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

Anne E. Marsden,[a] Florian D. Schubot,[b] Timothy L. Yahr[a]

Department of Microbiology, University of Iowa, Iowa City, Iowa, USA[a]; Department of Biological Sciences, Virginia Tech, Blacksburg, Virginia, USA[b]

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_{exoT} 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.

*P*seudomonas 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_{exoT}), 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 TGnnA sequences, functionally equivalent sites have been identified only in binding site 2 for the P_{exsC} 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 mono-hybrid 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). The

crystal structure of an AraC$_{NTD}$ 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 *Proteus mirabilis* UreR, *Pseudomonas putida* XylS, and *Citrobacter rodentium* 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. *Escherichia coli* 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. *E. coli* 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. *E. coli* strain Tuner (DE3) was maintained on LB agar with ampicillin (100 μg/ml). *P. aeruginosa* 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$_{DBD}$-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 86360966-8650714), which then were cloned into pAM45, 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 mutant templates as XbaI/SacI restriction fragments (primer pair 86360966-27369454) and cloning into the arabinose-inducible pJN105 expression vector. Plasmids expressing ExsA with combinations of two alanine substitutions at residues L140, K141, or L148 (pAM77, pAM79, or pAM82) were generated through QuikChange mutagenesis using a wild-type (WT; pEB124) template and primers containing the desired mutations (primers 84452013, 84452022, and 84452014; one primer per reaction). LexA$_{DBD}$-ExsA and NTD constructs with multiple alanine substitutions (pAM114, pAM116, pAM118, pAM103, pAM105, and pAM107) were generated by PCR amplification (primer pair 83085368-86360966) from their respective pEB124 derivatives and cloned as XbaI/SacI fragments into pAM45 for the LexA monohybrid or pJN105 for NTD dominant-negative assays.

Transcriptional reporter and immunoblot assays. *P. aeruginosa* strains were grown overnight at 37°C on VBM plates with appropriate antibiotics. *E. coli* strains were grown overnight at 30°C with shaking in 5 ml LB with appropriate antibiotics. The following day, the cultures were diluted to an A_{600} of 0.1 in Trypticase soy broth (supplemented with 100 mM monosodium glutamate and 1% glycerol) for *P. aeruginosa* or LB for *E. coli*. EGTA (2 mM), arabinose, and isopropyl-β-D-thiogalactopyranoside (IPTG) were added as necessary. When the culture A_{600} reached 1.0,

samples were collected and assayed for β-galactosidase activity as previously described (29). Miller units were calculated and reported as the averages from at least three independent experiments, with error bars representing the standard errors of the means (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) using GraphPad Prism, version 5.0c, for Mac OS X (GraphPad Software, La Jolla, CA). Whole-cell lysate samples were collected by pelleting 1.25 ml of culture (A_{600} = 1.0) and resuspending in 0.25 ml SDS-PAGE sample buffer. Samples were sonicated for 10 s and analyzed by SDS-PAGE and immunoblotting.

Protein purification. The *exsA* (L140A, L148A) coding sequence from pAM79 was PCR amplified using primers (42308574-7736236) to incorporate NdeI and BamHI restriction sites. The resulting product was ligated into the pET16b expression vector to introduce an N-terminal 10× histidine tag. *E. coli* 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_{600} of 0.1. The culture was grown with shaking at 30°C until the culture A_{600} reached 0.5. Additional ampicillin was added to a final concentration of 300 μg/ml, and ExsA$_{His}$ 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^{2+}-nitrilotriacetic acid (NTA) affinity chromatography as previously described (18). After elution from the Ni^{2+}-NTA resin, peak fractions were pooled and dialyzed overnight against 4 liters of ExsA-binding buffer (excluding imidazole) with 1 mM dithiothreitol (DTT) at 4°C. Protein concentrations were determined with the DC protein assay (Bio-Rad) using bovine serum albumin protein standards. Purified ExsA$_{His}$ and CTD$_{His}$ were prepared from previously constructed strains in the manner described above (18, 22).

