacZ}$ translational fusion reporter was generated by cloning the AatII/EcoRI restriction fragment from plasmid p*luxI-lacZ* (Urbanowski et al. 2004) into plasmid pAH125. The resulting plasmids were integrated at the λ attachment site of E. coli strains GS162 and/or GA2071 by an electroporation method as described previously (Haldimann and Wanner 2001).

The constitutive ExsA expression plasmid p2UY21 was created through the following series of subcloning steps. ExsA expression plasmid pEB102 was created by PCR amplifying the *exsA* gene from *P. aeruginosa* strain PA103 using NdeI/SacI-containing primers and cloning the resulting fragment into plasmid pUY30 (Urbanowski et al. 2007). Plasmid p2UY21 was created by cloning the 210 bp ApoI fragment from plasmid pMCTX-$P_{lacUV5mut\text{-}lacZ}$ (described below) into the MfeI/EcoRI sites of plasmid pEB102. Plasmid pMCTX-$P_{lacUV5mut\text{-}lacZ}$ was created by annealing complementary oligonucleotides (5' – AGCTTAGGCTTATCACTTTATGCTTCCGGCTCGTATAATGTGTG – 3' and 5' – AATTCACACATTATACGAGCCGGAAGCATAAAGTGATAAGCCTA -3') and cloning the resulting fragment into the HindIII-EcoRI sites of plasmid pMini-CTX-lacZ (Becher and Schweizer 2000). Constitutive LuxR expression plasmid p2UY21-*luxR* was created by cloning the NdeI-SacI fragment from pMU102 (Urbanowski et al. 2004) into plasmid p2UY21. The pGEX-*rpoD*(K593A,R596A,R599A) triple mutant σ^{70} expression plasmid as well as P_{exsC} promoter point mutant transcription templates were generated by QuickChange site-directed mutagenesis (Stratagene).

The carboxy-terminal hexahistidine-tagged α subunit expression vector pET24-$rpoA_{\text{HisCTD}}$ was created by PCR amplifying the *rpoA* gene from *P. aeruginosa* strain PA103 lacking its native stop codon by using NdeI-NotI-containing primers and cloning the resulting fragment into pET-24a (Novagen). β and β' prime subunit expression vectors pET24-*rpoB* and pET24-*rpoC* were created by PCR amplification of the *rpoB* or *rpoC* gene from *P. aeruginosa* strain PA103 by using NdeI-NotI containing primers and cloning the resulting fragment into pET-24a. The carboxy-terminal hexahistidine-tagged σ^{70} expression vector pET23-$rpoD_{\text{HisCTD}}$ was created by PCR amplification of the *rpoD* gene lacking its native stop codon from *P. aeruginosa* strain PA103 by using primers incorporating NdeI-HindIII restriction sites and cloning the resulting fragment into pET-

23b (Novagen). Point mutations in *rpoD* were introduced by QuickChange site-directed mutagenesis (Stratagene).

Purification of *P. aeruginosa* RNAP core enzyme, σ^{70}, and holoenzyme

Individual RNAP subunits were purified as described previously (Tang et al. 1996) with modification. *E. coli* Tuner (DE3) carrying pET24-*rpoA*$_{\text{HisCTD}}$ was grown at 37°C in 50 ml of Luria Broth containing 50 μg ml^{-1} kanamycin to an OD_{600}-0.7 at which time IPTG (1 mM) was added and the culture was incubated for an additional 3 h at 37°C. Bacteria were harvested by centrifugation and suspended in 4 ml of buffer A (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, and 5 mM imidazole). Cells were lysed via sonication on ice and unbroken cells were removed by centrifugation (15 min, 16,000 x g, 4°C). Solid ammonium sulfate (60% of saturation) was added and samples were allowed to precipitate for 15 min at 4°C with agitation. The precipitate was collected by centrifugation (20 min, 16,000 x g, 4°C) and resuspended in 10 ml of buffer B (20 mM Tris-HCl [pH 7.9], 6 M guanidine HCl, and 500 mM NaCl) containing 5 mM imidazole. Prior to Ni^{2+}-affinity chromatography (see below), the material was subjected to ultracentrifugation (30 min, 100,000 x g, 4°C) to remove particulates.

E. coli tuner (DE3) carrying pET23-*rpoD*$_{\text{HisCTD}}$ was grown at 37°C in 200 ml LB containing 200 μg ml^{-1} ampicillin to an OD_{600}-0.5 at which time IPTG (1 mM) was added and the culture was incubated for an additional 3 hr at 37°C. Bacteria were harvested by centrifugation and suspended in 5 ml buffer B containing 5 mM imidazole. Cells were lysed via sonication on ice and unbroken cells were removed by centrifugation (15 min, 38,000 x g, 4°C).

The α and σ subunits were purified from the soluble fractions described above by Ni^{2+}-affinity chromatography. Lysates was applied to a 1 ml HisTrap column (GE Healthcare) equilibrated with buffer B containing 5 mM imidazole, washed with 10 ml

buffer B containing 30 mM imidazole, and developed with a 10 ml linear imidazole gradient (30-500 mM) in buffer B. The elution peaks were established by SDS-PAGE. The purified α subunit was stored on ice for immediate use in core RNAP reconstitution. Purified σ^{70} was dialyzed overnight against buffer E (see below) at 4°C, subjected to ultracentrifugation (30 min, 100,000 x g, 4°C), and stored in 50% glycerol at -20°C.

The β and β' RNAP subunits were purified from *E. coli* inclusion bodies. *E. coli* Tuner (DE3) carrying either pET24-*rpoB* or pET24-*rpoC* was grown at 37°C in 1 L of LB containing 50 μg ml^{-1} kanamycin to an OD_{600}-0.5 at which time IPTG (1 mM) was added and the culture was incubated for an additional 3 h at 37°C. Bacteria were harvested by centrifugation and suspended in 16 ml of Buffer C (40 mM Tris-HCl [pH 7.9], 300 mM KCl, 10 mM EDTA, 1 mM DTT, and 1x Protease Inhibitor Cocktail [Roche]) containing 0.2 mg ml^{-1} lysozyme and 0.2% (w/v) sodium deoxycholate. The bacteria were incubated on ice for 20 min and lysed by sonication. Inclusion bodies were collected by centrifugation (30 min, 38,000 x g, 4°C) and washed with 16 ml buffer C containing 0.2% n-octyl-β-D-glucoside. Inclusion bodies were sonicated and centrifuged as above, followed by a final wash with 16 ml buffer C. Washed inclusion bodies were solubilized in 2 ml of buffer D (50 mM Tris-HCl [pH 7.9], 6 M guanidine-HCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM EDTA, 10 mM DTT, and 10% glycerol [v/v]) and incubated at 25°C for 10 min. The resulting material was subjected to ultracentrifugation (30 min, 100,000 x g, 4°C) and the soluble fraction was stored on ice for immediate use in core enzyme reconstitution.

RNAP core enzyme was reconstituted by mixing 0.3 mg purified α subunit, 1.5 mg purified β subunit, and 3 mg β' subunit in buffer D (2 ml) and dialyzing twice against 500 ml of buffer E (50 mM Tris-HCl [pH 7.9], 200 mM KCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 20% [v/v] glycerol) at 4°C with constant stirring. The resulting material was subjected to ultracentrifugation (30 min, 100,000 x g, 4°C) and the soluble fraction was applied to a 1 ml HiTrap Heparin HP

column (GE Healthcare) equilibrated with Buffer E. The column was washed with 10 ml Buffer E containing 0.4 M KCl and developed with a 10 ml linear KCl gradient (0.4-2 M) in Buffer E. The elution peaks were analyzed by SDS-PAGE and pure fractions containing stoichiometric core RNAP ($\alpha_2\beta\beta'$) were dialyzed against 1 L Buffer E containing 50% glycerol and stored at -20°C.

RNAP holoenzyme was reconstituted by mixing core RNAP (500 nM) and σ^{70} (1 μM) in 35 μl 1x transcription buffer (40 mM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 % Tween-20, and 0.5 mg ml^{-1} bovine serum albumin [BSA]) for 30 min at 25°C. The resulting holoenzyme was then used (1 μl) in a 20 μl transcription reaction.

in vitro transcription assays

Supercoiled transcription templates containing the P_{exsC} and P_{exsD} promoters were described previously (Brutinel et al. 2009b; Vakulskas et al. 2009). The pOM90-P_{exsC} template was generated by PCR-amplifying the P_{exsC} promoter (nucleotides -207 to +192 relative to the transcriptional start site) and cloning as an EcoRI fragment into pOM90 (Richet and Sogaard-Andersen 1994). The resulting template contains a fusion of the P_{exsC} promoter to the *rpoC* terminator on pOM90, and directs synthesis of a 261 base transcript. The pOM90-P_{trc180} and pOM90-P_{trc250} templates were generated by PCR-amplifying the P_{trc} promoter (nucleotides -61 to +109/179 relative to the transcriptional start site) from pTRCHIS-b (Invitrogen) and cloning as an EcoRI fragment into pOM90. The pOM90-P_{trc180} and pOM90-P_{trc250} templates fuse the P_{trc} promoter to the *rpoC* terminator from pOM90, and direct synthesis of 180 and 250 base transcripts, respectively. Finally, the pOM90-$P_{RE\#}$ template was generated by annealing complementary nucleotides and the resulting BamHI-EcoRI fragment was cloned into pOM90. The pOM90-$P_{RE\#}$ template fuses the $P_{RE\#}$ promoter to the *rrnb* T1 terminator on pOM90, and directs synthesis of a 135 base transcript.

Single-round transcription assays (20 µl final volume) were performed by incubating $ExsA_{His}$ (35 nM) with transcription templates (2 nM) at 25°C in 1x transcription buffer (40 mM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 % Tween-20, and 0.5 mg ml^{-1} BSA) containing the initiating nucleotides ATP and GTP (0.75 mM). After 10 min, 25 nM reconstituted *P. aeruginosa* RNAP holoenzyme was added, and open complexes were allowed to form for 1 min at 25°C in the presence of $ExsA_{His}$ or for 20 min at 25°C in the absence of $ExsA_{His}$. Elongation was allowed to proceed by the addition of the remaining nucleotides (0.25 mM ATP/GTP/CTP, 0.75 mM UTP, and 2.5 µCi [α-^{32}P]-CTP) in 1x transcription buffer containing heparin (final concentration 50 µg ml^{-1}). Reactions were stopped after 5 min at 25°C by the addition of 20 µl stop buffer (98% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Samples were heated to 95°C for 5 min and immediately incubated on ice before electrophoresis on 5% denaturing polyacrylamide gels.

Results

The carboxy-terminal domain of the RNAP α subunit is not required for ExsA-dependent transcriptional activation

Since ExsA activates transcription primarily through recruitment of RNAP (Vakulskas et al. 2009), and most transcriptional activators contact the carboxy-terminal domain of the RNAP α subunit (α-CTD), we tested the hypothesis that ExsA uses a similar mechanism. Previous studies have shown that ExsA activates transcription *in vitro* to similar levels using RNAP from either *P. aeruginosa* or *E. coli* (Vakulskas et al. 2009). To demonstrate that ExsA can activate transcription in *E. coli* from the P_{exsC}, P_{exsD}, and P_{exoT} promoters, ExsA was expressed in *E. coli* from a plasmid under the transcriptional control of a constitutive α-CTD-independent promoter and ExsA-dependent transcription was measured from transcriptional reporters consisting of ExsA-dependent promoters (P_{exsC}, P_{exsD}, and P_{exoT}) fused to *lacZ* and integrated at the *E. coli* λ

phage attachment site. Significant ExsA-dependent activation of all three promoters was observed relative to a control plasmid (Fig. 12A), demonstrating that ExsA is sufficient to activate transcription from P_{exsC}, P_{exsD}, and P_{exoT} as was previously shown for P_{exsC} in *E. coli* (Thibault et al. 2009).

To determine the role of the α-CTD we used an established *E. coli* assay in which the native α subunit (α-wt) or α lacking the C-terminal 239 amino acids (α-ΔCTD) were expressed from a plasmid such that their cellular concentrations are in vast excess over native α subunit expressed from the chromosome. This approach was necessary because *E. coli rpoA* deletion mutants are lethal. ExsA-dependent transcription following overexpression of α-ΔCTD was plotted as a percentage of activation observed with α-wt. As a control we also measured LuxR-dependent activation of a $P_{luxI\text{-}lacZ}$ transcriptional fusion (Antunes et al. 2007). LuxR is an activator known to require the α-CTD (Stevens et al. 1999). ExsA-dependent activation of the $P_{exsC\text{-}lacZ}$, $P_{exsD\text{-}lacZ}$, and $P_{exoT\text{-}lacZ}$ reporters in the presence of α-ΔCTD was ≥100% of that seen with α-wt indicating that ExsA does not require the α-CTD for transcriptional activation (Fig. 12B). Curiously, activation from the P_{exsC} promoter in the presence of α-ΔCTD was 125% of WT suggesting the α-CTD might have an inhibitory function at this promoter. In contrast, activation of the $P_{luxI\text{-}lacZ}$ reporter was reduced to ~33% of wild-type in the presence of α-ΔCTD.

ExsA-dependent transcription in *E. coli* is dependent on specific amino acids within region 4.2 of σ^{70}

A number of class II transcriptional activators interact with a basic amino acid region of σ^{70} region 4.2. Since the ExsA promoter-binding site overlaps a near-consensus -35 RNAP recognition hexamer, we hypothesized that ExsA recruits RNAP through an interaction with region 4.2 of the σ^{70} subunit. Lonetto et al. generated an *rpoD* plasmid expression library containing alanine point mutations in 16 non-essential positions of region 4.2 to test for activator specific defects in gene expression (Lonetto et al. 1998).

These experiments were performed in *E. coli* strain GA2071 where expression of chromosomal *rpoD* is tightly repressed. To measure ExsA activity, the $P_{exsC\text{-}lacZ}$ and $P_{exsD\text{-}lacZ}$ transcriptional reporters were introduced at the λ phage attachment site of *E. coli* strain GA2071 and the resulting strains were transformed with a plasmid expressing wild-type RpoD or the RpoD point mutants. Given the tendency for reversion to wild-type *rpoD* sequence (Lonetto et al. 1998) each expression plasmid was sequenced after being introduced into strain GA2071. ExsA was constitutively expressed from plasmid p2UY21 and the levels of ExsA protein in each were verified by western blot (Fig. 13A). Since the RpoD mutants in this library do not affect activator-independent transcription, the levels of ExsA protein were maintained at a constant level (Fig. 13A) (Lonetto et al. 1998). ExsA-dependent expression from the P_{exsC} and P_{exsD} reporters in the presence of RpoD mutants was plotted relative to wild-type RpoD (Fig. 13). The most drastic effect on ExsA-dependent activation of the $P_{exsC\text{-}lacZ}$ reporter resulted with the K593A, R596A and R599A substitutions, which exhibited 50%, 29%, and 25% the activity seen with native RpoD, respectively (Fig. 13B). Similarly, $P_{exsD\text{-}lacZ}$ reporter activity was also impaired by the K593A, R596A and R599A substitutions to 42%, 67%, and 36% the native RpoD levels (Fig. 13C).

