Self-Association Is Required for Occupation of Adjacent Binding Sites in *Pseudomonas aeruginosa* Type III Secretion System Promoters

Anne E. Marsden,* Florian D. Schubot,** Timothy L. Yahr*

Department of Microbiology, University of Iowa, Iowa City, Iowa, USA*; Department of Biological Sciences, Virginia Tech, Blacksburg, Virginia, USA**

ExsA is a member of the AraC/XylS family of transcriptional regulators and is required for expression of the *Pseudomonas aeruginosa* type III secretion system (T3SS). All *P. aeruginosa* T3SS promoters contain two adjacent binding sites for monomeric ExsA. The amino-terminal domain of ExsA (NTD) is thought to mediate interactions between the ExsA monomers bound to each site. Threading the NTD onto the AraC backbone revealed an α-helix that likely serves as the primary determinant for dimerization. In this study, we performed alanine scanning mutagenesis of the ExsA α-helix (residues 136 to 152) to identify determinants required for self-association. Residues L137, C139, L140, K141, and L148 exhibited self-association defects and were required for maximal activation by ExsA. Disruption of self-association resulted in decreased binding to T3SS promoters, particularly loss of binding by the second ExsA monomer. Removing the NTD or increasing the space between the ExsA-binding sites restored the ability of the second ExsA monomer to bind the P$_{exsC}$ promoter. This finding indicated that, in the absence of self-association, the NTD prevents binding by a second monomer. Similar findings were seen with the P$_{exsT}$ Promoter; however, binding of the second ExsA monomer in the absence of self-association also required the presence of a high-affinity site 2. Based on these data, ExsA self-association is necessary to overcome inhibition by the NTD and to compensate for low-affinity binding sites, thereby allowing for full occupation and activation of ExsA-dependent promoters. Therefore, ExsA self-association is indispensable and provides an attractive target for antivirulence therapies.

*Pseudomonas aeruginosa* is a ubiquitous environmental microorganism and an opportunistic pathogen responsible for a variety of acute and chronic infections. *P. aeruginosa* is notable for causing 7.5% of all hospital-acquired infections and severe complications for patients with cystic fibrosis (1). Individuals with compromised immune systems, including neutropenia due to chemotherapy, disruption of the epithelia due to severe burns, and cystic fibrosis patients, are especially vulnerable to *P. aeruginosa* infection. High rates of antimicrobial resistance in nosocomial isolates indicate a need for alternative control methods (2, 3).

Many Gram-negative, pathogenic bacteria contain a type III secretion system (T3SS) (4). This needle-like complex spans both membranes of the cell envelope and translocates effector proteins directly from the bacterial cytosol into the cytoplasm of a host cell. Numerous studies have shown that the T3SS is a major *P. aeruginosa* virulence determinant (5–12). The translocated effector proteins are important for interactions between the bacteria and host and promote host tissue destruction, disruption of the actin cytoskeleton, inhibition of phagocytosis, and induction of apoptosis (13). Expression of the *P. aeruginosa* T3SS is associated with poor clinical outcomes in humans. The relative risk of mortality from lower respiratory tract and systemic infections is significantly higher in patients colonized with strains expressing T3SS effector proteins (14). In patients with *P. aeruginosa* bacteremia, expression of T3SS effector proteins is associated with an increase in 30-day mortality (15). Likewise, secretion of T3SS effectors is associated with more severe disease (defined as death or relapse) in patients with ventilator-associated pneumonia (16).

T3SS gene expression is dependent on the transcriptional activator ExsA, which directly binds to and activates transcription from 10 distinct promoter regions (17). ExsA, a member of the AraC/XylS family of transcriptional regulators, contains two helix-turn-helix DNA-binding motifs located in the carboxy-terminal domain (CTD). ExsA-dependent promoters contain two binding sites centered at −44 and −65 relative to the start of transcription (referred to as binding sites 1 and 2, respectively), at which two ExsA monomers bind in a head-to-tail orientation (18, 19). On at least one of the T3SS promoters (P$_{exsT}$), occupation of the binding sites occurs in an ordered fashion whereby the first ExsA monomer binds to site 1 and then recruits a second ExsA monomer to binding site 2 (18). Whereas binding site 1 of all T3SS promoters contains conserved GnC and TGnA sequences, functionally equivalent sites have been identified only in binding site 2 for the P$_{exsT}$ promoter (19, 20). Occupation of site 2 in other promoters is not well understood but may depend on nonspecific interactions between ExsA and site 2 and/or self-association with the monomer bound to site 1.