Electrophoretic mobility shift assays (EMSAs). Specific promoter probes (~200 bp) for P$_{exsC}$, P$_{exsC+5}$ and P$_{exsC+10}$ (with binding sites 1 and 2 separated by 5 and 10 bp, respectively), P$_{exoT}$, P$_{exoT+5}$, P$_{exsC2-exoT1}$, and P$_{exsC2(+5)exoT1}$ and the nonspecific *algD* probe (160 bp from the *algD* promoter region) were generated by PCR amplification (primer pairs 22963127-49188917 and 31216338-43613960). The PCR products were end labeled with [γ-^{32}P]ATP, and EMSA reactions were performed as previously described (18). Briefly, reaction mixtures containing specific and/or nonspecific probes (0.25 nM), ExsA DNA-binding buffer (10 mM Tris [pH 7.5], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol), 25 ng/μl poly(dI-dC), and 100 μg/ml bovine serum albumin were incubated for 5 min at 25°C. ExsA$_{His}$, CTD$_{His}$, and ExsA$_{L140A,L148AHis}$ (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 glycine 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 NTDs 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
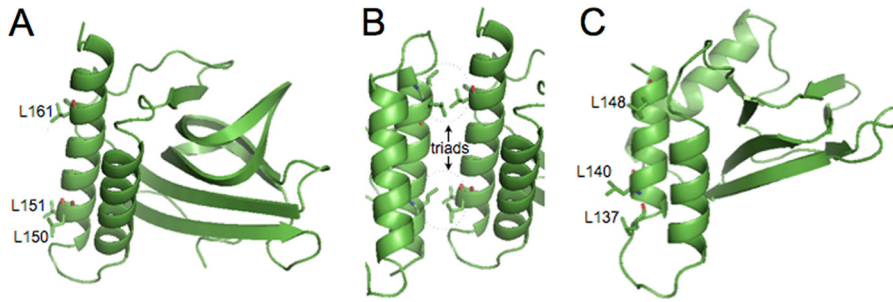
FIG 1 Structural model of the ExsA dimerization domain. (A and B) Solution structure of the AraC NTD dimer (PDB code 2ARC) showing leucine residues 150, 151, and 161 (A), which form interaction triads that stabilize the self-association interface (B). (C) PHYRE prediction of the ExsA NTD based on the 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.

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 interest can be used to test for protein-protein interactions in *E. coli* strain SU101, which carries a LexA-repressible $P_{sulA-lacZ}$ reporter. When expressed alone, the LexA$_{DBD}$ (pSR658) fails to dimerize and repress $P_{sulA-lacZ}$ reporter activity (Fig. 2B). In contrast, fusion of ExsA to the LexA$_{DBD}$ promotes dimerization and subsequent repression of $P_{sulA-lacZ}$ reporter activity (normalized to 100% repression). To determine if residues 136 to 152 are involved in self-association, the alanine scanning mutants were expressed as LexA$_{DBD}$-ExsA fusions from an IPTG-inducible vector. Since the stability of the alanine substitution mutants may differ between *P. aeruginosa* and *E. coli*, mutants that were not stably expressed in *P. aeruginosa* (L137A, L145A, and L146A) were examined in *E. coli*. The L137A and L145A, but not L146A, mutants

were stably expressed in *E. coli* as LexA$_{DBD}$-ExsA fusions (Fig. 2B). Alanine substitutions at residues L137, L140, and K141 resulted in small but significant defects in repression of $P_{sulA-lacZ}$ reporter activity, suggesting that these residues are required for maximal self-association.