The effects observed from the single amino acid substitution mutants were modest (2-3 fold) and likely reflect the fact that each of the individual positions represents only a small portion of the ExsA-σ^{70} interaction site. We predicted that ExsA-dependent transcription might result from synergistic interactions with each of the three amino acid positions. This proved to be true as the activity of the P_{exsC} and P_{exsD} reporters in the presence of a triple RpoD mutant (K593A, R596A, R599A) was only 15% of wild-type in both cases (Fig. 13). We did note that strain GA2071 expressing the RpoD triple mutant exhibited a 2-fold growth defect yet had equivalent ExsA levels compared to GA2071 expressing native RpoD (Fig. 13A).

ExsA-dependent transcription *in vitro* is dependent on *P. aeruginosa* σ^{70} region 4.2

To further characterize the role of σ^{70} region 4.2 the mutations from *E. coli rpoD* (K593A, R596A, and R599A) that affect ExsA-dependent transcription *in vivo* were introduced into *P. aeruginosa rpoD*. Native and mutant forms of *P. aeruginosa* RpoD were expressed in *E. coli* and purified under denaturing conditions by Ni^{2+}-affinity chromatography. Core RNAP was generated by expressing the *P. aeruginosa* α, β, and β' subunits in *E. coli*, purifying the individual purified components (Fig. 14A), and reconstituting σ-saturated RNAP holoenzyme with either native RpoD, RpoD-K593A, RpoD-R596A, RpoD-R599A, or the triple RpoD mutant. RNAP holoenzyme activity was normalized between the different RpoD-reconstituted polymerases by comparing the production of single-round in vitro transcription products from the P_{trc} promoter. Transcription from the P_{trc} promoter is not affected by the K593A, K596A, or K599A mutations in region 4.2 of σ^{70} (Lonetto et al. 1998).

Reconstituted RNAP holoenzmes were then assayed for ExsA-dependent transcription *in vitro* using supercoiled plasmid templates containing the P_{exsC} and P_{exsD} promoters fused to the *rpoC* terminator. Each of the templates generates a 261 nucleotide, terminated transcript. As expected, terminated transcripts were not observed with core RNAP alone. We initially tested the single amino acid substitutions in RpoD for ExsA-dependent activation of the P_{exsC} or P_{exsD} promoters but found that none had an activation defect greater than 50% of native RpoD (data not shown). This result was not surprising given that a similar observation was made when testing *in vivo* activation in *E. coli* (Fig. 13A). In contrast, the triple RpoD mutant produced far less *exsC* and *exsD* transcripts than did native RpoD (Fig. 14B,C). These combined results indicate that σ^{70} region 4.2 is required for ExsA-dependent activation of the P_{exsC} and P_{exsD} promoters both *in vivo* and *in vitro*.

The near-consensus -35 sequence at the P_{exsC} promoter is not required for ExsA-independent transcription

We previously demonstrated that the P_{exsC} promoter has low basal activity in the absence of ExsA (Vakulskas et al. 2009). To determine whether the putative -35 sequence is required for ExsA-independent promoter activity we generated P_{exsC} transcription templates containing point mutations at each of the -35 nucleotide positions. All of the nucleotide substitutions were changed to the complementary A/T or G/C nucleotide to maintain the G+C ratio and each, with the exception of G41T, were divergent from the σ^{70} consensus (Fig. 15A). The mutant promoters were assayed for ExsA-independent transcript levels and compared to the native P_{exsC} promoter and to a negative control containing a single point mutation in the established -10 Pribnow box (Fig. 15A). To account for subtle differences in template concentration and purity, the P_{exsC} transcripts were normalized to a constitutive transcript generated from a promoter located on the plasmid backbone (Vakulskas et al. 2009). Whereas the negative control (T8G) lacking a functional -10 hexamer exhibited a > 50-fold decrease in transcription compared to wild-type P_{exsC} (Fig. 15), the remaining point mutants had little (less than 2-fold) or no effect on ExsA-independent transcription (Fig. 15B-C). These data indicate that the putative -35 hexamer is not important for ExsA-independent transcription at the P_{exsC} promoter.

Although a near-consensus, but improperly spaced, -35 sequence is present at the P_{exsC} promoter, it is possible that a weak, unrecognizable -35 hexamer with a poor match to the σ^{70} consensus is present and optimally spaced (16/17 bp) from the -10 hexamer. Potential -35 hexamers spaced at either 16 or 17 bp would have the sequence AAAG<u>C</u>G or AAA<u>A</u>GC, respectively (matches to consensus are underlined). To test this hypothesis we constructed a single point mutant in the P_{exsC} promoter (A33G) such that the potential -35 hexamer spaced 16 bp (AA<u>G</u>G<u>C</u>G) from the -10 hexamer more closely resembles the -35 consensus sequence and the potential -35 hexamer spaced at 17 bp

(AAAGGC) would be a weaker match to consensus. The A33G mutation had no significant effect (<2 fold) compared to native P_{exsC}. These combined data suggest that the putative -35 sequence is not important for ExsA-independent transcription.

The P_{exsC} promoter sequence located immediately upstream of the -10 box resembles an extended -10 promoter (Fig. 15A). Extended -10 promoters contain the sequence TGnTATAAT and can function both in the presence or absence of a -35 hexamer (Barne et al. 1997; Mitchell et al. 2003). To determine whether the P_{exsC} promoter contains an extended -10 element we mutated the consensus TG sequence to AC (hereafter referred to as $P_{exsC\text{-}TG}$). As expected the mutant $P_{exsC\text{-}TG}$ promoter had a significant reduction in ExsA-independent transcription (5-fold) when compared to the native P_{exsC} promoter (Fig. 15B-C). These combined data suggest that the P_{exsC} promoter lacks a -35 hexamer, and that an extended -10 element may provide basal promoter activity.

The extended -10 element is important for ExsA-independent and ExsA–dependent promoter activity

Since the ExsA-independent activity of the P_{exsC} promoter requires an apparent extended -10 sequence, we asked whether ExsA-dependent activation had a similar requirement using *in vitro* transcription assays. $P_{exsC\text{-}TG}$ promoter activity was reduced 3-fold in the presence of ExsA demonstrating that the extended -10 element affects P_{exsC} to a similar extent in the presence and absence of ExsA (Fig. 16A-B). Note that ExsA-independent transcripts were not observed under these conditions due to the short RNAP incubation time (1 min) required to detect ExsA-dependent open complex formation in the linear range. To rule out the trivial explanation that the DNA-binding activity of ExsA is affected by the TG mutation we employed electrophoretic mobility shift assays (EMSAs) and found no significant difference in the binding affinity of ExsA for the $P_{exsC\text{-}TG}$ and native P_{exsC} promoters or in formation of shift complexes 1 and 2 (Fig. 16C).

Region 4.2 of σ^{70} is required for ExsA-dependent but not ExsA-independent transcription

Region 4.2 of σ^{70} recognizes the -35 hexamer and is essential for recognition of most bacterial promoters (Campbell et al. 2002). Region 4.2 is also a common target for AraC-family transcriptional activators. We have provided evidence that ExsA interacts with this region and that the putative -35 is not a determinant for RNAP recruitment at the P_{exsC} promoters. Based on these data we hypothesized that the P_{exsC} extended -10 element compensates for the lack of a functional -35 hexamer. To test this idea we employed *in vitro* transcription assays utilizing RNAP holoenzyme reconstituted with σ^{70} lacking the carboxy-terminal 43 amino acids (region 4.2). Deletion of region 4.2 renders promoters that are dependent upon -35 hexamers non-functional; however, this deletion has little effect on transcription initiation and elongation from extended -10 promoters (Kumar et al. 1993). The following promoters were used as controls for this experiment; (i) P_{trc} which contains a strong -35 hexamer and requires region 4.2 of σ^{70}, and (ii) $P_{RE\#}$, a synthetic promoter which lacks a -35 hexamer and does not require σ^{70} region 4.2, but is dependent upon an extended -10 element (Brosius et al. 1985; Kumar et al. 1993) (Fig. 17A). Although as previously reported (Lonetto et al. 1998) the truncated σ^{70} protein (hereafter referred to as $\sigma^{70\Delta4.2}$) has a slightly reduced affinity for core RNAP enzyme, the specific activity of $\sigma^{70\Delta4.2}$ at the $P_{RE\#}$ promoter is similar to that seen with native RNAP holoenzyme at $P_{RE\#}$ (Fig. 17B). In contrast, RNAP-$\sigma^{70\Delta4.2}$ generated significantly fewer transcripts from the P_{trc} promoter than did RNAP-σ^{70} (Fig. 17B). Consistent with our hypothesis that the P_{exsC} promoter lacks a functional -35 hexamer, RNAP-$\sigma^{70\Delta4.2}$ and RNAP-σ^{70} generated similar levels of P_{exsC} transcript (Fig 17B). In addition, the $P_{exsC\text{-}TG}$ and $P_{exsCT8G}$ mutants were essentially void of RNAP-$\sigma^{70\Delta4.2}$-dependent activity. Finally, we tested whether ExsA-dependent transcripts were produced from the P_{exsC} promoter using RNAP-$\sigma^{70\Delta4.2}$. Although ExsA-dependent transcription was drastically reduced with RNAP-$\sigma^{70\Delta4.2}$, a detectable transcript was made when reactions were allowed 1 min

for open complex formation. These same conditions do not support the detection of ExsA-independent transcription using wild-type σ^{70}-RNAP (Fig. 16A). It is unclear whether the weak ExsA-dependent transcription in the absence of region 4.2 represents additional contacts between ExsA and σ^{70} outside of region 4.2 or additional contacts between ExsA and other RNAP subunits.

Discussion

In the present study we found that recruitment of RNAP by ExsA does not require the CTD of the α subunit of RNAP, a common target for AraC-family regulators. Although these studies were performed in *E. coli*, we believe the findings would be identical in *P. aeruginosa*. Data supporting this claim include the following; (i) ExsA activates transcription from T3SS promoters in vitro to a similar extent with RNAP (normalized for specific activity) from either *E. coli* or *P. aeruginosa* (Vakulskas et al. 2009); (ii) the carboxy-terminal 90 amino acids of the α subunit from *E. coli* and *P. aeruginosa* share 86% identity; and (iii) heterologous activators known to require the α-CTD, including LuxR from *V. fischeri* (used in this study), can efficiently activate *E. coli* RNAP (Stevens et al. 1999). For these reasons, we believe that the involvement of the α-CTD in ExsA-dependent activation would have been detected in our experiments.

Interestingly, ExsA-dependent transcription from the P_{exsC} promoter was slightly elevated (125%) in the presence of α-ΔCTD as compared to full-length α subunit (Fig. 12B). A possible explanation for this finding is that the α-ΔCTD may bind the P_{exsC} promoter and antagonize ExsA function. In this scenario, the α-ΔCTD -P_{exsC} promoter interaction might sterically hinder the DNA-binding activity of ExsA or its ability to contact RNAP. The physiological relevance and role this phenomenon plays is unknown. We did not test the hypothesis that ExsA interacts with the α-NTD as *Egan et al.* have shown that an extremely diverse group of AraC family members do not require this domain for class II activation (Egan et al. 2000).

Using a plasmid-based mutant *rpoD* expression library, we found that ExsA requires the K593, R596, and R599 amino acids of σ^{70} for full activation of the P_{exsC} and P_{exsD} promoters (Fig. 13). These specific residues are some of the most frequently observed contact points for AraC-family members and unrelated transcriptional regulators (Table 8). Although ExsA-dependent activation defects greater than 2-fold were not routinely observed with a single point mutation in *rpoD*, a 6-fold defect was observed with the triple σ mutant (K597A, R600A, and R603A). We believe these data support one or more of the following conclusions. First, although expression of the chromosomal *rpoD* gene is suppressed, leaky expression of *rpoD* may result in higher levels of ExsA-dependent activation and would bias the data towards transcriptional activation defects smaller than those observed. Second, the available data suggest that RNAP-activator interaction regions most likely consist of several amino acids contacts (Lonetto et al. 1998). We believe our data support the latter conclusion since *in vitro* and *in vivo* studies with the triple σ mutant showed a cumulative effect on ExsA-dependent transcription (Fig. 13 and 14). Finally, ExsA may interact with amino acids in region 4.2 that we did not test, other regions in σ^{70}, and/or different RNAP subunits. The 16 amino acids in the mutant *rpoD* expression library we used were selected because those positions had little effect on activator-independent transcription (Lonetto et al. 1998). Some amino acids in region 4.2 were omitted from this library because alanine substitution resulted in unstable protein or because they are required for interaction with the -35 hexamer. It is therefore possible that other amino acids are also important for the interaction with ExsA. Finally, the finding that a σ^{70} derivative lacking region 4.2 ($\sigma^{70\Delta 4.2}$) is still capable of weak ExsA-dependent activation supports the hypothesis that ExsA interacts with several regions of σ^{70} and/or multiple RNAP subunits (Fig. 17B).

We provide evidence that site 1 of the ExsA consensus binding site, which closely resembles a -35 hexamer, is not sufficient for ExsA-independent expression from the P_{exsC} promoter. In support of this hypothesis, a previous study indicated that the -35

hexamer from P_{exsD} although a close match to the σ^{70} consensus, is a poor RNAP recognition site (Vakulskas et al. 2009). To further characterize the role of T3SS promoter -35 hexamers we generated point mutations at every position in the P_{exsC} -35 site and the resulting mutations had no significant effect (< 2-fold) on ExsA-independent transcription while control mutations in the -10 hexamer produced undetectable levels of transcript (Fig. 15). An explanation for this result is that an authentic -35 hexamer is located at a more favorable position (16 or 17 bp relative to -10) but has few matches to the consensus. We tested this hypothesis by creating a single point mutation in P_{exsC} (A33G), which should drastically increase or decrease ExsA-independent activation if the -35 hexamer is positioned 16 or 17 bp from the -10 hexamer, respectively (Moyle et al. 1991). No significant effect was observed with this mutant suggesting a -35 hexamer is not required for ExsA-independent transcription at the P_{exsC} promoter. Unfortunately, we were unable to assess the role of the -35 hexamer with respect to ExsA-dependent transcription as mutations in the -35 sequence are known to disrupt ExsA binding to site 1 (Brutinel et al. 2008). Consistent with the hypothesis that a -35 is not required for ExsA-independent transcription we identified a putative extended -10 element located upstream of the P_{exsC} promoter -10 hexamer. Extended -10 elements are 1 bp removed from the -10 hexamer with the consensus being TGnTATAAT (Kumar et al. 1993). We made point a mutation within this element and observed a significant effect on both ExsA-dependent and ExsA-independent transcription (Fig. 15-16). EMSA experiments demonstrated the extended -10 mutation had no affect on the binding of ExsA to the promoter Fig. 16C). These data indicate that the P_{exsC} promoter contains an extended -10 promoter that might partially compensate for the lack of a functional -35 hexamer. The P_{exsC} promoter is the only ExsA-dependent promoter that contains an extended -10 element that matches consensus. It is tempting to speculate that the extended -10 element is important in maintaining a housekeeping level of the *exsCEBA* transcript. The fact that the extended -10 element is required for maximal P_{exsC} promoter activity, however,

prevented us from testing this hypothesis. Nevertheless, 5' RACE promoter mapping experiments suggest that *exsCEBA* transcript is detectable in an *exsA* mutant (Vakulskas et al. 2009) suggesting that P_{exsC} exhibits some level of basal activity.