Although ExsA is a monomer in solution, the available evidence suggests that self-association plays an important role in promoter binding (18). ExsA self-association occurs via the amino-terminal domain (NTD) as measured by a LexA-based monohybrid assay (21). The isolated carboxy-terminal domain (CTD), containing only the DNA-binding motifs, lacks cooperative binding properties and exhibits a 4-fold reduction in both binding affinity and activation of T3SS promoters (21, 22). The NTD of AraC family members is not well conserved but often is involved in binding regulatory molecules and/or oligomerization. Like ExsA, the NTD of AraC is required for self-association (23).

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crystal structure of an AraC<sub>NTD</sub> dimer indicates that self-association occurs at an antiparallel, coiled-coil interface with leucines from both monomers forming triads at each end of the interface in a head-to-head orientation (24, 25). Conserved leucines in several other AraC family proteins, including <i>Proteus mirabilis</i> UreB, <i>Pseudomonas putida</i> YfL3, and <i>Citrobacter rodentium</i> RegA, also are involved in protein self-association (26–28).

In this study, we identified a putative α-helix in the ExsA NTD that contains residues involved in self-association. We propose that leucine residues 137, 140, and 148 form interaction triads at each end of an anti-parallel interface through a mechanism analogous to that seen with AraC. Additionally, we find that ExsA self-association serves two distinct roles to promote occupation of the distal site by a second ExsA monomer: (i) self-association relieves NTD-mediated inhibition of site 2 occupation, and (ii) self-association facilitates occupation of low-affinity binding sites.

**Materials and Methods**

**Bacterial strains and culture conditions.** The bacterial strains used for this study are listed in Table S1 in the supplemental material. <i>Escherichia coli</i> DH5α was used for cloning purposes and maintained on Luria-Bertani (LB) agar plates with gentamicin (15 μg/ml), tetracycline (12 μg/ml), or ampicillin (100 μg/ml) as appropriate. <i>E. coli</i> strains SU101 and SU202 were used for the LexA monohybrid and two-hybrid assays and maintained on LB agar with tetracycline (12 μg/ml) or ampicillin (100 μg/ml) as appropriate. <i>E. coli</i> strain Tuner (DE3) was maintained on LB agar with ampicillin (100 μg/ml). <i>P. aeruginosa</i> PA103 strains were maintained on Vogel-Bonner minimal media (VBM) with gentamicin (100 μg/ml) as necessary.

**Plasmid construction and mutagenesis.** The reporter fusions and plasmids used in this study are listed in Table S1 in the supplemental material. Site-directed mutagenesis was performed with the QuikChange multisite-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Single primers (20 to 40 bp) were designed as listed in Table S2 and used to amplify the pEB124 exsA expression vector while incorporating the desired substitutions (pAM33-44 and pAM83-85). LexA<sub>Δ218-Δ235</sub>-ExsA mutants (pAM51-62 and pAM119-121) were generated using pEB124 mutant templates in PCRs to amplify exsA as XbaI/SacI restriction fragments (primer pair 86360966-86507014), which were then ligated into pM455, a pSR658 derivative with XbaI and SacI restriction sites in the multiple cloning site, generated by QuikChange mutagenesis with primer pair 82218921-82218920. ExsA NTD mutants (pAM63-74 and pAM108-110) were generated by amplifying nucleotides 1 to 540 from pEB124 as XbaI/SacI restriction fragments by PCR amplification (primer pair 82218921-82218920). ExsA NTD mutants were cloned as N-terminal 10X histidine tag.

**Protein purification.** The exsA (L1410A, L148A) coding sequence from pAM79 was PCR amplified using primers (42308574-7736236) to incorporate Ndel and BamHI restriction sites. The resulting product was ligated into the pET16b expression vector to introduce an N-terminal 10X histidine tag. <i>E. coli</i> Tuner (DE3) was transformed with the resulting expression vector (pAM145). An overnight culture grown at 37°C on LB agar containing ampicillin (100 μg/ml) was used to inoculate 500 ml LB containing ampicillin (100 μg/ml) to an initial A<sub>600</sub> of 0.1. The culture was grown with shaking at 30°C until the culture A<sub>600</sub> reached 0.5. Ammonium was added to a final concentration of 300 μg/ml and ExsA<sub>L1410A</sub> expression was induced by IPTG addition (1 mM). After an additional 4-h incubation period, cells were harvested by centrifugation (10 min at 6,000 × g, 4°C) and resuspended in 30 ml ExsA-binding buffer containing 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 0.5% Tween 20, 20 mM imidazole, and 3 protease inhibitor cocktail tablets (complete mini, EDTA-free protease inhibitor cocktail; Roche Applied Science). Cells were disrupted by passage through a Microfluidizer. Lysates were cleared by two rounds of centrifugation (10 min at 20,000 × g, 4°C) and subjected to Ni<sup>2+</sup>-nitrilotriacetic acid (NTA) affinity chromatography as previously described (18). After elution from the Ni<sup>2+</sup>-NTA resin, peak fractions were pooled and dialyzed overnight against 4 liters of ExsA-binding buffer (excluding imidazole) with 1 mM diethiothreitol (DTT) at 4°C. Protein concentrations were determined with the DC protein assay (Bio-Rad) using bovine serum albumin protein standards. Purified ExsA<sub>L1410A</sub> and CTD<sub>His</sub> were prepared from previously constructed strains in the manner described above (18, 22).