A previous study found that the isolated NTD has dominant-negative activity when coexpressed with full-length ExsA (22). This activity was presumed to result from a nonproductive interaction between the NTD and full-length ExsA. Using the LexA 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, L145, 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 expression 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 NTD$_{(L137A)}$, NTD$_{(L145A)}$, and NTD$_{(L146A)}$. 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
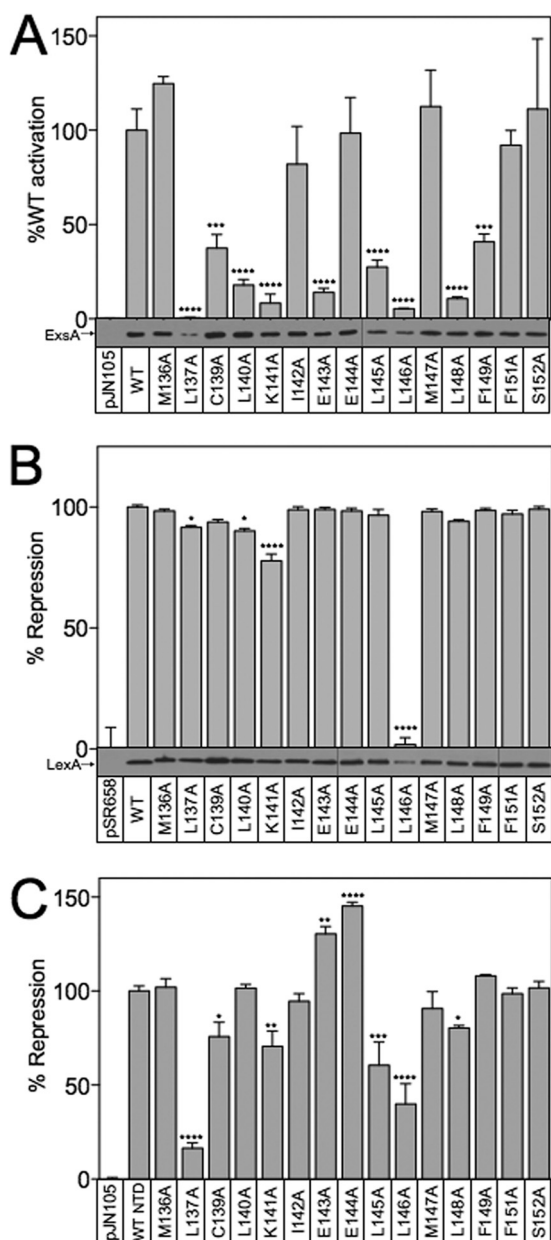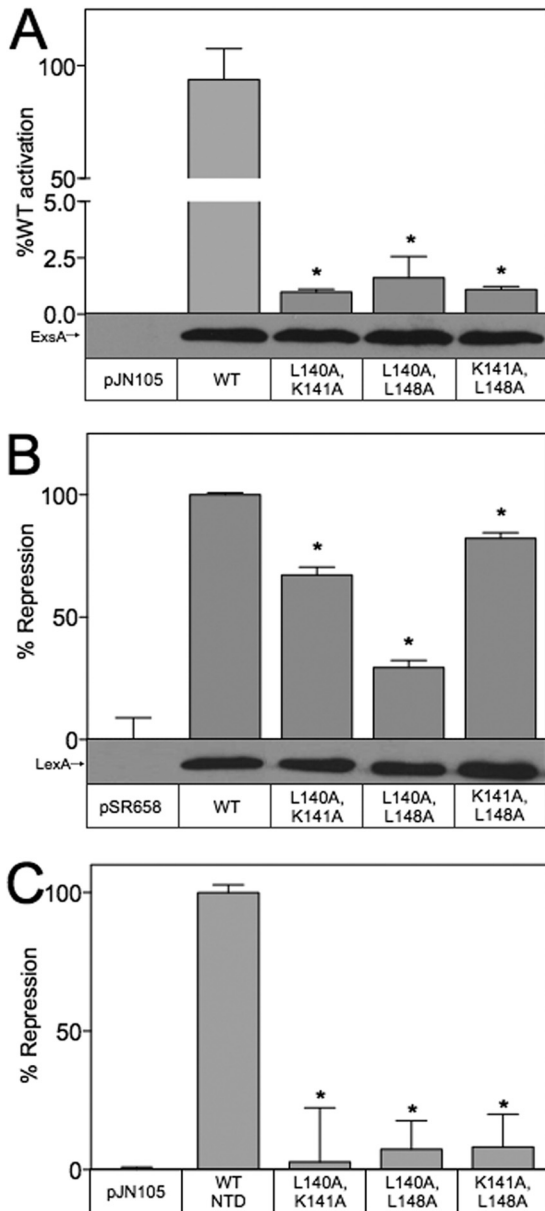
FIG 2 Characterization of alanine scanning mutants located in the ExsA self-association helix. (A) An *exsA* mutant (PA103 *exsA*::Ω) was transformed with a vector control (pJN105) or vectors expressing WT ExsA or the indicated alanine scanning mutants. Protein expression was induced by addition of 0.01% arabinose. Cells were cultured under inducing conditions (+EGTA) for T3SS gene expression and assayed for $P_{exoT-lacZ}$ activity, reported as percent WT activation. (B) *E. coli* strain SU101 was transformed with a vector control (pSR658) expressing $LexA_{DBD}$ alone, $LexA_{DBD}$-ExsA (WT), or the indicated $LexA_{DBD}$-ExsA alanine scanning mutants. Protein expression was induced by addition of 0.1 mM IPTG. Self-association was assayed by measuring $P_{sulA-lacZ}$ reporter activity and reported as percent repression, with strains carrying pSR658 or $LexA_{DBD}$-ExsA representing 0 and 100% repression, respectively. (C) The PA103 Δ*exsD* mutant expressing wild-type *exsA* was transformed with a vector control (pJN105), the ExsA NTD (WT NTD), or the indicated ExsA NTD alanine scanning mutants. Protein expression was induced by addition of 0.5% arabinose. Activation of the $P_{exsD-lacZ}$ reporter is reported as percent repression, with strains carrying pJN105 or WT NTD representing 0 and 100% repression, respectively. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$.