We propose a model in which ExsA recruits RNAP to an extended -10 promoter (P_{exsC}) by contacting σ^{70} region 4.2. In support of our model, we created a truncated σ^{70} lacking region 4.2 ($\sigma^{70\Delta 4.2}$) and found that the resulting holoenzyme was able to transcribe from P_{exsC} in the absence of ExsA. Interestingly, ExsA was still able to activate transcription with $\sigma^{70\Delta 4.2}$-RNAP albeit very poorly (Fig. 17). The general contention is that an RNAP subunit other than α and σ^{70} is likely involved in transcriptional activation by a number of AraC-like regulators (Bhende and Egan 1999; Egan et al. 2000; Holcroft and Egan 2000; Jair et al. 1995). Our data may support this claim and are worthy of further study.

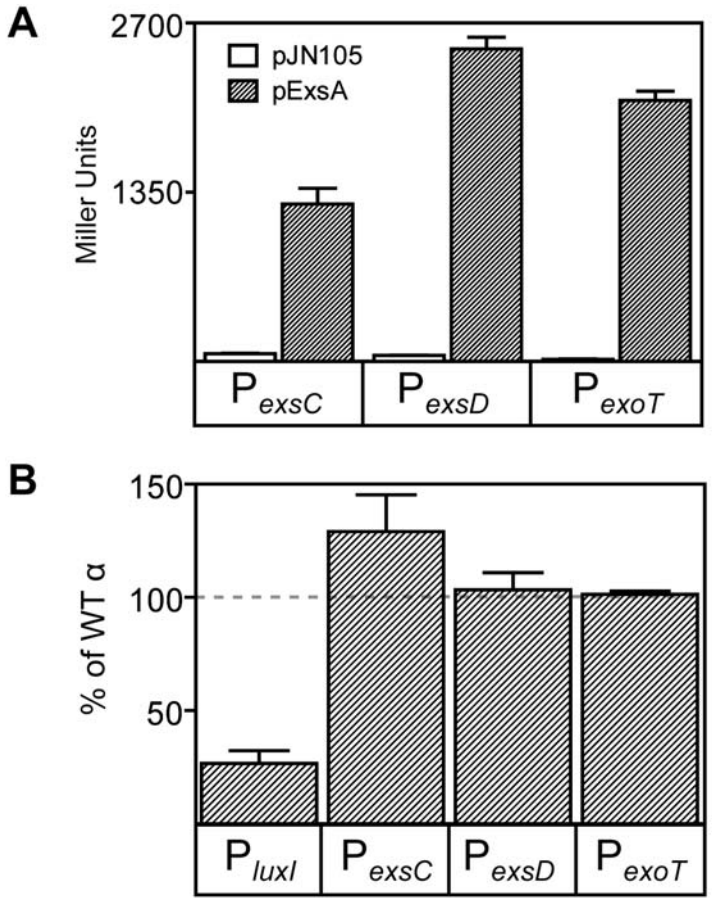

FIGURE 12. The RNAP α-CTD is not required for ExsA-dependent activation of transcription. (A) *E. coli* strain GS162 carrying the indicated transcriptional reporters was transformed with a vector control (pJN105) or a constitutive ExsA expression plasmid (p2UY21, labeled pExsA in the Figure). The resulting strains were grown in LB to an OD_{600}-0.6 and assayed for β-galactosidase activity. (B) *E. coli* strain GS162 carrying the indicated reporter and ExsA expression plasmid p2UY21 or p2UY21-*luxR* was transformed with a plasmid expressing the wild-type α or α-CTD subunit. The resulting strains were grown in LB to an OD_{600}-0.6 and assayed for β-galactosidase activity. The reported values (Miller Units [A] or % activity in the presence of wild-type α subunit [B]) represent the average of three independent experiments, and error bars represent the standard error of the means.

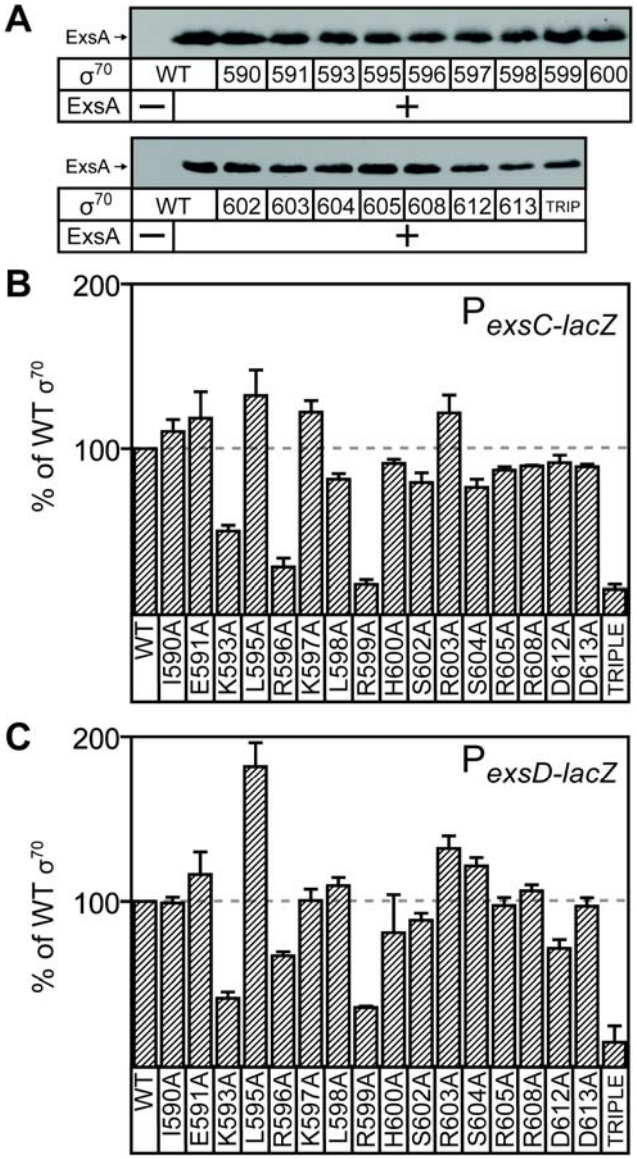

FIGURE 13. ExsA-dependent transcription is dependent on several amino acids in region 4.2 of *E. coli* σ^{70}. (A) ExsA immunoblots demonstrating that steady state expression levels are similar in each of the strains used below. (B-C) *E. coli* strain GA2071 (tightly suppressed for native σ^{70} expression) carrying the $P_{exsC\text{-}lacZ}$ or $P_{exsD\text{-}lacZ}$ transcriptional reporters and the p2UY21 ExsA expression plasmid was transformed with a wild-type σ^{70} expression plasmid or an σ^{70} expression plasmid carrying the indicated point mutation in region 4.2. The resulting strains were grown in LB to an OD_{600}-0.6 and assayed for β-galactosidase activity. The reported values (% activity in the presence of wild-type σ^{70} subunit) are the average of three independent experiments, and error bars represent the standard error of the means.

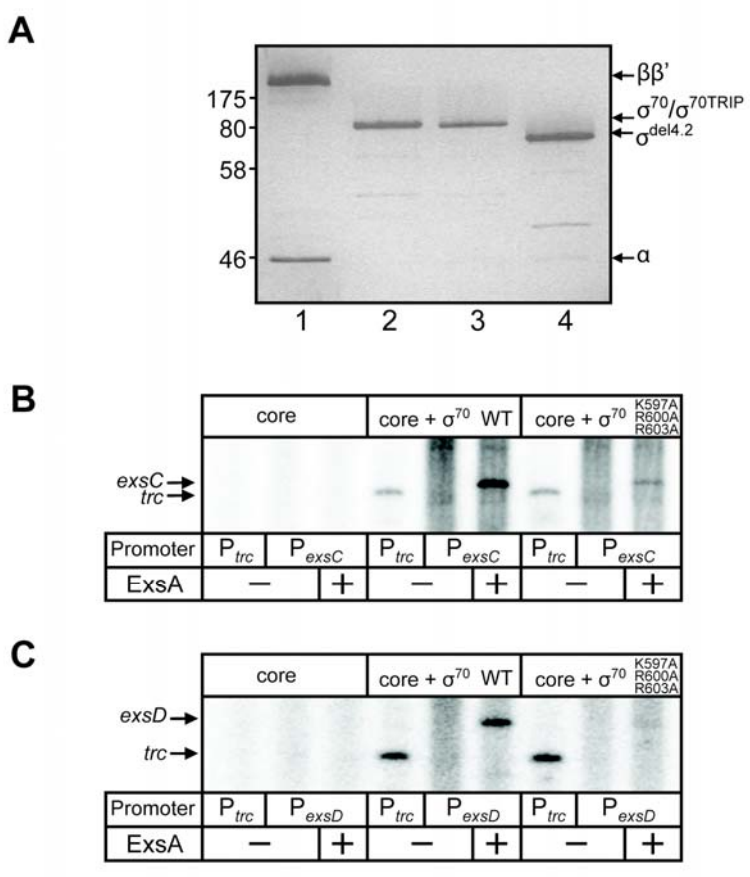

FIGURE 14. ExsA-dependent transcription *in vitro* is dependent on *P. aeruginosa* σ^{70} region 4.2 (A) Silver-stained SDS-polyacrylamide gel of purified and reconstituted core polymerase subunits α, β, and β' (lane 1), native σ^{70} (lane 2), σ^{70} carrying the K597A, R596A, and R599A amino acid substitutions (lane 3), and σ^{70} lacking region 4.2 (lane 4). (B-C) Single-round *in vitro* transcription assays. $ExsA_{His}$ (35 nM) was incubated with 2 nM supercoiled P_{exsC} or P_{exsD} promoter template (pOM90-P_{exsC} or pOM90-P_{exsD}) at 25ºC in presence of ATP and GTP. After 10 min, *P. aeruginosa* core RNAP, σ^{70}-RNAP, or σ^{70} (K597A/R596A/R599A)-RNAP was added (25 nM of each, the activity of σ-saturated enzymes was normalized with P_{trc}) and the reaction mixture was incubated for 1 min at 25ºC. Heparin and substrate nucleotides (including 2.5 μ Ci [α32P]-CTP) were immediately added, and the reaction mixture was incubated for 5 min at 25ºC. Reactions were terminated, and the resulting products were electrophoresed on a 5% denaturing polyacrylamide-urea gel and subjected to phosphorimaging. The ExsA-dependent terminated transcripts (261 nt) from the P_{exsC} or P_{exsD} promoters and the run-off transcripts (250 or 180 nt) from the P_{trc} promoter are indicated.

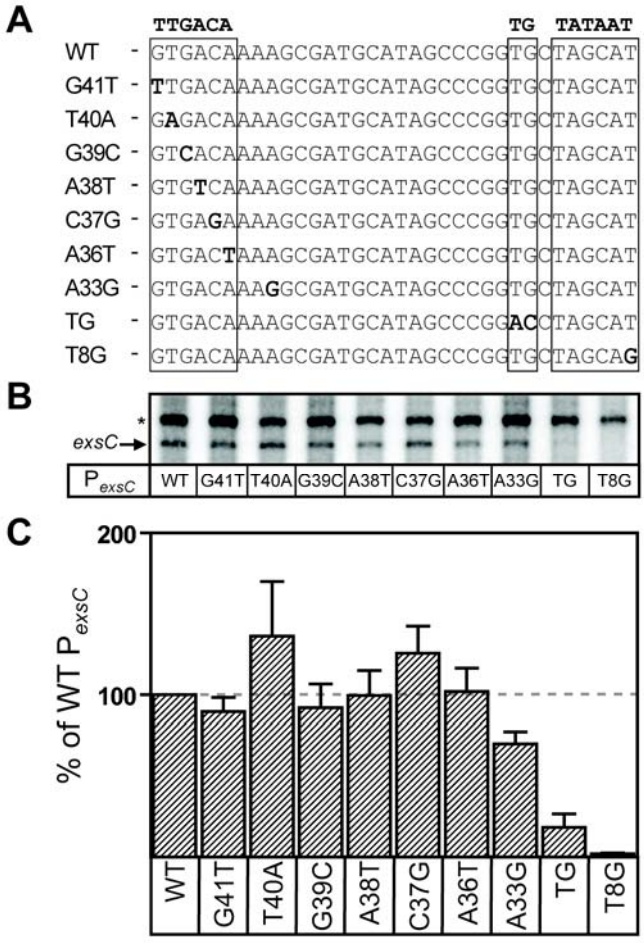

FIGURE 15. The near-consensus -35 hexamer at the P_{exsC} promoter is not required for ExsA-independent transcription. (A) Diagram showing the mutant P_{exsC} promoter derivatives used in this experiment. The -35, extended -10, and -10 elements are boxed and the individual point mutations are in bold typeface. (B) Single-round *in vitro* transcription assays showing ExsA-independent transcription from P_{exsC} derivatives containing -35 (G41T, T40A, G39C, A38T, C37G, A36T, and A33G), extended -10 (TG), and -10 (T8G) point mutations. Reactions were performed as described previously (Fig. 14) except open complexes were allowed to form for 20 min in the absence of ExsA. (C) Quantification of the *in vitro* transcription data shown in panel B. The amount of *exsC* transcript produced in each experiment was normalized to an ExsA-independent transcript (Vakulskas et al. 2009) produced from a weak promoter on the minicircle backbone. The reported values are the averages of three independent experiments, and error bars represent the standard error of the means.