**Electrophoretic mobility shift assays (EMSAs).** Specific promoter probes (ca. 200 bp) for P<sub>exoT</sub> and P<sub>exsC</sub> (with binding sites 1 and 2 separated by 5 and 10 bp, respectively) were added to a total volume of 20 μl with 1 mM dithiothreitol (DTT) at 4°C. After an additional 4-h incubation period, cells were harvested by centrifugation (10 min at 6,000 × g, 4°C) and resuspended in 30 ml ExsA-binding buffer containing 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 0.5% Tween 20, 20 mM imidazole, and 3 protease inhibitor cocktail tablets (complete mini, EDTA-free protease inhibitor cocktail; Roche Applied Science). Cells were disrupted by passage through a Microfluidizer. Lysates were cleared by two rounds of centrifugation (10 min at 20,000 × g, 4°C) and subjected to Ni<sup>2+</sup>-nitrilotriacetic acid (NTA) affinity chromatography as previously described (18). Briefly, reaction mixtures containing specific and/or nonspecific probes (0.25 mM), ExsA DNA-binding buffer (10 mM Tris [pH 7.5], 50 mM KCl, 1 mM EDTA, 1 mM diethiothreitol, 5% glycerol), 25 μg/ml poly(dI-dC), and 100 μg/ml bovine serum albumin were incubated for 5 min at 25°C. ExsA<sub>L1410A</sub>CTD<sub>His</sub> and ExsA<sub>L1410A,L148A</sub>CTD<sub>His</sub> (concentration determined empirically as indicated in the figure legends) were added to a total volume of 20 μl and incubated for 15 min at 25°C. Samples were analyzed by electrophoresis on 5% polyacrylamide gel gels (10 mM Tris [pH 7.5], 380 mM glycine, 1 mM EDTA) at 4°C. Imaging was performed using a FLA-7000 phosphorimager (Fujifilm) and Multi-Gauge v3.0 software (Fujifilm).

**Results**

**Self-association of ExsA is required for activation of T3SS promoters.** The AraC dimeric interface consists of an α-helix derived from each monomer that forms an antiparallel, coiled-coil interface stabilized by leucine triads (leucines 150, 151, and 161) positioned at each end of the coiled coil (Fig. 1A and B) (24, 25). Although the AraC and ExsA NDVs lack significant sequence identity (11%) at the primary amino acid level, threading the ExsA NTD onto the crystal structure of the AraC NTD suggested structural conservation (Fig. 1C). In particular, residues 136 to 152 of...
ExsA are equivalent to the α-helix in AraC that constitutes the dimeric interface. To determine whether residues 136 to 152 are required for ExsA function, we performed alanine scanning mutagenesis. An arabinose-inducible ExsA expression vector (pEB124) carrying single alanine substitutions at residues 136 to 152 (excluding A138 and A150) was introduced into an exsA mutant carrying an ExsA-dependent transcriptional reporter (P_{exoT-lacZ}) integrated at the chromosomal CTX phage attachment site. The resulting strains were cultured under inducing conditions for T3SS expression (low Ca^{2+}, generated by addition of EGTA to the growth medium [+EGTA]) and assayed for expression of the P_{exoT-lacZ} reporter. Alanine substitutions at residues L137, C139, L140, K141, E143, L145, L146, L148, and F149 resulted in less than 50% activation of the P_{exoT-lacZ} reporter compared to that of wild-type ExsA (Fig. 2A). Activation by the remaining ExsA mutants did not significantly differ from that of wild-type ExsA. To determine whether the alanine substitutions altered protein stability, whole-cell extracts were immunoblotted for ExsA. Alanine substitutions at residues L137, L145, and L146 resulted in decreased steady-state expression levels (Fig. 2A), accounting for the severe activation defects of these mutant proteins (<1%, 28%, and 6% wild-type activation, respectively). The remaining mutants were stably expressed, suggesting that residues C139, L140, K141, E143, L148, and F149 are required for maximal ExsA activity.