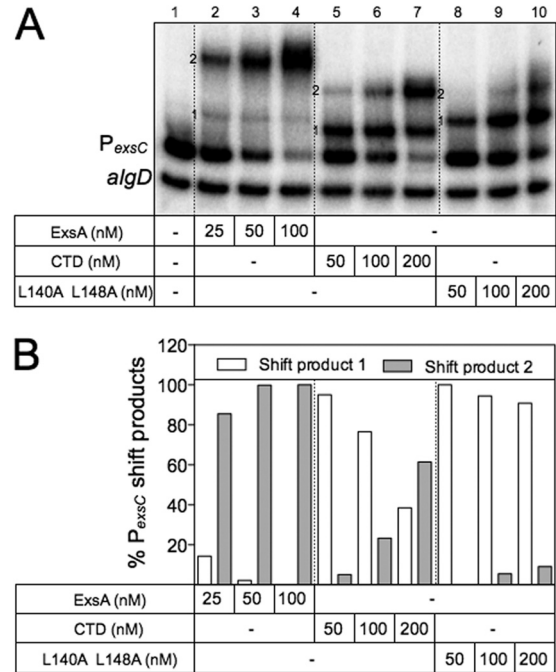with productive contacts with promoter DNA and/or RNA polymerase.

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 $P_{exoT-lacZ}$ 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 $P_{exoT-lacZ}$ 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 $ExsA_{L140A,L148A}$ mutant 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 $ExsA_{His}$ to the P_{exsC} promoter probes 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 P_{exsC} 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_{His} 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 $ExsA_{L140A,L148AHis}$ 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_{His} and $ExsA_{L140A,L148AHis}$ indicate that self-association facilitates efficient formation of shift product 2 but also revealed the surprising finding that $ExsA_{L140A,L148AHis}$ is more defective than CTD_{His}.

ExsA self-association suppresses the inhibitory activity of the NTD. The ability of CTD_{His} to efficiently occupy both binding sites on the P_{exsC} promoter probe seemed contrary to our model

FIG 4 Binding properties of ExsA, CTD, and the ExsA$_{L140,L148A}$ double mutant using radiolabeled probes derived from P$_{exsC}$. 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 ExsA$_{L140,L148A}$ 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 (P$_{exsC}$ 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.