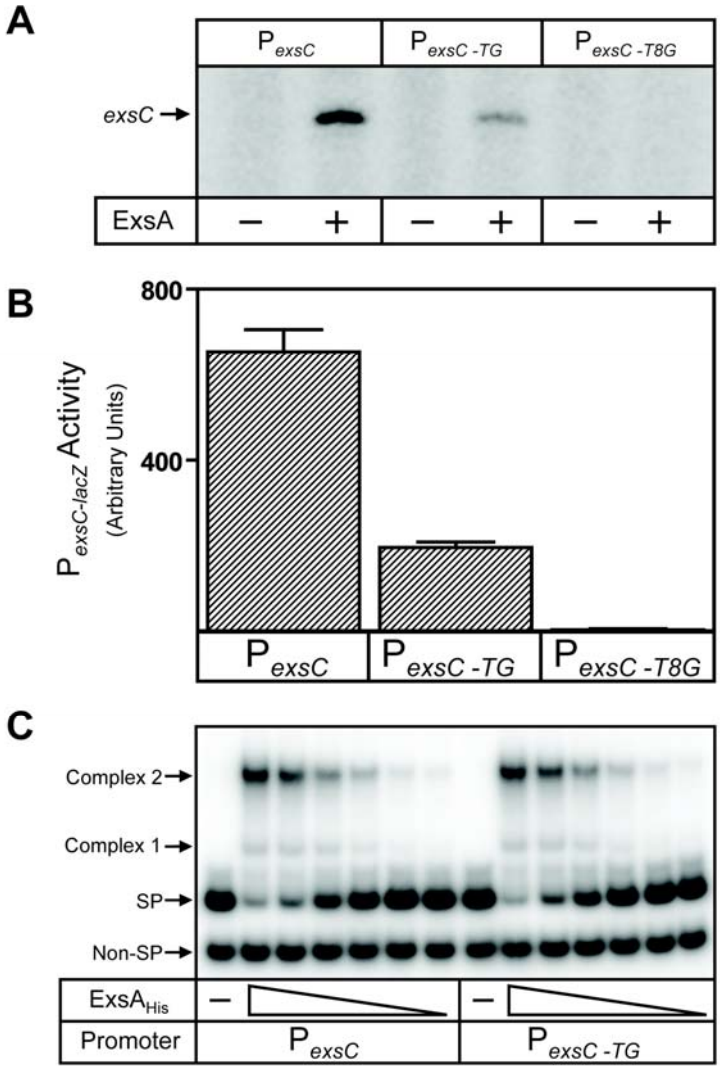

FIGURE 16. The extended -10 element is important for ExsA-independent and ExsA-dependent promoter activity. (A-B) Single-round *in vitro* transcription assays and quantification of the corresponding transcripts from the P_{exsC}, $P_{exsC\text{-}TG}$, and $P_{exsCT8G}$ promoters. Experiments were performed as described in Fig. 14 and allowing 1' for open complex formation in both absence and presence of ExsA. The reported values are the averages of three independent experiments, and error bars represent the standard error of the means. (C) Electrophoretic mobility shift assays (EMSAs) of the P_{exsC} and $P_{exsC\text{-}TG}$ promoter probes. Specific (SP) and non-specific (Non-SP) probes (0.25 nM each) were incubated in the absence of $ExsA_{His}$ (-) or with increasing concentrations of $ExsA_{His}$ (1.125-36 nM; 2-fold dilutions) for 15 min followed by electrophoresis and phosphorimaging. $ExsA_{His}$-dependent shift products 1 and 2 are indicated.

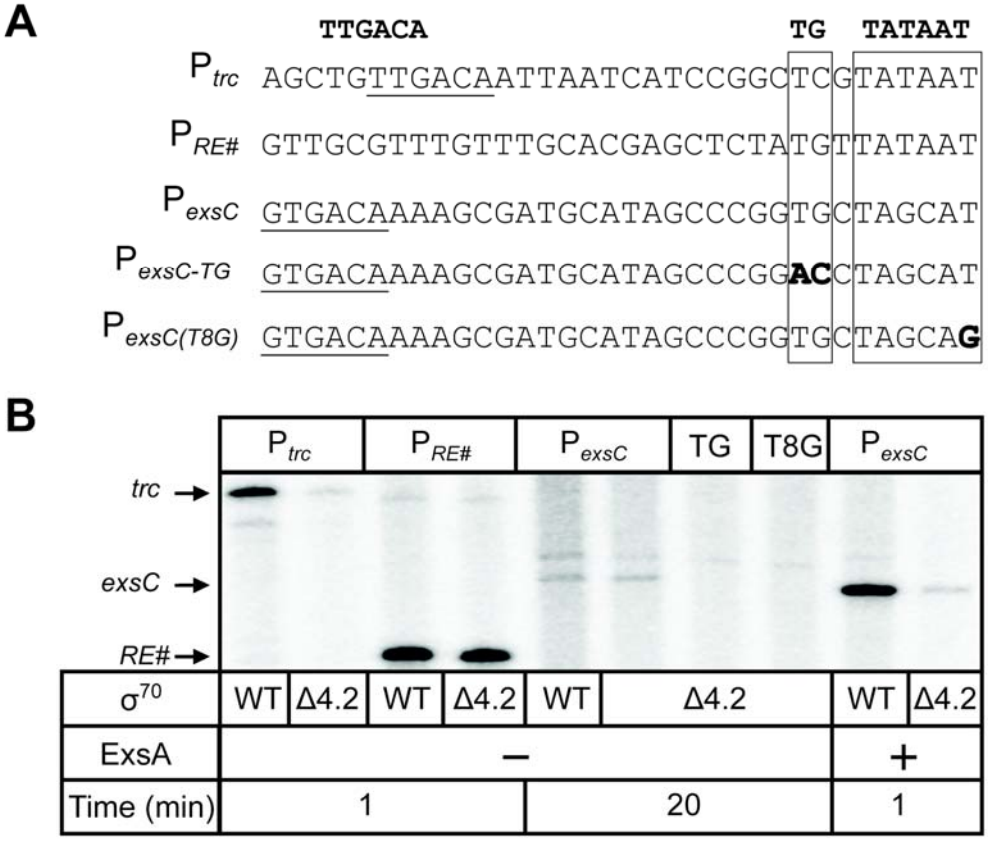

FIGURE 17. Region 4.2 of σ^{70} is required for ExsA-dependent but not ExsA-independent transcription. (A) Diagram of transcription templates used in this experiment. The -35 elements (underlined), extended -10 elements (boxed), -10 elements (boxed), and point mutations (bold typeface) are indicated. (B) Single-round *in vitro* transcription assays were performed with σ^{70} and $\sigma^{70\Delta4.2}$ reconstituted RNAP holoenzymes normalized for specific activity using the $P_{RE\#}$ extended -10 promoter (lanes 3 and 4). Reactions were performed as described previously (Fig. 14) and open complexes were allowed to form for 1 min (lanes 1-4,9-10) or 20 min (lanes 5-8) as indicated.

TABLE 5

Bacterial strains and plasmids used in Chapter IV

Strain or plasmid	Relevant characteristics	Reference
Pseudomonas aeruginosa strains		
PA103	wild-type parental strain	(Frank et al. 1994)
Escherichia coli strains		
DH5α	*recA* cloning strain	(Hanahan 1983)
GS162	wild-type strain carrying *ΔlacU169*	(Stauffer et al. 1981)
SA1751	thermoinducible Int expression from the cryptic prophage for minicircle recombination	(Choy and Adhya 1993)
GA2071	*rpoD* suppression strain	(Lonetto et al. 1998)
BL21 (DE3) Tuner	protein purification	(Novagen)
BW25141	maintenance of *pir*-dependent plasmids	(Haldimann and Wanner 2001)
Plasmids		
pREiiα	*rpoA* expression vector	(Blatter et al. 1994)
pGS490	*rpoA* expression vector with a stop codon at 239	(Jourdan and Stauffer 1999)
pJN105	arabinose inducible expression vector	(Newman and Fuqua 1999)
pUY30	arabinose inducible expression vector	(Urbanowski et al. 2007)
pMini-CTX-lacZ	vector for single-copy integration of *lacZ* reporters onto the *P. aeruginosa* chromosomal *attB* site	(Hoang et al. 2000)
pMCTX-$P_{lacUV5mut\text{-}lacZ}$	transcriptional fusion of the $P_{lacUV5mut\text{-}lacZ}$ promoter to *lacZ*	this study
p2UY21-*exsA*	plasmid that constitutively expresses *exsA*	this study
p2UY21-*luxR*	plasmid that constitutively expresses *luxR*	this study
pMU102	*luxR* expression vector	(Urbanowski et al. 2004)
pAH125	vector for single-copy integration of *lacZ* reporters onto the *E. coli* λ attachment site	(Haldimann and Wanner 2001)
p*luxI-lacZ*	translational fusion of the P_{luxI} promoter to *lacZ*	(Urbanowski et al. 2004)
pAH125-$P_{luxI\text{-}lacZ}$	translational fusion of the P_{luxI} promoter to *lacZ*	this study
pAH125-$P_{exsC\text{-}lacZ}$	transcriptional fusion of the P_{exsC} promoter to *lacZ*	this study
pAH125-$P_{exsD\text{-}lacZ}$	transcriptional fusion of the P_{exsD} promoter to *lacZ*	this study
pAH125-$P_{exoT\text{-}lacZ}$	transcriptional fusion of the P_{exoT} promoter to *lacZ*	this study
pGEX-*rpoD* and its derivatives	plasmid that constitutively expresses *rpoD* or one of 16 alanine point mutations	(Lonetto et al. 1998)
pGEX-$rpoD^{(K593A,R596A,R599A)}$	*rpoD* expression plasmid carrying the K593A, R596A, and R599A mutations	this study
pET-23b	protein expression vector that includes a carboxy-terminal His_6 tag	(Novagen)
pET23-$rpoD_{HisCTD}$ and its derivatives	RpoD expression vector with a carboxy-terminal His_6 tag	this study
pET-24a	protein expression vector that includes a carboxy-terminal His_6 tag	(Novagen)
pET24-$rpoA_{HisCTD}$	untagged RpoA expression vector	this study
pET24-*rpoB*	untagged RpoB expression vector	this study
pET24-*rpoC*	untagged RpoC expression vector	this study
pOM90	*in vitro* transcription template	(Richet and Sogaard-Andersen 1994)
pOM90-P_{exsC}	*in vitro* transcription template containing the P_{exsC} promoter	this study
pOM90-P_{exsD}	*in vitro* transcription template containing the P_{exsD} promoter	(Vakulskas et al. 2009)
pSA508-P_{exsC} and its derivatives	P_{exsC} template vector; yielding minicircle $pMCP_{exsC}$	(Vakulskas et al. 2009), this study
pTRCHIS-b	source of P_{trc} promoter	(Invitrogen)
pOM90-$P_{trc(250)}$	*in vitro* transcription template containing the P_{trc} promoter	this study
pOM90-$P_{trc(180)}$	*in vitro* transcription template containing the P_{trc} promoter	this study
pOM90-$P_{RE\#}$	*in vitro* transcription template the $P_{RE\#}$ promoter containing	this study
pET23-*rpoD* (1-574)	RpoD expression vector lacking region 4.2	this study

TABLE 6

Primers used in Chapter IV

Primer ID	Primer Sequence
44122038	CATGGCCATATGAAAAACATAAATGCCGAC
44122037	CATGGCGAGCTCTTAATTTTTAAAGTATGG
39530603	GCGACGCGGTACCATGAAGGACGTCCTGCAGCTCATCC
49188917	TGATGAATTCGCCTCCTAAAGCTCAGCGCATGC
48669731	CAGATCGAAGCGGCGGCGCTGGCCAAACTGGCTCACCCGAGCCGT
48669730	ACGGCTCGGGTGAGCCAGTTTGGCCAGCGCCGCCGCTTCGATCTG
43812190	CCGAGCCATATGTCCGGAAAAGCGCAA
43812191	GGCAGGAAGCTTCTCGTCGAGGAAGGAGCG
46001014	CAGATCGAAGCCGCGGCGTTGCGCAAG
46001013	CTTGCGCAACGCCGCGGCTTCGATCTG
47437714	TCGCGACGGATGGGCCAGCTTGCGCAA
47437715	TTGCGCAAGCTGGCCCATCCGTCGCGA
47437713	CGCTCCTTCCTCGCCGAGAAGCTTGCG
47437712	CGCAAGCTTCTCGGCGAGGAAGGAGCG
48432036	GCCGCGGCGTTGGCCAAGCTGGCCCAT
48432035	ATGGGCCAGCTTGGCCAACGCCGCGGC
46775590	GCCACCCATATGCAGAGTTCGGTAAATGAGTT
46775589	GCCTACGCGGCCGCGGCGGCAGTGGCCTTGTCGTCTTTCTTA
46775588	GCCACCCATATGGCTTACTCATACACTGAGAAAAAACG
46775587	GCCTACGCGGCCGCGGCTTATTCGGTTTCCAGTTCGATGTCG
47100507	GCCACCCATATGAAAGACTTGCTTAATCTGTTGAA
46775585	GCCTACGCGGCCGCGGCTTAGTTACCGCTCGAGTTCAGCGCTT
35048925	ATACTGGAATTCTGCGGTTCCCCCCC
35048926	ACGAATGAATTCCCACATCGGCCTCCAGCAAC
43648443	AAGAAAAGTCTCTCATTGACAAAAGCGATGC
43648442	GCATCGCTTTTGTCAATGAGAGACTTTTCTT
48552525	AAAGTCTCTCAGAGACAAAAGCGAG
48552524	CTCGCTTTTGTCTCTGAGAGACTTT
48552527	AAGTCTCTCAGTCACAAAAGCGAGG
48552526	CCTCGCTTTTGTGACTGAGAGACTT
43648441	AAAAGTCTCTCAGTGTCAAAAGCGATGCATA
43648440	TATGCATCGCTTTTGACACTGAGAGACTTTT
43648439	AAAGTCTCTCAGTGAGAAAAGCGATGCATAG
43648438	CTATGCATCGCTTTTCTCACTGAGAGACTTT
48552529	TCTCTCAGTGACTAAAGCGAGGCAT
48552528	ATGCCTCGCTTTAGTCACTGAGAGA
43648437	TCTCTCAGTGACAAAGGCGATGCATAGCCCG
43648436	CGGGCTATGCATCGCCTTTGTCACTGAGAGA
48552531	GGCATAGCCCGGACCTAGCATGCGCT
48552530	AGCGCATGCTAGGTCCGGGCTATGCC
43579324	AGCCCGGTGCTAGCAGGCGCTGAGCTTTAGG
43579323	CCTAAAGCTCAGCGCCTGCTAGCACCGGGCT
25444818	CTGCGAATTCAACGGTTCTGGCAAATATTC
25444816	CCGCGAATTCGGTTTATTCCTCCTTATTTAATCG
25444814	CTATGAATTCGAGTGCCCACACAGATTTC
48495914	GATCCTCGTTGCGTTTGTTTGCACGAGCTCTATGTTATAATTTCCTAAGCTTG
48495913	AATTCAAGCTTAGGAAATTATAACATAGAGTCGTGCAAACAAACGCAACGAG
48495915	GGCAGGAAGCTTCGACTGCATGGTGGAGTC