To determine whether the defect for activation of the P_{exoT-lacZ} reporter resulted from impaired self-association, we employed the LexA monohybrid assay. This system was previously used to demonstrate self-association of full-length ExsA (21). LexA is a transcriptional repressor consisting of an amino-terminal DNA-binding domain (DBD) and a carboxy-terminal self-association domain. Self-association is critical for LexA DNA-binding activity (30). Replacement of the self-association domain with a protein of two-hybrid assay, we confirmed that the NTD does indeed interact with full-length ExsA (see Fig. S1 in the supplemental material). We then used the dominant-negative activity of the NTD to further assess self-association of the alanine mutants. Each of the alanine substitutions was introduced into the NTD and tested for inhibition of P_{exoT-lacZ} reporter activity in the presence of full-length ExsA. This strain contained a deletion of the anti-activator ExsD to avoid changes in ExsA activity that might result from alanine substitutions that alter ExsA-ExsD interactions. Compared to the high levels of activation observed with the vector control (0% repression), overexpression of the ExsA NTD strongly inhibited activation of the P_{exoT-lacZ} reporter (normalized to 100% repression) (Fig. 2C). Alanine substitutions at residues L137, C139, K141, E143, L146, and L148 resulted in reduced repression by the NTD, indicative of self-association defects. Although an immunoblot was performed to assess protein stability, NTD repression was undetectable, leaving uncertainty as to whether reduced repression resulted from decreased self-association, lack of protein stability, or a combination of the two effects. Substitutions at residues L137, L145, and L146 resulted in decreased protein expression for full-length ExsA in P. aeruginosa (Fig. 2A), a finding that likely is translatable to ExsA (L145A), NTD, and NTD_{L145A}. Therefore, excluding residues L145 and L146, the LexA monohybrid and NTD dominant-negative assays indicate that residues L137, C139, L140, K141, and L148 are involved in ExsA self-association. With the exception of the L137A mutant, which was not stably expressed, the remaining mutants were all defective for ExsA activation (Fig. 2A), supporting the hypothesis that ExsA self-association is required for ExsA activation of T3SS promoters. Alanine substitutions at residues E143 and E144 resulted in significantly increased dominant-negative activity, suggesting that these substitutions result in a stronger interaction with full-length ExsA. The E143A and E144A mutants, however, did not have increased P_{exoT-lacZ} reporter activity (Fig. 2A), an expected consequence of stronger self-association. These substitutions may result in stronger self-association but interfere

FIG 1 Structural model of the ExsA dimerization domain. (A and B) Solution structure of the AraC NTD dimer (PDB code 2ARC) showing leucine residues 150, 151, and 161 (A), which form interaction triads that stabilize the self-association interface (B). (C) PHyre prediction of the ExsA NTD based on the AraC NTD structure. Leucine residues 137, 140, and 148 in ExsA have orientation and spacing similar to those of the critical leucine residues in AraC.
with productive contacts with promoter DNA and/or RNA polymerase.

Several single-amino-acid substitutions in the ExsA self-association helix resulted in significant but modest decreases in self-association. Complete disruption of AraC self-association requires four amino acid substitutions (25). For this reason, we constructed double alanine substitution mutants expecting to exacerbate activation and self-association defects. The L140A, K141A, and L148A substitutions were chosen based on the severity of their activation (Fig. 2A) and self-association (Fig. 2B and C) defects, stable expression in *P. aeruginosa* and *E. coli*, and a bias toward leucine residues, which are required for AraC self-association. Mutants were tested for complementation of the *exoT-lacZ* reporter activity in an *exsA* mutant, self-association in the LexA monohybrid assay, and dominant-negative activity. Each of the double mutants had severe defects in activation of the *exoT-lacZ* reporter (<1% wild-type activity) compared to the single-alanine substitutions (Fig. 3A). Self-association defects also were evident in the LexA monohybrid assay, with the combination of L140A and L148A substitutions resulting in the strongest defect (<30% wild-type repression [Fig. 3B]). Dramatic decreases (<10% wild-type activity) in dominant-negative activity were observed for each of the combined substitution mutants (Fig. 3C). Immunoblotting revealed that each of the double mutants was stably expressed in the context of full-length ExsA in *P. aeruginosa* and as LexA fusions in *E. coli* (Fig. 3A and B). These data further demonstrate that disruption of ExsA self-association correlates with decreased ExsA-dependent transcription.