FIG 3 Self-association is required for ExsA-dependent transcription. (A) An *exsA* mutant (PA103 *exsA*::Ω) was transformed with a vector control (pJN105), 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 P$_{exoT-lacZ}$ activity is reported as percent WT activation. (B) Self-association in the LexA monohybrid was assayed by measuring the activation of the P$_{sulA-lacZ}$ reporter. *E. coli* strain SU101 was transformed with a vector control (pSR658), LexA$_{DBD}$-ExsA expression vector (WT), or the LexA$_{DBD}$-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 LexA$_{DBD}$-ExsA representing 0 and 100% repression, respectively. (C) The dominant-negative activity of the ExsA NTD was used to measure self-association. The PA103 Δ*exsD* mutant was transformed with a vector control (pJN105), the ExsA NTD (NTD), or the indicated mutants. Protein expression was induced by addition of 0.5% arabinose. P$_{exsD-lacZ}$ activity was determined and is reported as percent repression, with strains carrying pJN105 or WT NTD representing 0 and 100%, respectively. *, $P < 0.00005$.
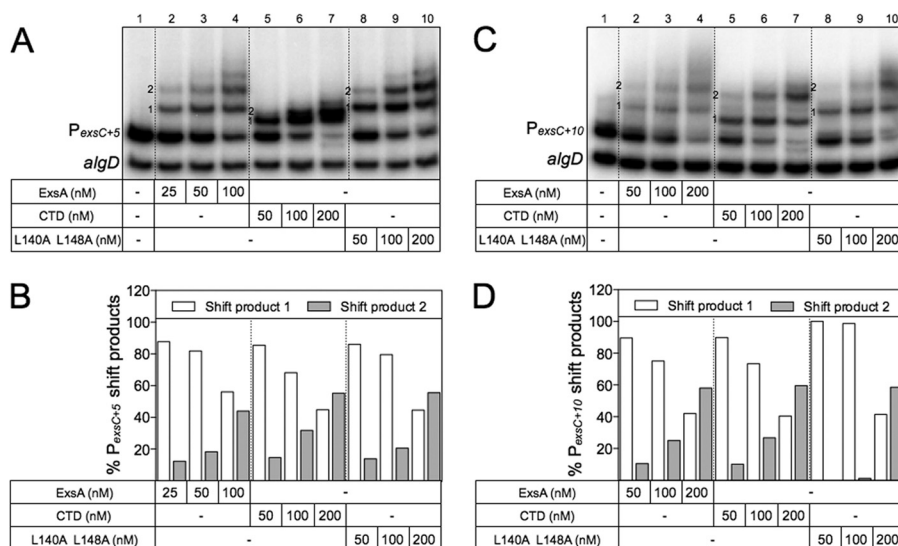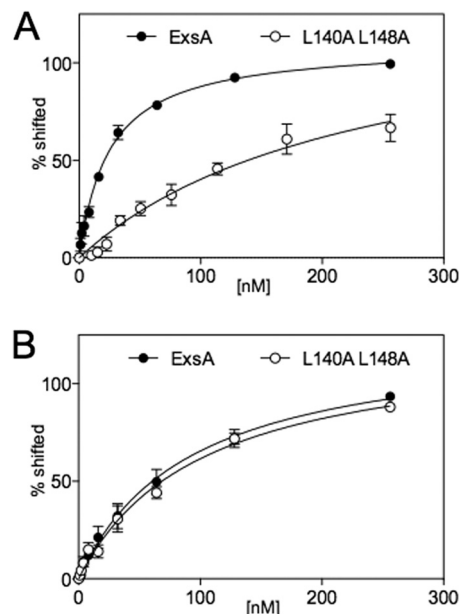
that self-association is required for maximal occupation of sites 1 and 2. One model accounting for this discrepancy is that the NTD of ExsA bound to the first site prevents binding by the second ExsA monomer, and that self-association between the two monomers is required to provide access to the second site (Fig. 5A and B). In the case of CTD$_{His}$, which lacks the NTD, this inhibitory mechanism is absent, allowing for occupation