TABLE 7

Construction of plasmids used in Chapter IV

Figure	Product	Primer Pair	Cloning vector
Fig. 12B	p2UY21-LuxR	44122038-44122037	p2UY21
Fig. 12, 13	pAH125-P_{exsC}	39530603-49188917	pAH125
Fig. 13	pGEX-*rpoD* (K593A, R596A, R599A)	48669731-48669730	pGEX-*rpoD*
Fig. 14-17	$RpoD_{HisCTD}$	43812190-43812191	pET-23b
Fig. 14	RpoD (K597A)	46001014-46001013	pET23$RpoD_{HisCTD}$
Fig. 14	RpoD (R603A)	47437714-47437715	pET23$RpoD_{HisCTD}$
Fig. 14	RpoD (D616A)	47437713-47437712	pET23$RpoD_{HisCTD}$
Fig. 14	RpoD (K597A,R603A)	46001014-46001013	pET23$RpoD_{HisCTD}$ (R603A)
Fig. 14	RpoD (K597A, R600A, R603A)	48432036-48432035	pET23$RpoD_{HisCTD}$ (K597A,R603)
Fig. 14-17	$RpoA_{HisCTD}$	46775590-46775589	pET-24a
Fig. 14-17	RpoB	46775588-46775587	pET-24a
Fig. 14-17	RpoC	47100507-46775585	pET-24a
Fig. 14	pOM90-P_{exsC}	35048925-35048926	pOM90
Fig. 15	P_{exsC} (G41T)	43648443-43648442	pSA508-P_{exsC}
Fig. 15	P_{exsC} (T40A)	48552525-48552524	pSA508-P_{exsC}
Fig. 15	P_{exsC} (G39C)	48552527-48552526	pSA508-P_{exsC}
Fig. 15	P_{exsC} (A38T)	43648441-43648440	pSA508-P_{exsC}
Fig. 15	P_{exsC} (C37G)	43648439-43648438	pSA508-P_{exsC}
Fig. 15	P_{exsC} (A36T)	48552529-48552528	pSA508-P_{exsC}
Fig. 15	P_{exsC} (A33G)	43648437-43648436	pSA508-P_{exsC}
Fig. 15-17	P_{exsC} (TG)	48552531-48552530	pSA508-P_{exsC}
Fig. 15-17	P_{exsC} (T8G)	43579324-43579323	pSA508-P_{exsC}
Fig. 17	pOM90-$P_{trc(250)}$	25444818-25444816	pOM90
Fig. 17	pOM90-$P_{trc(179)}$	25444818-25444814	pOM90
Fig. 17	pOM90-$P_{RE\#}$	48495914-48495913	pOM90
Fig. 17	RpoD (1-574)	43812190-48495915	pET-23b

CHAPTER V

GENERAL DISCUSSION

ExsA Stoichiometry and Ligand Response

Oligmerization (when present) by an AraC-family member is mediated by a self-interaction domain that is separate from the DNA-binding domain (Gallegos et al. 1997). In the present study I determined that ExsA is monomeric in solution (Fig. 4). I furthermore show that ExsA binds DNA at T3SS promoters forming complexes that have 1 or 2 ExsA molecules bound (Fig 4). Current research indicates that the stoichiometric state of individual AraC-family members does not change when in solution or when DNA-bound. AraC, for example, forms a dimer that persists when free in solution and when DNA-bound (LaRonde-LeBlanc and Wolberger 2000). In contrast, AraC-family members like MarA exist as monomers both when in solution and when DNA-bound (Rhee et al. 1998). These combined data indicate that the stoichiometric state of ExsA may be unique among AraC-family regulators. Data that conflict with this hypothesis, however, include monohybrid experiments which demonstrate that ExsA can self-associate, and that multimerization is mediated by the amino-terminal domain (Brutinel et al. 2009a). One potential explanation for this discrepancy is that monomeric ExsA, which is fully able to bind DNA and activate transcription *in vitro*, represents a physiologically irrelevant form of the protein, and is an *in vitro* artifact. Alternatively, it may be possible that ExsA does not readily dimerize and that by binding DNA, either in the context of the native protein or as a LexsA-fusion, the local concentration of ExsA is increased thereby promoting self-association. Resolving this discrepancy will undoubtedly provide insight into the mechanism in which ExsA interacts with itself, DNA, and ExsD.

To further characterize the mechanism by which ExsD antagonizes ExsA activity, I performed chromatin immunoprecipitation assays *in vivo*. Using this assay I demonstrated that ExsD inhibits the binding of ExsA to DNA at the P_{exsC} and P_{exsD}

promoters (Fig. 5). Recent data suggests that ExsD also disrupts the self-association properties of ExsA (Brutinel et al. 2009a). Anti-activators from other systems (non-AraC targets) function by one of three mechanisms; preventing self-association of the activator (Chai et al. 2001), occluding the DNA binding domain from interacting with DNA (Navarro-Aviles et al. 2007), or inducing conformational changes that alter the structure of the DNA binding domain (Chen et al. 2007). My data best support a mechanism in which ExsD inhibits the DNA-binding activity of ExsA, as the amount of promoter-bound ExsA was significantly lower when ExsD was present (Brutinel et al. 2009a). Of the AraC-family members that are known to interact with a ligand, ExsA is the first example where DNA-binding activity is lost. AraC, for example, binds arabinose and causes the AraC dimer to occupy a different set of DNA binding sites (Gallegos et al. 1997). In contrast, AraC-family members like RhaS and UreR, have dramatically increased affinity for DNA in the presence of rhamnose and urea, respectively (Gallegos et al. 1997). The loss of DNA-binding activity by an activator-ligand interaction is a unique mechanism that may be shared by homologous AraC-family members that regulate T3SSs such as MxiE from *S. flexneri*.

Mechanism of ExsA-Dependent Transcription

Functional diversity seems to be the hallmark of AraC-family regulators as they can have dramatically different ligands, stoichiometries, and DNA-binding sites; it seems hardly surprising that the transcriptional activation mechanism is no exception. The mechanism of transcription is known for very few AraC-family proteins. The prototypical family member, AraC, recruits RNAP to promoters and enhances isomerization to an open complex (Zhang et al. 1996). In the present study I present evidence that ExsA strongly recruits (5 to 10-fold) RNAP to T3SS promoters and has only a modest effect on the isomerization to an open complex (< 2-fold). I propose that the difference in transcriptional activation mechanisms between AraC-family proteins

may be due to differences in promoter structures and activator-DNA binding sites. For example, the AraC-dependent P_{BAD} promoter contains σ^{70}-dependent -35 (TAGATC) and -10 (GATCCT) RNAP recognition sites that are separated by 17 bp. The available data suggests that RNAP recognizes the P_{BAD} -35 hexamer (albeit weakly) in the absence of AraC. Additionally, the -10 hexamer is located properly with respect to the transcription start site, and the unwinding of DNA during open complex formation occurs from -10 to +1 (Zhang et al. 1996). These data suggest that the -35 and -10 hexamers are functional, and that the poor AraC-independent activity from P_{BAD} is most likely because the -35 and -10 are poor matches (both 3/6) to the σ^{70} consensus.

In contrast, my data indicates that T3SS promoter consensus -35 sites are not RNAP recognition determinants. For example, I have established that the P_{exsD} promoter near-consensus -10 hexamer (TAGAAT) is properly spaced with respect to the P_{exsD} transcription start site (Fig. 8, 10). Functionally, I have determined that DNA unwinds at the P_{exsD} -10 site during open complex formation (Fig. 10). The P_{exsD} putative -35 hexamer (TTGAAA), however, is spaced 21 bp from the -10 site; an increase of 4 bp compared to the σ^{70} consensus (Fig. 3). Furthermore, I have determined that the P_{exsD} -35 hexamer is a poor recognition site even in the context of ideal promoter spacing (Fig. 6). A simple interpretation of this data is that whereas the function of AraC is to overcome relatively weak -35 and -10 sites, the function of ExsA is to overcome the complete lack of a -35 site. The following data support this hypothesis: (i) whereas mutations in the -35 site of σ^{70} promoters affect closed complex formation, mutations in the -10 site of σ^{70} promoters affect isomerization to an open complex (Paget and Helmann 2003); and, (ii) ExsA strongly promotes closed complex formation while AraC strongly promotes both closed and open complex formation (Fig. 11)(Zhang et al. 1996). A more complex interpretation of the data, however, is that ExsA and AraC activate transcription by fundamentally different mechanisms. This interpretation seems unlikely as AraC, ExsA, and a host of other AraC-family members interact with the same amino acids in σ^{70}

region 4.2 (Table 8). As the transcriptional activation mechanisms of additional AraC-family members become available, perhaps some of these questions can be answered.

ExsA-RNAP Interaction

In chapter IV I present data that indicates ExsA interacts with region 4.2 of σ^{70}. The three amino acids I determined to be important for ExsA-σ^{70}-RNAP interaction are some of the most common positions identified in studies of both related and unrelated transcriptional activators (Table 8). It is curious, however, that the interaction of ExsA with region 4.2 of σ^{70} functions primarily in the recruitment of RNAP. Most dimeric AraC family members are known to both recruit RNAP and promote isomerization to the open complex, and these activities are thought to result from individual contacts with α-CTD and σ^{70}, respectively (Egan 2002; Gallegos et al. 1997; Zhang et al. 1996). The best example of an activator (non AraC-family) that interacts with σ^{70} region 4.2, cI, increases the isomerization rate at the P_{RM} promoter (Dove et al. 2000; Hawley and McClure 1982). The cI example is somewhat paradoxical since it has been well established that σ^{70} region 4.2 interacts directly with DNA at the -35 position to facilitate the initial binding of RNAP to the promoter (Campbell et al. 2002). In fact, the observation that ExsA recruits RNAP through contacts with σ^{70} region 4.2 seems to better support the known function of region 4.2. I believe the most likely explanation for these discrepancies is that protein-protein interactions with σ^{70} region 4.2 can harbor effects on both closed and open complex formation. In support of this claim a single point mutation (R596H) in σ^{70} region 4.2 changes the mechanism of cI activation to an enhancement on closed complex formation, while having almost no effect on the isomerization rate to an open complex (Dove et al. 2000). We furthermore believe that the nature of the specific interaction between σ^{70} region 4.2 and transcriptional activator does not drastically differ whether it enhances closed or open complex formation. *Dove et al*. have suggested that the promoter sequence and location of the activator binding site may play the most important part in determining the mechanism of transcriptional activation by an activator (Dove et

al. 2000). Further studies analyzing the structure of activator-RNAP complexes are needed to address this curiosity.

Model for Activation of T3SS Gene Expression by ExsA

My data and the published literature combined have led me to propose the following model for how ExsA activates transcription from T3SS promoters. Monomeric ExsA preferentially binds to site 1 at T3SS promoters. Monomeric ExsA bound to site 1 recruits a second molecule to site 2 by interacting with the NTD. Through specific interactions with region 4.2 of σ^{70}, ExsA then recruits RNAP to T3SS promoters and open complexes are readily formed.

The available data suggests that in cases where two or more activator molecules bind to DNA, the promoter-proximal molecule contacts σ^{70}. Examples of this phenomenon include the fumarate/nitrate reductase regulator FNR and the catabolite activator protein CRP (Blake et al. 2002; Busby and Ebright 1999). Since ExsA interacts with region 4.2 of σ^{70} independent of the α-CTD, I predict that the promoter-proximal ExsA monomer interacts with σ^{70}, and that the promoter-distal ExsA monomer stabilizes this interaction. A problem with this hypothesis, however, is that mutations in ExsA binding site 2 completely disrupt ExsA-dependent activation. Mutations in ExsA binding site 2 do not significantly affect ExsA binding to site 1 (Brutinel et al. 2008), and therefore the promoter-distal ExsA molecule must be important for ExsA-dependent transcriptional activation. A potential explanation for this discrepancy is that interaction with σ^{70} region 4.2 is necessary but not sufficient for ExsA-dependent transcriptional activation. An alternative hypothesis is that although a specific interaction between ExsA monomers at T3SS promoters is not required for ExsA-dependent transcriptional activation (Brutinel et al. 2009b), binding of a second ExsA monomer to site 2 may bend DNA to allow ExsA bound at site 1 to efficiently contact σ^{70} region 4.2. This is an attractive hypothesis since ExsA exhibits DNA bending, a phenomenon known to be

required for the mechanism of some transcriptional activators (Kerppola and Curran 1997).

I also provide a model that explains how ExsA-independent transcription at the P_{exsC} promoter occurs in the absence of a -35 hexamer. In this model, σ^{70}-RNAP binds the P_{exsC} promoter at an extended -10 element and this is sufficient to provide basal transcription in the absence of ExsA. This data also seems to indicate that a maintenance level of T3SS gene expression occurs in an ExsA-independent fashion. This interpretation introduces a paradox, as ExsA strongly activates its own expression and it is therefore unclear whether basal transcription from the P_{exsC} promoter is required for T3SS gene expression. If so, basal P_{exsC} promoter activity may be required for the maintenance of ExsA in the cell, or the initial expression of the *exsCEBA* operon in a newly divided cell. The former hypothesis seems unlikely, as the basal level of P_{exsC} promoter activity in an *exsA* mutant is at least 10-fold lower than P_{exsC} activity in an uninduced wild-type cell (Dasgupta et al. 2004). Whether ExsA-independent P_{exsC} promoter activity plays a role in the initial expression of *exsCEBA* in a newly divided cell would depend on whether the number of ExsA molecules (if any) acquired from the parent cell is sufficient to activate the P_{exsC} promoter.

Future Directions

The available data suggests that ExsA bound to both sites 1 and 2 is required for transcriptional activation of T3SS promoters (Brutinel et al. 2008). Since ExsA most likely directly interacts with σ^{70} region 4.2, I predict that ExsA bound to site 1 is responsible for this interaction based on its proximity to the -10 hexamer. This hypothesis is supported with data from the dimeric Crp and FNR regulators in which the promoter-proximal molecule contacts σ^{70} region 4.2 (Blake et al. 2002; Busby and Ebright 1999). Alternatively, ExsA bound to site 1 may not interact with RNAP, and solely functions to recruit an additional ExsA monomer to site 2. The ExsA molecule

bound to site 2 would then contact region 4.2 of σ^{70} to activate transcription. This hypothesis would explain why ExsA bound to both sites 1 and 2 is required for transcription, but seems improbable given the distance between site 2 and the -10 hexamer. A potential way to test this hypothesis would involve isolating both ExsA mutants that are unable to interact with region 4.2 of σ^{70}, and alleles of ExsA that bind altered recognition sites. Using heterodimers containing the afore-mentioned ExsA mutants and wild-type ExsA, the role of each ExsA molecule in transcription activation can be determined. This question remains perhaps the most interesting facet of ExsA-dependent transcriptional activation as it potentially sets it apart from other AraC-family members.