**Self-association of ExsA is required for maximal promoter occupation.** ExsA-dependent promoters contain two adjacent binding sites for monomeric ExsA (18). Although nucleotide determinants have been defined for binding site 1, site 2 is poorly conserved, and it is unclear whether occupation of site 2 is driven by specific protein–DNA interactions and/or self-association with the ExsA monomer bound to site 1 (19). To differentiate between these possibilities, we compared the DNA-binding properties of ExsA to the self-association-defective ExsA<sub>L140A,L148A</sub> and the isolated ExsA DNA-binding domain (CTD), which lacks the NTD and should be unable to self-associate. Each was purified from *E. coli* as a histidine-tagged fusion protein. In electrophoretic mobility shift assays (EMSA), binding of ExsA<sub>His</sub> to the CTD<sub>His</sub> promoter probe results in the appearance of two distinct shift products representing occupation of site 1 only and both sites 1 and 2 (18). Binding to the CTD<sub>His</sub> promoter, however, occurs in a highly cooperative manner such that shift product 2 is most readily detected (Fig. 4A and B, lanes 2 to 4). Binding by CTD<sub>Hsl</sub> resulted in preferential formation of shift product 1 when used at lower concentrations (lane 5) and formation of both shift products 1 and 2 at higher concentrations (lane 7). Binding by ExsA<sub>L140A,L148A</sub> also resulted in preferential formation of shift product 1 at lower concentrations (lane 8), but formation of shift product 2 remained inefficient even when using higher protein concentrations (lane 10). The combined findings with CTD<sub>Hsl</sub> and ExsA<sub>L140A,L148A</sub> indicate that self-association facilitates efficient formation of shift product 2 but also revealed the surprising finding that ExsA<sub>L140A,L148A</sub> is more defective than CTD<sub>Hsl</sub>.

**ExsA self-association suppresses the inhibitory activity of the NTD.** The ability of CTD<sub>Hsl</sub> to efficiently occupy both binding sites on the P<sub>exc</sub> promoter probe seemed contrary to our model
that self-association is required for maximal occupation of sites 1 and 2. One model accounting for this discrepancy is that the NTD of ExsA bound to the first site prevents binding by the second ExsA monomer, and that self-association between the two monomers is required to provide access to the second site (Fig. 5A and B). In the case of CTDHis, which lacks the NTD, this inhibitory mechanism is absent, allowing for occupation of both sites (Fig. 5C). Conversely, the inability of ExsAL140A,L148AHis to self-associate prevents efficient occupation of the second site (Fig. 5D). We reasoned that physically separating binding sites 1 and 2 might relieve the inhibitory activity of the NTD and allow for more efficient formation of shift product 2 by ExsAL140A,L148AHis (Fig. 5E). To test this idea, promoter probes PexsC and algD were used as probes, and EMSAs were performed with wild-type ExsAHis, CTDHis, and ExsAL140A,L148AHis. All three proteins generated shift products 1 and 2 when using the PexsC probe, and in each case the formation of shift product 2 reached ~50% of the total shifted material (Fig. 6A to D). In particular, ExsAL140A,L148AHis binding to PexsC was significantly improved relative to that for the native PexsC probe. The apparent dissociation constant (Kd) for ExsAHis binding to the native PexsC probe differed by 10-fold (25 and 230 nM, respectively) (Fig. 7A; also see Fig. S2). In contrast, the dissociation constants for ExsAHis and ExsAL140A,L148AHis binding to the PexsC probe were

FIG 3 Self-association is required for ExsA-dependent transcription. (A) An exsA mutant (PA103 exsA::H) was transformed with a vector control (pN105), an ExsA expression vector (WT), or a vector expressing pairwise L140A, K141A, and L148A substitutions in ExsA. Protein expression was induced by addition of 0.01% arabinose. Cells were cultured under inducing conditions (+EGTA) for T3SS gene expression, and PexoT activity is reported as percent WT activation. (B) Self-association in the LexA monohybrid was measured by the activation of the PexoT::lacZ reporter. E. coli strain SU101 was transformed with a vector control (pSR658), LexADBD-ExsA expression vector (WT), or the LexADBD-ExsA mutants. Protein expression was induced by addition of 0.1 mM IPTG. Reporter activity was measured in Miller units and is reported as percent repression, with strains carrying pSR658 or LexADBD-ExsA representing 0 and 100% repression, respectively. *, P < 0.00005.

FIG 4 Binding properties of ExsA, CTD, and the ExsAL140A,L148A double mutant using radiolabeled probes derived from PexsC. The negative control, a nonspecific probe from the algD promoter region, was included in each binding reaction. The indicated concentrations of ExsA, CTD, and the ExsAL140A,L148A double mutant were incubated with the promoter probes (0.05 nM each) for 15 min at 25°C. Binding reactions were analyzed by native polyacrylamide gel electrophoresis and phosphorimaging. The positions of the unshifted probes (PexsC and algD) and shift products 1 and 2 are indicated. (B) Quantification of shift products 1 and 2 as a percentage of the total shifted product.
nearly identical (91 and 100 nM, respectively) (Fig. 7B). These findings suggest that increasing the distance between the binding sites allows for efficient binding even in the absence of self-association (Fig. 5E) and support the hypotheses that the NTD inhibits binding to the second site and that self-association suppresses the inhibitory activity of the NTD.