of both sites (Fig. 5C). Conversely, the inability of ExsA$_{L140A,L148AHis}$ 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 ExsA$_{L140A,L148AHis}$ (Fig. 5E). To test this idea, P$_{exsC}$ sites 1 and 2 were separated by 5 or 10 bp, resulting in promoter probes P$_{exsC+5}$ and P$_{exsC+10}$, respectively (see Fig. S3 in the supplemental material), and EMSAs were performed with wild-type ExsA$_{His}$, CTD$_{His}$, and ExsA$_{L140A,L148AHis}$. All three proteins generated shift products 1 and 2 when using the P$_{exsC+5}$ and P$_{exsC+10}$ promoter probes, and in each case the formation of shift product 2 reached ~50% of the total shifted material (Fig. 6A to D). In particular, ExsA$_{L140A,L148AHis}$ binding to P$_{exsC+5}$ was significantly improved relative to that for the native P$_{exsC}$ probe. The apparent dissociation constant (K_d) for ExsA$_{His}$ and ExsA$_{L140A,L148AHis}$ binding to the native P$_{exsC+5}$ probe differed by 10-fold (25 and 230 nM, respectively) (Fig. 7A; also see Fig. S2). In contrast, the dissociation constants for ExsA$_{His}$ and ExsA$_{L140A,L148AHis}$ binding to the P$_{exsC+5}$ promoter probe were
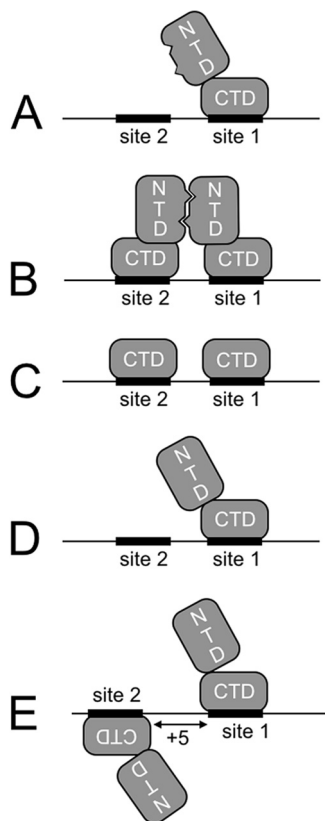
FIG 5 Model for inhibition of DNA binding by the NTD of ExsA. (A) When bound to the first site, the NTD of the first ExsA molecule inhibits binding of the incoming monomer to the second site. (B) Self-association of WT ExsA with a second monomer through the NTD relieves the block on occupation of the 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).
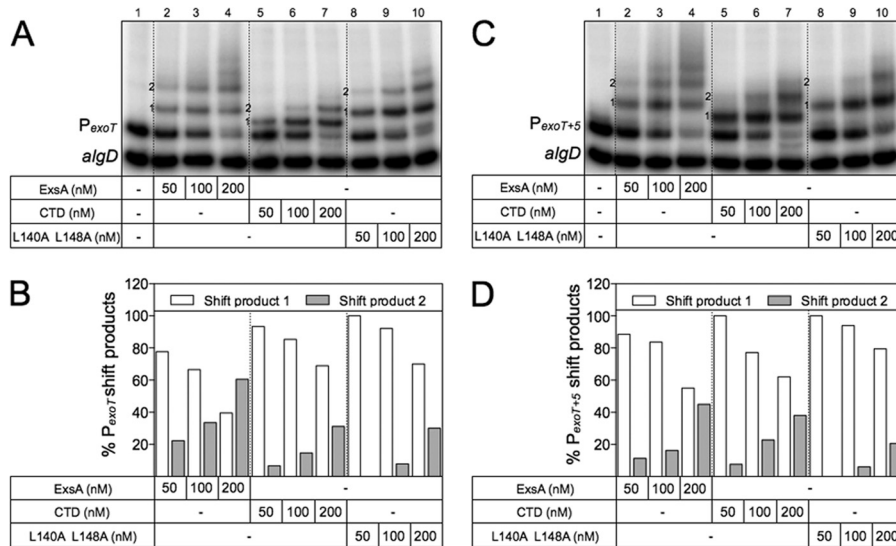