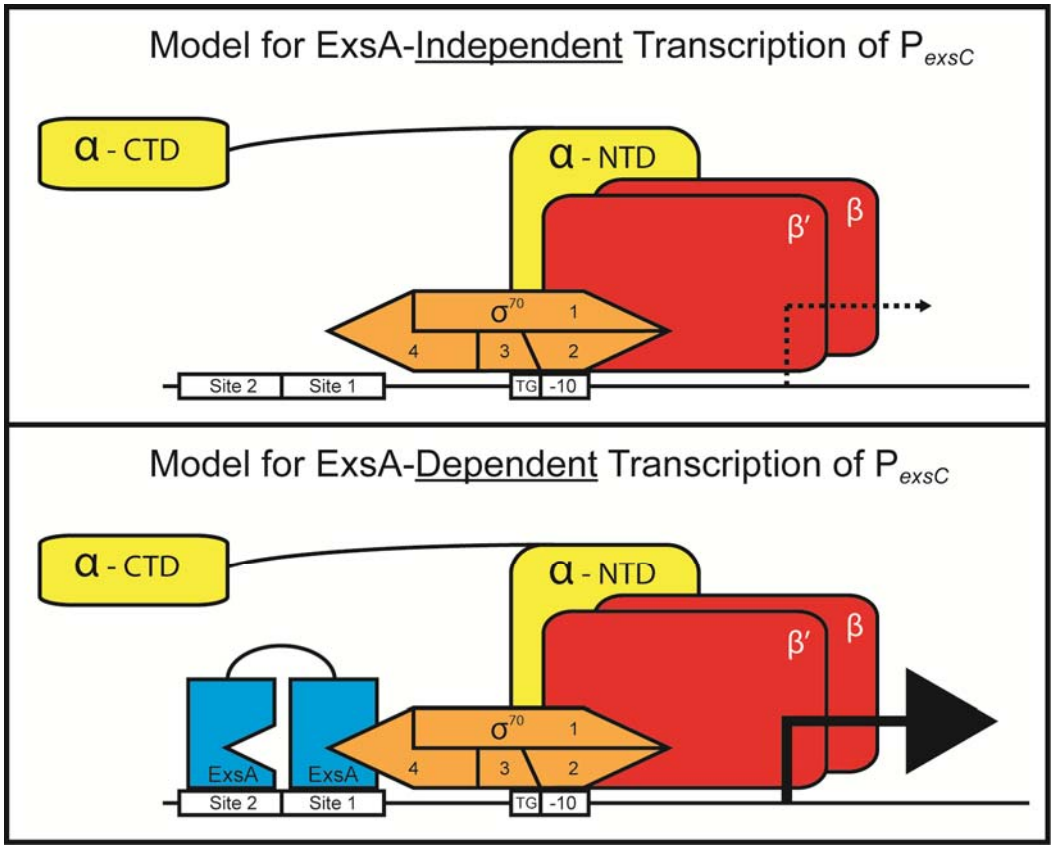

FIGURE 18. Model for transcription activation of the P_{exsC} promoter. In the absence of ExsA, the σ^{70} subunit of RNAP recognizes the extended -10 and -10 elements, and weakly activates transcription. An ExsA monomer then binds site 1 and recruits a second molecule to site 2. The promoter-proximal ExsA molecule (bound to site 1) then recruits RNAP by an interaction with region 4.2 of σ^{70}.

TABLE 8

Transcription activators that interact with region 4.2 of σ^{70}

Regulator	Amino Acids	Organism	Reference
AraC Family			
ExsA	**K593**, **R596**, **R599**	*P. aeruginosa*	(this study)
AraC	K591, **K593**, **E596**	*E. coli*	(Hu and Gross 1985) (Lonetto et al. 1998) (Travers 1974)
RhaS	**K593**, **R599**	*E. coli*	(Bhende and Egan 2000) (Wickstrum and Egan 2004)
RhaR	**K593**, L595, **R599**, R608	*E. coli*	(Wickstrum and Egan 2004)
MelR	**R596**, **R599**	*E. coli*	(Grainger et al. 2004)
Ada*	**K593**, K597, R603	*E. coli*	(Landini and Busby 1999)
Unrelated regulators			
CRP	**K593**, K597, **R599** R603	*E. coli*	(Lonetto et al. 1998) (Rhodius and Busby 2000)
FNR	**K593**, **R596**, R603	*E. coli*	(Lonetto et al. 1998)
LuxR	E591, L595, **R596**, K597, S602, R603	*V. fischeri*	(Johnson et al. 2003)
GcvA	E591, **K593**, L595, H600, S602, R603	*E. coli*	(Stauffer and Stauffer 2005)
CI	R588, **K593**, **R596**	Phage λ	(Kuldell and Hochschild 1994) (Li et al. 1994) (Nickels et al. 2002)

REFERENCES

Akman SA, Doroshow JH, and Dizdaroglu M. 1990. Base modifications in plasmid DNA caused by potassium permanganate. Arch Biochem Biophys 282(1):202-205.

Allan B, and Kropinski AM. 1987. DNA-dependent RNA polymerase from Pseudomonas aeruginosa. Biochem Cell Biol 65(9):776-782.

Antunes LC, Schaefer AL, Ferreira RB, Qin N, Stevens AM, Ruby EG, and Greenberg EP. 2007. Transcriptome analysis of the Vibrio fischeri LuxR-LuxI regulon. J Bacteriol 189(22):8387-8391.

Badea L, Beatson SA, Kaparakis M, Ferrero RL, and Hartland EL. 2009. Secretion of flagellin by the LEE-encoded type III secretion system of enteropathogenic Escherichia coli. BMC Microbiol 9:30.

Barbieri JT, and Sun J. 2004. Pseudomonas aeruginosa ExoS and ExoT. Rev Physiol Biochem Pharmacol 152:79-92.

Barne KA, Bown JA, Busby SJ, and Minchin SD. 1997. Region 2.5 of the Escherichia coli RNA polymerase sigma70 subunit is responsible for the recognition of the 'extended-10' motif at promoters. Embo J 16(13):4034-4040.

Becher A, and Schweizer HP. 2000. Integration-proficient Pseudomonas aeruginosa vectors for isolation of single-copy chromosomal lacZ and lux gene fusions. BioTechniques 29(5):948-950, 952.

Bendiak GN, and Ratjen F. 2009. The approach to Pseudomonas aeruginosa in cystic fibrosis. Semin Respir Crit Care Med 30(5):587-595.

Berka RM, Gray GL, and Vasil ML. 1981. Studies of phospholipase C (heat-labile hemolysin) in Pseudomonas aeruginosa. Infect Immun 34(3):1071-1074.

Bernhards RC, Jing X, Vogelaar NJ, Robinson H, and Schubot FD. 2009. Structural evidence suggests that antiactivator ExsD from Pseudomonas aeruginosa is a DNA binding protein. Protein Sci 18(3):503-513.

Bhende PM, and Egan SM. 1999. Amino acid-DNA contacts by RhaS: an AraC family transcription activator. J Bacteriol 181(17):5185-5192.

Bhende PM, and Egan SM. 2000. Genetic evidence that transcription activation by RhaS involves specific amino acid contacts with sigma 70. J Bacteriol 182(17):4959-4969.

Bird TH, Grimsley JK, Hoch JA, and Spiegelman GB. 1996. The Bacillus subtilis response regulator Spo0A stimulates transcription of the spoIIG operon through modification of RNA polymerase promoter complexes. Journal of molecular biology 256(3):436-448.

Blake T, Barnard A, Busby SJ, and Green J. 2002. Transcription activation by FNR: evidence for a functional activating region 2. J Bacteriol 184(21):5855-5861.

Blatter EE, Ross W, Tang H, Gourse RL, and Ebright RH. 1994. Domain organization of RNA polymerase alpha subunit: C-terminal 85 amino acids constitute a domain capable of dimerization and DNA binding. Cell 78(5):889-896.

Blaylock B, Riordan KE, Missiakas DM, and Schneewind O. 2006. Characterization of the Yersinia enterocolitica type III secretion ATPase YscN and its regulator, YscL. J Bacteriol 188(10):3525-3534.

Bleves S, Soscia C, Nogueira-Orlandi P, Lazdunski A, and Filloux A. 2005. Quorum sensing negatively controls type III secretion regulon expression in Pseudomonas aeruginosa PAO1. J Bacteriol 187(11):3898-3902.

Bowser TE, Bartlett VJ, Grier MC, Verma AK, Warchol T, Levy SB, and Alekshun MN. 2007. Novel anti-infection agents: small-molecule inhibitors of bacterial transcription factors. Bioorg Med Chem Lett 17(20):5652-5655.

Brosius J, Erfle M, and Storella J. 1985. Spacing of the -10 and -35 regions in the tac promoter. Effect on its in vivo activity. J Biol Chem 260(6):3539-3541.

Brutinel ED, Vakulskas CA, Brady KM, and Yahr TL. 2008. Characterization of ExsA and of ExsA-dependent promoters required for expression of the Pseudomonas aeruginosa type III secretion system. Mol Microbiol 68(3):657-671.

Brutinel ED, Vakulskas CA, and Yahr TL. 2009a. ExsD inhibits expression of the Pseudomonas aeruginosa type III secretion system by disrupting ExsA self-association and DNA binding activity. J Bacteriol.

Brutinel ED, Vakulskas CA, and Yahr TL. 2009b. Functional domains of ExsA, the transcriptional activator of the Pseudomonas aeruginosa type III secretion system. J Bacteriol 191(12):3811-3821.

Burgess RR, and Jendrisak JJ. 1975. A procedure for the rapid, large-scall purification of Escherichia coli DNA-dependent RNA polymerase involving Polymin P precipitation and DNA-cellulose chromatography. Biochemistry 14(21):4634-4638.

Burgess RR, Travers AA, Dunn JJ, and Bautz EK. 1969. Factor stimulating transcription by RNA polymerase. Nature 221(5175):43-46.

Busby S, and Ebright RH. 1999. Transcription activation by catabolite activator protein (CAP). J Mol Biol 293(2):199-213.

Cacalano G, Kays M, Saiman L, and Prince A. 1992. Production of the Pseudomonas aeruginosa neuraminidase is increased under hyperosmolar conditions and is regulated by genes involved in alginate expression. J Clin Invest 89(6):1866-1874.

Campbell EA, Muzzin O, Chlenov M, Sun JL, Olson CA, Weinman O, Trester-Zedlitz ML, and Darst SA. 2002. Structure of the bacterial RNA polymerase promoter specificity sigma subunit. Mol Cell 9(3):527-539.

Chai Y, Zhu J, and Winans SC. 2001. TrlR, a defective TraR-like protein of Agrobacterium tumefaciens, blocks TraR function in vitro by forming inactive TrlR:TraR dimers. Mol Microbiol 40(2):414-421.

Chen G, Jeffrey PD, Fuqua C, Shi Y, and Chen L. 2007. Structural basis for antiactivation in bacterial quorum sensing. Proc Natl Acad Sci U S A 104(42):16474-16479.

Chopra I. 2007. Bacterial RNA polymerase: a promising target for the discovery of new antimicrobial agents. Curr Opin Investig Drugs 8(8):600-607.

Choy HE, and Adhya S. 1993. RNA polymerase idling and clearance in gal promoters: use of supercoiled minicircle DNA template made in vivo. Proc Natl Acad Sci U S A 90(2):472-476.

Cornelis GR. 2002a. The Yersinia Ysc-Yop 'type III' weaponry. Nat Rev Mol Cell Biol 3(10):742-752.

Cornelis GR. 2002b. The Yersinia Ysc-Yop virulence apparatus. Int J Med Microbiol 291(6-7):455-462.

Cowell BA, Twining SS, Hobden JA, Kwong MS, and Fleiszig SM. 2003. Mutation of lasA and lasB reduces Pseudomonas aeruginosa invasion of epithelial cells. Microbiology 149(Pt 8):2291-2299.

Cowell BA, Willcox MD, Herbert B, and Schneider RP. 1999. Effect of nutrient limitation on adhesion characteristics of Pseudomonas aeruginosa. J Appl Microbiol 86(6):944-954.

Crouch Brewer S, Wunderink RG, Jones CB, and Leeper KV, Jr. 1996. Ventilator-associated pneumonia due to Pseudomonas aeruginosa. Chest 109(4):1019-1029.

Cunha BA. 2002. Pseudomonas aeruginosa: resistance and therapy. Semin Respir Infect 17(3):231-239.

Dacheux D, Goure J, Chabert J, Usson Y, and Attree I. 2001. Pore-forming activity of type III system-secreted proteins leads to oncosis of Pseudomonas aeruginosa-infected macrophages. Mol Microbiol 40(1):76-85.

Dasgupta N, Lykken GL, Wolfgang MC, and Yahr TL. 2004. A novel anti-anti-activator mechanism regulates expression of the Pseudomonas aeruginosa type III secretion system. Mol Microbiol 53(1):297-308.

Davis CA, Bingman CA, Landick R, Record MT, Jr., and Saecker RM. 2007. Real-time footprinting of DNA in the first kinetically significant intermediate in open complex formation by Escherichia coli RNA polymerase. Proc Natl Acad Sci U S A 104(19):7833-7838.

Dominquez-Cuevas P, and Marques, S. 2004. Compiling 70-Dependent Promoters. In, Ramos, J-L, Pseudomonas: Volume 2: Virulence and Gene Regulation:319-344.

Dove SL, and Hochschild A. 1998. Conversion of the omega subunit of Escherichia coli RNA polymerase into a transcriptional activator or an activation target. Genes Dev 12(5):745-754.

Dove SL, Huang FW, and Hochschild A. 2000. Mechanism for a transcriptional activator that works at the isomerization step. Proc Natl Acad Sci U S A 97(24):13215-13220.

Egan SM. 2002. Growing repertoire of AraC/XylS activators. J Bacteriol 184(20):5529-5532.

Egan SM, Pease AJ, Lang J, Li X, Rao V, Gillette WK, Ruiz R, Ramos JL, and Wolf RE, Jr. 2000. Transcription activation by a variety of AraC/XylS family activators does not depend on the class II-specific activation determinant in the N-terminal domain of the RNA polymerase alpha subunit. J Bacteriol 182(24):7075-7077.

Engel J, and Balachandran P. 2009. Role of Pseudomonas aeruginosa type III effectors in disease. Curr Opin Microbiol 12(1):61-66.

Feltman H, Schulert G, Khan S, Jain M, Peterson L, and Hauser AR. 2001. Prevalence of type III secretion genes in clinical and environmental isolates of Pseudomonas aeruginosa. Microbiology 147(Pt 10):2659-2669.

Fierer J, Taylor PM, and Gezon HM. 1967. Pseudomonas aeruginosa epidemic traced to delivery-room resuscitators. N Engl J Med 276(18):991-996.

Fleiszig SM, Wiener-Kronish JP, Miyazaki H, Vallas V, Mostov KE, Kanada D, Sawa T, Yen TS, and Frank DW. 1997. Pseudomonas aeruginosa-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. Infect Immun 65(2):579-586.

Franchi L, Amer A, Body-Malapel M, Kanneganti TD, Ozoren N, Jagirdar R, Inohara N, Vandenabeele P, Bertin J, Coyle A et al. . 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. Nat Immunol 7(6):576-582.