We had previously observed that ExsA triggers significant DNA bending upon binding to sites 1 and 2 on the P_{exsC} probe binding to the P_{exsC} (A) and P_{exsC+5} (B) promoter probes. The percentage of shifted probe (y axis) was plotted as a function of protein concentration (x axis). The reported values are averages from three independent experiments.

FIG 6 Binding properties of ExsA, CTD, and the ExsA_{L140,L148A} double mutant using radiolabeled probes derived from P_{exsC} (A) and P_{exsC+5} (C). The indicated concentrations of ExsA, CTD, and the ExsA_{L140,L148A} double mutant were incubated with the promoter probes (0.05 nM each) for 15 min at 25°C and then analyzed by native polyacrylamide gel electrophoresis and phosphorimaging. The positions of the unshifted probes (P_{exsC}, P_{exsC+5}, and algD) and shift products 1 and 2 are indicated. (A and C) Quantification of shift products 1 and 2 as a percentage of the total shifted product is indicated for the P_{exsC} (B) and P_{exsC+5} (D) probes.

FIG 7 Apparent equilibrium binding constant for ExsA and ExsA_{L140,L148A} binding to the P_{exsC} (A) and P_{exsC+5} (B) promoter probes. The percentage of shifted probe (y axis) was plotted as a function of protein concentration (x axis). The reported values are averages from three independent experiments.

FIG 5 Model for inhibition of DNA binding by the NTD of ExsA. (A) When bound to the first site, the NTD of the first ExsA molecule inhibits binding of the incoming monomer to the second site. (B) Self-association of WT ExsA with a second monomer through the NTD relieves the block on occupation of the second site. (C) Lacking the NTD, the isolated DNA-binding domain of ExsA (CTD) is able to occupy both sites 1 and 2 in the absence of self-association. (D and E) The self-association defect of the L140A-L148A mutant prevents occupation of the second site (D), and that defect can be suppressed by separating sites 1 and 2 by 5 bp (E).
compared to other ExsA-dependent promoters (18). This is evidenced by the significant retardation of shift product 2 in the EMSAs (Fig. 4A, lane 2 to 4). Shift product 2 formed by ExsAl140A,l148A, however, had enhanced mobility (lanes 8 to 10), which likely results from altered DNA bending and suggests that self-association is required for maximal DNA bending by ExsA. When binding sites 1 and 2 were separated by 5 or 10 nucleotides, the mobility of the EMSA products formed by ExsA and ExsAl140A,l148A were identical (Fig. 6A and C, lanes 2 to 4 versus 8 to 10), suggesting that optimal bending requires both self-association and a specific spacing requirement between sites 1 and 2.

ExsA self-association compensates for low-affinity ExsA interactions with binding site 2. In addition to self-association, protein-DNA interactions also contribute to occupation of the second binding site in the P exsa promoter (20). In other ExsA-dependent promoters, however, binding site 2 is poorly defined, but the available data suggest that ExsA has a lower affinity for binding site 2 than site 1 (18). To examine the role of self-association in a promoter lacking a high-affinity site 2, we examined binding to a P exsa promoter probe. Whereas 200 nM ExsAHis formed more shift product 2 than shift product 1 on the P exsa promoter probe (Fig. 8A and B, lanes 2 to 4), the same concentration of CTDHis and ExsAl140A,l148A preferentially generated shift product 1 (Fig. 8A and B, lanes 5 to 10). These data are similar to our findings for ExsAHis and ExsAl140A,l148Ahis binding to the P exsa promoter probe (Fig. 4A and B) and further support our conclusion that self-association is required for maximal occupation of site 2.

In contrast to our findings with the P exsa+5 Promoter probe (Fig. 6A and B), however, separation of P exsa+5 binding sites 1 and 2 by 5 bp (P exsaT+5) did not result in increased generation of shift product 2 by ExsAl140A,l148Ahis (Fig. 8C and D, lanes 8 to 10). One possible explanation is that the weaker affinity of ExsA for P exsa site 2 relative to P exsa site 2 is unable to support efficient occupation by ExsAl140A,l148Ahis even when the inhibitory activity of the NTD is negated by increasing the spacing between sites 1 and 2. To test this possibility, we replaced P exsaT site 2 with the higher-affinity site 2 from P exsaC. Whereas high concentrations of ExsAHis and CTDHis preferentially resulted in formation of shift product 2 on the hybrid P exsaC-exaT1 promoter probe, ExsAl140A,l148Ahis preferentially generated shift product 1 (Fig. 9A and B). In agreement with our model that the NTD inhibits site 2 binding, separation of sites 1 and 2 on the P exsaC-exaT1 probe by 5 bp [P exsaC+(+5)exaT1] resulted in nearly equal levels of shift products 1 and 2 by ExsAl140A,l148Ahis (Fig. 9C and D, lanes 8 to 10).