Frank DW. 1997. The exoenzyme S regulon of Pseudomonas aeruginosa. Mol Microbiol 26(4):621-629.

Frank DW, and Iglewski BH. 1991. Cloning and sequence analysis of a trans-regulatory locus required for exoenzyme S synthesis in Pseudomonas aeruginosa. J Bacteriol 173(20):6460-6468.

Frank DW, Nair G, and Schweizer HP. 1994. Construction and characterization of chromosomal insertional mutations of the Pseudomonas aeruginosa exoenzyme S trans-regulatory locus. Infect Immun 62(2):554-563.

Gaal T, Ross W, Blatter EE, Tang H, Jia X, Krishnan VV, Assa-Munt N, Ebright RH, and Gourse RL. 1996. DNA-binding determinants of the alpha subunit of RNA polymerase: novel DNA-binding domain architecture. Genes Dev 10(1):16-26.

Gallegos MT, Schleif R, Bairoch A, Hofmann K, and Ramos JL. 1997. Arac/XylS family of transcriptional regulators. Microbiol Mol Biol Rev 61(4):393-410.

Geffers C, Zuschneid I, Sohr D, Ruden H, and Gastmeier P. 2004. [Microbiological isolates associated with nosocomial infections in intensive care units: data of 274 intensive care units participating in the German Nosocomial Infections Surveillance System (KISS)]. Anasthesiol Intensivmed Notfallmed Schmerzther 39(1):15-19.

Goehring UM, Schmidt G, Pederson KJ, Aktories K, and Barbieri JT. 1999. The N-terminal domain of Pseudomonas aeruginosa exoenzyme S is a GTPase-activating protein for Rho GTPases. J Biol Chem 274(51):36369-36372.

Goure J, Pastor A, Faudry E, Chabert J, Dessen A, and Attree I. 2004. The V antigen of Pseudomonas aeruginosa is required for assembly of the functional PopB/PopD translocation pore in host cell membranes. Infect Immun 72(8):4741-4750.

Grainger DC, Webster CL, Belyaeva TA, Hyde EI, and Busby SJ. 2004. Transcription activation at the Escherichia coli melAB promoter: interactions of MelR with its DNA target site and with domain 4 of the RNA polymerase sigma subunit. Mol Microbiol 51(5):1297-1309.

Grana D, Gardella T, and Susskind MM. 1988. The effects of mutations in the ant promoter of phage P22 depend on context. Genetics 120(2):319-327.

Gross CA, Chan CL, and Lonetto MA. 1996. A structure/function analysis of Escherichia coli RNA polymerase. Philos Trans R Soc Lond B Biol Sci 351(1339):475-482.

Ha U, and Jin S. 2001. Growth phase-dependent invasion of Pseudomonas aeruginosa and its survival within HeLa cells. Infect Immun 69(7):4398-4406.

Ha UH, Kim J, Badrane H, Jia J, Baker HV, Wu D, and Jin S. 2004. An in vivo inducible gene of Pseudomonas aeruginosa encodes an anti-ExsA to suppress the type III secretion system. Mol Microbiol 54(2):307-320.

Hahn HP. 1997. The type-4 pilus is the major virulence-associated adhesin of Pseudomonas aeruginosa--a review. Gene 192(1):99-108.

Haldimann A, and Wanner BL. 2001. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. J Bacteriol 183(21):6384-6393.

Hanahan D. 1983. Studies on transformation of Escherichia coli with plasmids. Journal of molecular biology 166(4):557-580.

Harley CB, and Reynolds RP. 1987. Analysis of E. coli promoter sequences. Nucleic Acids Res 15(5):2343-2361.

Harrison GP, Mayo MS, Hunter E, and Lever AM. 1998. Pausing of reverse transcriptase on retroviral RNA templates is influenced by secondary structures both 5' and 3' of the catalytic site. Nucleic Acids Res 26(14):3433-3442.

Hauser AR. 2009. The type III secretion system of Pseudomonas aeruginosa: infection by injection. Nat Rev Microbiol 7(9):654-665.

Hawley DK, and McClure WR. 1982. Mechanism of activation of transcription initiation from the lambda PRM promoter. Journal of molecular biology 157(3):493-525.

Hawley DK, and McClure WR. 1983. Compilation and analysis of Escherichia coli promoter DNA sequences. Nucleic Acids Res 11(8):2237-2255.

Hendrickson W, and Schleif R. 1985. A dimer of AraC protein contacts three adjacent major groove regions of the araI DNA site. Proc Natl Acad Sci U S A 82(10):3129-3133.

Heurlier K, Williams F, Heeb S, Dormond C, Pessi G, Singer D, Camara M, Williams P, and Haas D. 2004. Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in Pseudomonas aeruginosa PAO1. J Bacteriol 186(10):2936-2945.

Hoang TT, Kutchma AJ, Becher A, and Schweizer HP. 2000. Integration-proficient plasmids for Pseudomonas aeruginosa: site-specific integration and use for engineering of reporter and expression strains. Plasmid 43(1):59-72.

Hocquet D, Berthelot P, Roussel-Delvallez M, Favre R, Jeannot K, Bajolet O, Marty N, Grattard F, Mariani-Kurkdjian P, Bingen E et al. . 2007. Pseudomonas aeruginosa may accumulate drug resistance mechanisms without losing its ability to cause bloodstream infections. Antimicrob Agents Chemother 51(10):3531-3536.

Hogardt M, Roeder M, Schreff AM, Eberl L, and Heesemann J. 2004. Expression of Pseudomonas aeruginosa exoS is controlled by quorum sensing and RpoS. Microbiology 150(Pt 4):843-851.

Holcroft CC, and Egan SM. 2000. Roles of cyclic AMP receptor protein and the carboxyl-terminal domain of the alpha subunit in transcription activation of the Escherichia coli rhaBAD operon. J Bacteriol 182(12):3529-3535.

Hovey AK, and Frank DW. 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(15):4427-4436.

Hsu LM. 2009. Monitoring abortive initiation. Methods (San Diego, Calif 47(1):25-36.

Hu JC, and Gross CA. 1985. Mutations in the sigma subunit of E. coli RNA polymerase which affect positive control of transcription. Mol Gen Genet 199(1):7-13.

Ichikawa JK, English SB, Wolfgang MC, Jackson R, Butte AJ, and Lory S. 2005. Genome-wide analysis of host responses to the Pseudomonas aeruginosa type III secretion system yields synergistic effects. Cell Microbiol 7(11):1635-1646.

Igarashi K, Fujita N, and Ishihama A. 1991. Identification of a subunit assembly domain in the alpha subunit of Escherichia coli RNA polymerase. J Mol Biol 218(1):1-6.

Jair KW, Martin RG, Rosner JL, Fujita N, Ishihama A, and Wolf RE, Jr. 1995. Purification and regulatory properties of MarA protein, a transcriptional activator of Escherichia coli multiple antibiotic and superoxide resistance promoters. J Bacteriol 177(24):7100-7104.

Jansson AL, Yasmin L, Warne P, Downward J, Palmer RH, and Hallberg B. 2006. Exoenzyme S of Pseudomonas aeruginosa is not able to induce apoptosis when cells express activated proteins, such as Ras or protein kinase B/Akt. Cell Microbiol 8(5):815-822.

Jarrell K, and Kropinski AM. 1977. The chemical composition of the lipopolysaccharide from Pseudomonas aeruginosa strain PAO and a spontaneously derived rough mutant. Microbios 19(76):103-116.

Johnson DC, Ishihama A, and Stevens AM. 2003. Involvement of region 4 of the sigma70 subunit of RNA polymerase in transcriptional activation of the lux operon during quorum sensing. FEMS Microbiol Lett 228(2):193-201.

Jourdan AD, and Stauffer GV. 1999. GcvA-mediated activation of gcvT-lacZ expression involves the carboxy-terminal domain of the alpha subunit of RNA polymerase. FEMS Microbiol Lett 181(2):307-312.

Kang CI, Kim SH, Kim HB, Park SW, Choe YJ, Oh MD, Kim EC, and Choe KW. 2003. Pseudomonas aeruginosa bacteremia: risk factors for mortality and influence of delayed receipt of effective antimicrobial therapy on clinical outcome. Clin Infect Dis 37(6):745-751.

Kang PJ, Hauser AR, Apodaca G, Fleiszig SM, Wiener-Kronish J, Mostov K, and Engel JN. 1997. Identification of Pseudomonas aeruginosa genes required for epithelial cell injury. Mol Microbiol 24(6):1249-1262.

Kazmierczak BI, and Engel JN. 2002. Pseudomonas aeruginosa ExoT acts in vivo as a GTPase-activating protein for RhoA, Rac1, and Cdc42. Infect Immun 70(4):2198-2205.

Kerppola TK, and Curran T. 1997. The transcription activation domains of Fos and Jun induce DNA bending through electrostatic interactions. Embo J 16(10):2907-2916.

Klarmann GJ, Schauber CA, and Preston BD. 1993. Template-directed pausing of DNA synthesis by HIV-1 reverse transcriptase during polymerization of HIV-1 sequences in vitro. J Biol Chem 268(13):9793-9802.

Kuldell N, and Hochschild A. 1994. Amino acid substitutions in the -35 recognition motif of sigma 70 that result in defects in phage lambda repressor-stimulated transcription. J Bacteriol 176(10):2991-2998.

Kumar A, Malloch RA, Fujita N, Smillie DA, Ishihama A, and Hayward RS. 1993. The minus 35-recognition region of Escherichia coli sigma 70 is inessential for initiation of transcription at an "extended minus 10" promoter. J Mol Biol 232(2):406-418.

Kumar A, and Moran CP, Jr. 2008. Promoter activation by repositioning of RNA polymerase. J Bacteriol 190(9):3110-3117.

Landini P, and Busby SJ. 1999. The Escherichia coli Ada protein can interact with two distinct determinants in the sigma70 subunit of RNA polymerase according to promoter architecture: identification of the target of Ada activation at the alkA promoter. J Bacteriol 181(5):1524-1529.

LaRonde-LeBlanc N, and Wolberger C. 2000. Characterization of the oligomeric states of wild type and mutant AraC. Biochemistry 39(38):11593-11601.

Lee VT, Smith RS, Tummler B, and Lory S. 2005. Activities of Pseudomonas aeruginosa effectors secreted by the Type III secretion system in vitro and during infection. Infect Immun 73(3):1695-1705.

Lerm M, Schmidt G, and Aktories K. 2000. Bacterial protein toxins targeting rho GTPases. FEMS Microbiol Lett 188(1):1-6.

Li M, Moyle H, and Susskind MM. 1994. Target of the transcriptional activation function of phage lambda cI protein. Science 263(5143):75-77.

Li Z, and Demple B. 1994. SoxS, an activator of superoxide stress genes in Escherichia coli. Purification and interaction with DNA. J Biol Chem 269(28):18371-18377.

Liedberg H, and Lundeberg T. 1989. Silver coating of urinary catheters prevents adherence and growth of Pseudomonas aeruginosa. Urol Res 17(6):357-358.

Linares JF, Lopez JA, Camafeita E, Albar JP, Rojo F, and Martinez JL. 2005. Overexpression of the multidrug efflux pumps MexCD-OprJ and MexEF-OprN is associated with a reduction of type III secretion in Pseudomonas aeruginosa. J Bacteriol 187(4):1384-1391.

Liu S, Yahr TL, Frank DW, and Barbieri JT. 1997. Biochemical relationships between the 53-kilodalton (Exo53) and 49-kilodalton (ExoS) forms of exoenzyme S of Pseudomonas aeruginosa. J Bacteriol 179(5):1609-1613.

Lonetto MA, Rhodius V, Lamberg K, Kiley P, Busby S, and Gross C. 1998. Identification of a contact site for different transcription activators in region 4 of the Escherichia coli RNA polymerase sigma70 subunit. J Mol Biol 284(5):1353-1365.

Lyczak JB, Cannon CL, and Pier GB. 2002. Lung infections associated with cystic fibrosis. Clin Microbiol Rev 15(2):194-222.

Lykken GL, Chen G, Brutinel ED, Chen L, and Yahr TL. 2006. Characterization of ExsC and ExsD self-association and heterocomplex formation. J Bacteriol 188(19):6832-6840.

Maresso AW, Deng Q, Pereckas MS, Wakim BT, and Barbieri JT. 2007. Pseudomonas aeruginosa ExoS ADP-ribosyltransferase inhibits ERM phosphorylation. Cell Microbiol 9(1):97-105.

Marlovits TC, Kubori T, Sukhan A, Thomas DR, Galan JE, and Unger VM. 2004. Structural insights into the assembly of the type III secretion needle complex. Science 306(5698):1040-1042.

Martin DW, Schurr MJ, Mudd MH, and Deretic V. 1993. Differentiation of Pseudomonas aeruginosa into the alginate-producing form: inactivation of mucB causes conversion to mucoidy. Mol Microbiol 9(3):497-506.

Martin RG, Jair KW, Wolf RE, Jr., and Rosner JL. 1996. Autoactivation of the marRAB multiple antibiotic resistance operon by the MarA transcriptional activator in Escherichia coli. J Bacteriol 178(8):2216-2223.

Martin RG, and Rosner JL. 2001. The AraC transcriptional activators. Curr Opin Microbiol 4(2):132-137.

Mathew R, and Chatterji D. 2006. The evolving story of the omega subunit of bacterial RNA polymerase. Trends Microbiol 14(10):450-455.

Mathew R, Mukherjee R, Balachandar R, and Chatterji D. 2006. Deletion of the rpoZ gene, encoding the omega subunit of RNA polymerase, results in pleiotropic surface-related phenotypes in Mycobacterium smegmatis. Microbiology 152(Pt 6):1741-1750.

McCaw ML, Lykken GL, Singh PK, and Yahr TL. 2002. ExsD is a negative regulator of the Pseudomonas aeruginosa type III secretion regulon. Mol Microbiol 46(4):1123-1133.

McClure WR. 1980. Rate-limiting steps in RNA chain initiation. Proc Natl Acad Sci U S A 77(10):5634-5638.

Mena KD, and Gerba CP. 2009. Risk assessment of Pseudomonas aeruginosa in water. Rev Environ Contam Toxicol 201:71-115.

Miao EA, Ernst RK, Dors M, Mao DP, and Aderem A. 2008. Pseudomonas aeruginosa activates caspase 1 through Ipaf. Proc Natl Acad Sci U S A 105(7):2562-2567.

Mitchell JE, Zheng D, Busby SJ, and Minchin SD. 2003. Identification and analysis of 'extended -10' promoters in Escherichia coli. Nucleic Acids Res 31(16):4689-4695.

Mota LJ. 2006. Type III secretion gets an LcrV tip. Trends Microbiol 14(5):197-200.

Moyle H, Waldburger C, and Susskind MM. 1991. Hierarchies of base pair preferences in the P22 ant promoter. J Bacteriol 173(6):1944-1950.

Mueller CA, Broz P, Muller SA, Ringler P, Erne-Brand F, Sorg I, Kuhn M, Engel A, and Cornelis GR. 2005. The V-antigen of Yersinia forms a distinct structure at the tip of injectisome needles. Science 310(5748):674-676.

Mulcahy H, O'Callaghan J, O'Grady EP, Adams C, and 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. Infect Immun 74(5):3012-3015.

Navarro-Aviles G, Jimenez MA, Perez-Marin MC, Gonzalez C, Rico M, Murillo FJ, Elias-Arnanz M, and Padmanabhan S. 2007. Structural basis for operator and antirepressor recognition by Myxococcus xanthus CarA repressor. Mol Microbiol 63(4):980-994.

Newman JR, and Fuqua C. 1999. Broad-host-range expression vectors that carry the L-arabinose-inducible Escherichia coli araBAD promoter and the araC regulator. Gene 227(2):197-203.

Nickels BE, Dove SL, Murakami KS, Darst SA, and Hochschild A. 2002. Protein-protein and protein-DNA interactions of sigma70 region 4 involved in transcription activation by lambdacI. Journal of molecular biology 324(1):17-34.

Noura, Salih KM, Jusuf NH, Hamid AA, and Yusoff WM. 2009. High prevalence of Pseudomonas species in soil samples from Ternate Island-Indonesia. Pak J Biol Sci 12(14):1036-1040.

Olds JW, Kisch AL, Eberle BJ, and Wilson JN. 1972. Pseudomonas aeruginosa respiratory tract infection acquired from a contaminated anesthesia machine. Am Rev Respir Dis 105(4):629-632.

Orchard K, and May GE. 1993. An EMSA-based method for determining the molecular weight of a protein--DNA complex. Nucleic Acids Res 21(14):3335-3336.

Ottmann C, Yasmin L, Weyand M, Veesenmeyer JL, Diaz MH, Palmer RH, Francis MS, Hauser AR, Wittinghofer A, and Hallberg B. 2007. Phosphorylation-independent interaction between 14-3-3 and exoenzyme S: from structure to pathogenesis. EMBO J 26(3):902-913.

Paget MS, and Helmann JD. 2003. The sigma70 family of sigma factors. Genome biology 4(1):203.

Parsot C, Ageron E, Penno C, Mavris M, Jamoussi K, d'Hauteville H, Sansonetti P, and Demers B. 2005. A secreted anti-activator, OspD1, and its chaperone, Spa15, are involved in the control of transcription by the type III secretion apparatus activity in Shigella flexneri. Mol Microbiol 56(6):1627-1635.

Pastor A, Chabert J, Louwagie M, Garin J, and Attree I. 2005. PscF is a major component of the Pseudomonas aeruginosa type III secretion needle. FEMS Microbiol Lett 253(1):95-101.

Patel P, Whittier S, and Frank E. 2002. Person-to-person transmission of Pseudomonas pneumonia in the community: documentation by pulsed-field electrophoresis. South Med J 95(6):653-656.

Pedersen SS, Hoiby N, Espersen F, and Koch C. 1992. Role of alginate in infection with mucoid Pseudomonas aeruginosa in cystic fibrosis. Thorax 47(1):6-13.

Phillips RM, Six DA, Dennis EA, and Ghosh P. 2003. In vivo phospholipase activity of the Pseudomonas aeruginosa cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors. J Biol Chem 278(42):41326-41332.

Plano GV. 2004. Modulation of AraC family member activity by protein ligands. Mol Microbiol 54(2):287-290.

Pollack M. 1983. The role of exotoxin A in pseudomonas disease and immunity. Rev Infect Dis 5 Suppl 5:S979-984.

Pollack M, and Anderson SE, Jr. 1978. Toxicity of Pseudomonas aeruginosa exotoxin A for human macrophages. Infect Immun 19(3):1092-1096.

Pukatzki S, Kessin RH, and Mekalanos JJ. 2002. The human pathogen Pseudomonas aeruginosa utilizes conserved virulence pathways to infect the social amoeba Dictyostelium discoideum. Proc Natl Acad Sci U S A 99(5):3159-3164.

Rahme LG, Ausubel FM, Cao H, Drenkard E, Goumnerov BC, Lau GW, Mahajan-Miklos S, Plotnikova J, Tan MW, Tsongalis J et al. . 2000. Plants and animals share functionally common bacterial virulence factors. Proc Natl Acad Sci U S A 97(16):8815-8821.

Ren T, Zamboni DS, Roy CR, Dietrich WF, and Vance RE. 2006. Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathog 2(3):e18.

Rhee S, Martin RG, Rosner JL, and Davies DR. 1998. A novel DNA-binding motif in MarA: the first structure for an AraC family transcriptional activator. Proc Natl Acad Sci U S A 95(18):10413-10418.

Rhodius VA, and Busby SJ. 2000. Transcription activation by the Escherichia coli cyclic AMP receptor protein: determinants within activating region 3. Journal of molecular biology 299(2):295-310.

Richards MJ, Edwards JR, Culver DH, and Gaynes RP. 2000. Nosocomial infections in combined medical-surgical intensive care units in the United States. Infect Control Hosp Epidemiol 21(8):510-515.

Richet E, and Sogaard-Andersen L. 1994. CRP induces the repositioning of MalT at the Escherichia coli malKp promoter primarily through DNA bending. Embo J 13(19):4558-4567.

Rietsch A, and Mekalanos JJ. 2006. Metabolic regulation of type III secretion gene expression in Pseudomonas aeruginosa. Mol Microbiol 59(3):807-820.

Rietsch A, Vallet-Gely I, Dove SL, and Mekalanos JJ. 2005. ExsE, a secreted regulator of type III secretion genes in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 102(22):8006-8011.

Sandkvist M. 2001. Type II secretion and pathogenesis. Infect Immun 69(6):3523-3535.

Sato H, Feix JB, and Frank DW. 2006. Identification of superoxide dismutase as a cofactor for the pseudomonas type III toxin, ExoU. Biochemistry 45(34):10368-10375.

Sato H, Frank DW, Hillard CJ, Feix JB, Pankhaniya RR, Moriyama K, Finck-Barbancon V, Buchaklian A, Lei M, Long RM et al. . 2003. The mechanism of action of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU. EMBO J 22(12):2959-2969.

Schleif R. 2003. AraC protein: a love-hate relationship. Bioessays 25(3):274-282.

Schoehn G, Di Guilmi AM, Lemaire D, Attree I, Weissenhorn W, and Dessen A. 2003. Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in Pseudomonas. EMBO J 22(19):4957-4967.

Scotto-Lavino E, Du G, and Frohman MA. 2006. Amplification of 5' end cDNA with 'new RACE'. Nature protocols 1(6):3056-3061.

Seredick SD, and Spiegelman GB. 2004. The Bacillus subtilis response regulator Spo0A stimulates sigmaA-dependent transcription prior to the major energetic barrier. J Biol Chem 279(17):17397-17403.

Severinova E, Severinov K, Fenyo D, Marr M, Brody EN, Roberts JW, Chait BT, and Darst SA. 1996. Domain organization of the Escherichia coli RNA polymerase sigma 70 subunit. J Mol Biol 263(5):637-647.

Shafikhani SH, and Engel J. 2006. Pseudomonas aeruginosa type III-secreted toxin ExoT inhibits host-cell division by targeting cytokinesis at multiple steps. Proc Natl Acad Sci U S A 103(42):15605-15610.

Shafikhani SH, Morales C, and Engel J. 2008. The Pseudomonas aeruginosa type III secreted toxin ExoT is necessary and sufficient to induce apoptosis in epithelial cells. Cell Microbiol 10(4):994-1007.

Shakhnovich EA, Hung DT, Pierson E, Lee K, and Mekalanos JJ. 2007. Virstatin inhibits dimerization of the transcriptional activator ToxT. Proc Natl Acad Sci U S A 104(7):2372-2377.

Shen DK, Filopon D, Chaker H, Boullanger S, Derouazi M, Polack B, and Toussaint B. 2008. High-cell-density regulation of the Pseudomonas aeruginosa type III secretion system: implications for tryptophan catabolites. Microbiology 154(Pt 8):2195-2208.

Stauffer GV, Plamann MD, and Stauffer LT. 1981. Construction and expression of hybrid plasmids containing the Escherichia coli glyA genes. Gene 14(1-2):63-72.

Stauffer LT, and Stauffer GV. 2005. GcvA interacts with both the alpha and sigma subunits of RNA polymerase to activate the Escherichia coli gcvB gene and the gcvTHP operon. FEMS Microbiol Lett 242(2):333-338.

Stevens AM, Fujita N, Ishihama A, and Greenberg EP. 1999. Involvement of the RNA polymerase alpha-subunit C-terminal domain in LuxR-dependent activation of the Vibrio fischeri luminescence genes. J Bacteriol 181(15):4704-4707.

Strauch M, Webb V, Spiegelman G, and Hoch JA. 1990. The SpoOA protein of Bacillus subtilis is a repressor of the abrB gene. Proc Natl Acad Sci U S A 87(5):1801-1805.

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

Sun YH, Rolan HG, and Tsolis RM. 2007. Injection of flagellin into the host cell cytosol by Salmonella enterica serotype Typhimurium. J Biol Chem 282(47):33897-33901.

Sundin C, Wolfgang MC, Lory S, Forsberg A, and Frithz-Lindsten E. 2002. Type IV pili are not specifically required for contact dependent translocation of exoenzymes by Pseudomonas aeruginosa. Microb Pathog 33(6):265-277.

Tamura M, Ajayi T, Allmond LR, Moriyama K, Wiener-Kronish JP, and Sawa T. 2004. Lysophospholipase A activity of Pseudomonas aeruginosa type III secretory toxin ExoU. Biochem Biophys Res Commun 316(2):323-331.

Tang H, Kim Y, Severinov K, Goldfarb A, and Ebright RH. 1996. Escherichia coli RNA polymerase holoenzyme: rapid reconstitution from recombinant alpha, beta, beta', and sigma subunits. Methods Enzymol 273:130-134.

Thibault J, Faudry E, Ebel C, Attree I, and Elsen S. 2009. Anti-activator ExsD forms a 1:1 complex with ExsA to inhibit transcription of type III secretion operons. J Biol Chem 284(23):15762-15770.

Travers A. 1974. RNA polymerase--promoter interactions: some general principles. Cell 3(2):97-104.

Urbanowski ML, Brutinel ED, and Yahr TL. 2007. Translocation of ExsE into Chinese hamster ovary cells is required for transcriptional induction of the Pseudomonas aeruginosa type III secretion system. Infect Immun 75(9):4432-4439.

Urbanowski ML, Lostroh CP, and Greenberg EP. 2004. Reversible acyl-homoserine lactone binding to purified Vibrio fischeri LuxR protein. J Bacteriol 186(3):631-637.

Urbanowski ML, Lykken GL, and Yahr TL. 2005. A secreted regulatory protein couples transcription to the secretory activity of the Pseudomonas aeruginosa type III secretion system. Proc Natl Acad Sci U S A 102(28):9930-9935.

Vakulskas CA, Brady KM, and Yahr TL. 2009. Mechanism of Transcriptional Activation by Pseudomonas aeruginosa ExsA. J Bacteriol 191(21):6654-6664.

Vallis AJ, Yahr TL, Barbieri JT, and Frank DW. 1999. Regulation of ExoS production and secretion by Pseudomonas aeruginosa in response to tissue culture conditions. Infect Immun 67(2):914-920.

Veesenmeyer JL, Hauser AR, Lisboa T, and Rello J. 2009. Pseudomonas aeruginosa virulence and therapy: evolving translational strategies. Crit Care Med 37(5):1777-1786.

Ventre I, Goodman AL, Vallet-Gely I, Vasseur P, Soscia C, Molin S, Bleves S, Lazdunski A, Lory S, and Filloux A. 2006. Multiple sensors control reciprocal expression of Pseudomonas aeruginosa regulatory RNA and virulence genes. Proc Natl Acad Sci U S A 103(1):171-176.

Vogel HJ, and Bonner DM. 1956. Acetylornithinase of Escherichia coli: partial purification and some properties. J Biol Chem 218(1):97-106.

Warne SE, and deHaseth PL. 1993. Promoter recognition by Escherichia coli RNA polymerase. Effects of single base pair deletions and insertions in the spacer DNA separating the -10 and -35 regions are dependent on spacer DNA sequence. Biochemistry 32(24):6134-6140.

Weldon JE, Rodgers ME, Larkin C, and Schleif RF. 2007. Structure and properties of a truely apo form of AraC dimerization domain. Proteins 66(3):646-654.

Wickstrum JR, and Egan SM. 2004. Amino acid contacts between sigma 70 domain 4 and the transcription activators RhaS and RhaR. J Bacteriol 186(18):6277-6285.

Woestyn S, Allaoui A, Wattiau P, and Cornelis GR. 1994. YscN, the putative energizer of the Yersinia Yop secretion machinery. J Bacteriol 176(6):1561-1569.

Wolfgang MC, Lee VT, Gilmore ME, and Lory S. 2003. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. Dev Cell 4(2):253-263.

Wu W, Badrane H, Arora S, Baker HV, and Jin S. 2004. MucA-mediated coordination of type III secretion and alginate synthesis in Pseudomonas aeruginosa. J Bacteriol 186(22):7575-7585.

Wu W, and Jin S. 2005. PtrB of Pseudomonas aeruginosa suppresses the type III secretion system under the stress of DNA damage. J Bacteriol 187(17):6058-6068.

Yahr TL, and Frank DW. 1994. Transcriptional organization of the trans-regulatory locus which controls exoenzyme S synthesis in Pseudomonas aeruginosa. J Bacteriol 176(13):3832-3838.

Yahr TL, Hovey AK, Kulich SM, and Frank DW. 1995. Transcriptional analysis of the Pseudomonas aeruginosa exoenzyme S structural gene. J Bacteriol 177(5):1169-1178.

Yahr TL, Vallis AJ, Hancock MK, Barbieri JT, and Frank DW. 1998. ExoY, an adenylate cyclase secreted by the Pseudomonas aeruginosa type III system. Proc Natl Acad Sci U S A 95(23):13899-13904.

Yahr TL, and Wolfgang MC. 2006. Transcriptional regulation of the Pseudomonas aeruginosa type III secretion system. Mol Microbiol 62(3):631-640.

York K, Kenney TJ, Satola S, Moran CP, Jr., Poth H, and Youngman P. 1992. Spo0A controls the sigma A-dependent activation of Bacillus subtilis sporulation-specific transcription unit spoIIE. J Bacteriol 174(8):2648-2658.

Zhang X, Reeder T, and Schleif R. 1996. Transcription activation parameters at ara pBAD. J Mol Biol 258(1):14-24.

Zheng Z, Chen G, Joshi S, Brutinel ED, Yahr TL, and Chen L. 2007. Biochemical characterization of a regulatory cascade controlling transcription of the Pseudomonas aeruginosa type III secretion system. J Biol Chem 282(9):6136-6142.