To determine whether our findings from the in vitro DNA-binding studies correlated with ExsA activity in vivo, we generated transcriptional reporters consisting of the P exsaC, P exsaC+5, and P exsaT+5 promoters driving lacZ expression. Whereas ExsA activated the P exsaC-lacZ and P exsaT-lacZ reporters to high levels, ExsAl140A,l148A demonstrated a significant defect, confirming that self-association is required for activation by ExsA in vivo (Fig. 10). Neither ExsA nor ExsAl140A,l148A was able to activate the P exsaC+5-lacZ reporter, an expected result, since the separation of the ExsA-binding sites by five nucleotides should place the ExsA monomers on opposite faces of the DNA (Fig. 5E; also see Fig. S4 in the supplemental material) and prevent self-association (in the case of WT ExsA) and/or interactions with RNAP. Therefore, it was interesting that ExsA was capable of activating the P exsaT+5-lacZ reporter and that self-association was required for this activity. This finding seemed contrary to our previous data indicating that occupation of both binding sites is required for activation by ExsA (18). We previously proposed that, in some cases, site 2 occupation involves nonspecific protein-DNA interactions, because site 2 is poorly conserved among the 10 ExsA-dependent promoter regions (20). Based on these combined observations, we propose a model that takes into consideration the relative affinity of ExsA for itself (i.e., self-association) and for site 2 binding. If the Kd of ExsA for site 2 binding were lower than the Kd for self-association, then ExsA would preferentially occupy site 2 even when separated by 5
bp from site 1, resulting in low levels of reporter activity (similar to the scenario shown in Fig. 5E). Conversely, if the $K_d$ of ExsA for site 2 binding were higher than the $K_d$ for self-association, then ExsA would preferentially self-associate even when sites 1 and 2 are separated by 5 bp (see Fig. S4). In the latter case, the association of the second ExsA monomer with DNA would involve nonspecific interactions, maintaining the proper positioning of ExsA such that it can recruit RNA polymerase to activate transcription.

**DISCUSSION**

Our previous observation that the isolated DNA-binding domain of ExsA (i.e., the CTD) has reduced cooperative DNA-binding activity relative to that of full-length ExsA led to the hypothesis that NTD-mediated self-association is required for maximal activity (22). In this study, we used structural modeling to compare ExsA to AraC. AraC self-association occurs through two antiparallel $\alpha$-helices, with one helix contributed by each monomer (Fig. 1A and B). Leucine triads formed by L150, L151, and L161 anchor both ends of the interface, and hydrogen bonds formed between polar side chains located in the core of the interface further stabilize the interaction (25). Alignment of AraC and ExsA revealed low sequence similarity and no obvious candidates for leucine triad formation. Secondary structure predictions, however, suggested that AraC and ExsA have a similar composition of $\alpha$-helices and $\beta$-sheets. In particular, ExsA maintains the coiled-coil region of AraC that is involved in self-association. ExsA residues L137, L140, and L148 are involved in self-association and may be oriented in a manner amenable for leucine triad formation between the monomers. Residues L140 and L148 clearly play a role in ExsA self-association, as substitutions in either residue result in reduced self-association and promoter activation (Fig. 2). Although one would expect the L137 residue to be surface exposed, the importance of L137 is less clear, since the L137A mutant was not stably expressed in *P. aeruginosa*. Disruption of the local structure or an altered interaction with the adjacent $\alpha$-helix could explain the instability of the L137A mutant protein.

Abrogation of AraC dimer formation requires substitution of four residues (L150K, L151K, N154A, and L161S) in the self-association helix (24). Similarly positioned leucine residues are found in *Proteus mirabilis* UreR and *Pseudomonas putida* XylS (26, 28). Although individual UreR L147A, L148A, or L158A mutations result in decreased promoter activation, a combination of these mutations is required to completely disrupt transcriptional activation (28). Likewise, disruption of XylS L193 and L194 results in loss of both promoter activation and self-association. The first two leucines of the AraC, UreR, and XylS triads align with F151 and F152 of *Vibrio cholerae* ToxT. An F151 alanine substitution

**FIG 9** Binding properties of ExsA, CTD, and ExsA L140,L148A to PexsC2-exoT1 promoter probes. The indicated concentrations of ExsA, CTD, and ExsA L140,L148A were incubated with the PexsC2-exoT1 (A) or PexsC2+5-exoT1 (C) promoter probe (0.05 nM each) for 15 min at 25°C. Binding reactions were analyzed by native polyacrylamide gel electrophoresis and phosphorimaging. The positions of shift products 1 and 2 are indicated. Quantification of shift products 1 and 2 as a percentage of the total shift product is indicated for PexsC2-exoT1 (B) and PexsC2+5-exoT1 (D) promoter regions.
results in a monomeric form of ToxT that is unable to bind the tcpA promoter to wild-type levels, resulting in loss of murine intestinal colonization (31). For ExsA, pairwise combinations of the L140A, K141A, and L148A substitutions were sufficient to generate mutants with significant defects in self-association and promoter activation (Fig. 3). These mutants were chosen based on their defects in activation and self-association and stable expression in P. aeruginosa. Based on the defect exhibited by ExsA1{L140A,L148A}, the corresponding mutations recently were introduced into Versinia pestis LcrE, a close homolog of ExsA that exists as a dimer. As expected, the LcrE{L136A,L144A} mutant is largely monomeric, preferentially binds to a single site in electrophoretic mobility shift assays, and is significantly impaired for T3SS promoter activation (32).

All residues found to be important for self-association also were required for maximal activation of the P_{exoT-lacZ} reporter. In addition to L140 and L148, several other residues also seem to play an important role in self-association. The C139A and K141A mutants were significantly impaired for activation (~50% of wild-type activity) and appeared to be involved in self-association. While neither residue directly contributes to the leucine triad, the fact that these residues flank L140 suggests that they create a microenvironment amenable to leucine triad formation by affecting the orientation of L140. A few substitutions (E143A and F149A) did not activate the P_{exoT-lacZ} reporter to wild-type levels but lacked phenotypes in the self-association assays. Substitutions at these positions could directly or indirectly (i) alter interactions with the ExsD anti-activator (i.e., higher-affinity ExsA-ExsD binding), (ii) impair interactions with RNA polymerase and/or promoter DNA, or (iii) change the overall structural integrity of the protein. Interestingly, the E143A and E144A substitutions had a higher level of repression in the NTD dominant-negative assay relative to wild-type ExsA. Both of these substitutions neutralize charged residues that might inhibit self-association. Although self-association appears stronger for both mutants, reduced activation by the E143A and E144A mutants suggests that the residues also are required for DNA binding and/or promoter activation.

The self-association assays used in this study have several limitations. Although the LexA monohybrid assay was used previously to detect ExsA self-association, glycerol gradient centrifugation of purified ExsA found it to be predominantly monomeric in solution (18, 21). The LexA-based assay, therefore, appears to be extremely sensitive, revealing an interaction that cannot be seen biochemically. This may explain why single substitutions resulted in weaker phenotypes in this assay, even when using pairwise substitutions in L140, K141, and L148. The NTD dominant-negative assay measures the ability of plasmid-expressed NTD to inhibit activation by chromosomally encoded ExsA. Low levels of promoter activity correspond to a positive interaction between ExsA and the NTD. Immunoblots were unable to detect NTD protein expression, however, leaving the possibility that some substitutions resulted in unstable proteins, accounting for the low levels of promoter activity.

Binding site 2 in the P_{exoT} promoter is poorly defined but is thought to represent a low-affinity site for ExsA (18, 20). Unlike ExsA, binding by both ExsA_{L140A,L148A} and CTD to the P_{exoT} probe resulted in preferential formation of shift product 1 (Fig. 8A and B). Separating P_{exoT} binding sites 1 and 2 by 5 or 10 bp, however, did not result in increased formation of shift product 2. The latter finding is contrary to our findings with the P_{exoC} promoter probe and consistent with the idea that P_{exoT} Site 2 represents a low-affinity target for ExsA. Therefore, we propose that another role for self-association is to stabilize the interaction between the ExsA monomer bound to site 1 and the second monomer bound to the low-affinity site 2. Consistent with this model, replacement of the low-affinity P_{exoC} Site 2 with a high-affinity site (site 2 from P_{exoT}) supported formation of shift product 2 that reached levels similar to those of shift product 1, even in the absence of self-association. The importance of this function appears dispensable at the P_{exoC} promoter and may be more important for ExsA-dependent promoters like P_{exoT} with a low-affinity site 2.

One model to explain the role of self-association in counteracting the inhibitory effects of the NTD is that the NTD of a DNA-bound monomer of ExsA prevents access to the binding site, and a conformational change induced by self-association with the incoming monomer is required to allow binding. These findings have implications for further studies regarding inhibition by the anti-activator ExsD. ExsD prevents ExsA self-association and ExsA-dependent promoter activation, properties that are mediated by the ExsA NTD and CTD, respectively (21). However, ExsD interacts only with the NTD of ExsA, prompting the following question: how does ExsD affect DNA binding by the CTD? This study may partially answer this question by establishing the necessity of self-association for promoter binding; however, the ability of the self-association mutant to form shift product 1 indicates this is not the sole means of ExsD-mediated inhibition. The potential use of therapeutics that target virulence has become an attractive alternative to the use of bactericidal agents due to the expectation that inhibition of virulence factors would not lead to resistant organisms. The findings of this study suggest ExsA self-association is another potential target of novel inhibitors.

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