FIG 7 Apparent equilibrium binding constant for ExsA and $ExsA_{L140,L148A}$ binding to the P_{exsC} (A) and P_{exsC+5} (B) promoter probes. The percentage of shifted probe (y axis) was plotted as a function of protein concentration (x axis). The reported values are averages from three independent experiments.

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

FIG 6 Binding properties of ExsA, CTD, and the $ExsA_{L140,L148A}$ double mutant using radiolabeled probes derived from P_{exsC+5} (A) and $P_{exsC+10}$ (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+5}, $P_{exsC+10}$, 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+5} (B) and $P_{exsC+10}$ (D) probes.

FIG 8 Binding properties of ExsA, CTD, and ExsA$_{L140,L148A}$ to P$_{exoT}$ promoter probes. The indicated concentrations of ExsA, CTD, and the ExsA$_{L140,L148A}$ double mutant were incubated with radiolabeled P$_{exoT}$ (A) or P$_{exoT+5}$ (C) promoter probes (0.05 nM each) and the nonspecific *algD* probe 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. (B and D) Quantification of shift products 1 and 2 as a percentage of the total shift product for the P$_{exoT}$ (B) and P$_{exoT+5}$ (D) promoter probes.

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 ExsA$_{L140A,L148A}$, 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 ExsA$_{L140A,L148A}$ 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$_{exsC}$ 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$_{exoT}$ promoter probe. Whereas 200 nM ExsA$_{His}$ formed more shift product 2 than shift product 1 on the P$_{exoT}$ promoter probe (Fig. 8A and B, lanes 2 to 4), the same concentration of CTD$_{His}$ and ExsA$_{L140A,L148AHis}$ preferentially generated shift product 1 (Fig. 8A and B, lanes 5 to 10). These data are similar to our findings for ExsA$_{His}$ and ExsA$_{L140A,L148AHis}$ binding to the P$_{exsC}$ 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$_{exsC+5}$ promoter probe (Fig. 6A and B), however, separation of P$_{exoT}$ binding sites 1 and 2 by 5 bp (P$_{exoT+5}$) did not result in increased generation of shift product 2 by ExsA$_{L140A,L148AHis}$ (Fig. 8C and D, lanes 8 to 10). One possible explanation is that the weaker affinity of ExsA for P$_{exoT}$ site 2 relative to P$_{exsC}$ site 2 is unable to support efficient occupation by ExsA$_{L140A,L148AHis}$, 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$_{exoT}$ site 2 with the higher-affinity site 2 from P$_{exsC}$. Whereas high concentrations of ExsA$_{His}$ and CTD$_{His}$ preferentially resulted in formation of shift product 2 on the hybrid P$_{exsC2-exoT1}$ promoter probe, ExsA$_{L140A,L148AHis}$ 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$_{exsC2-exoT1}$ probe by 5 bp [P$_{exsC2(+5)exoT1}$] resulted in nearly equal levels of shift products 1 and 2 by ExsA$_{L140A,L148AHis}$ (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$_{exsC}$, P$_{exsC+5}$, P$_{exoT}$, and P$_{exoT+5}$ promoters driving *lacZ* expression. Whereas ExsA activated the P$_{exsC-lacZ}$ and P$_{exoT-lacZ}$ reporters to high levels, ExsA$_{L140A,L148A}$ demonstrated a significant defect, confirming that self-association is required for activation by ExsA *in vivo* (Fig. 10). Neither ExsA nor ExsA$_{L140A,L148A}$ was able to activate the P$_{exsC+5-lacZ}$ 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$_{exoT+5-lacZ}$ 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 K_d of ExsA for site 2 binding were lower than the K_d for self-association, then ExsA would preferentially occupy site 2 even when separated by 5
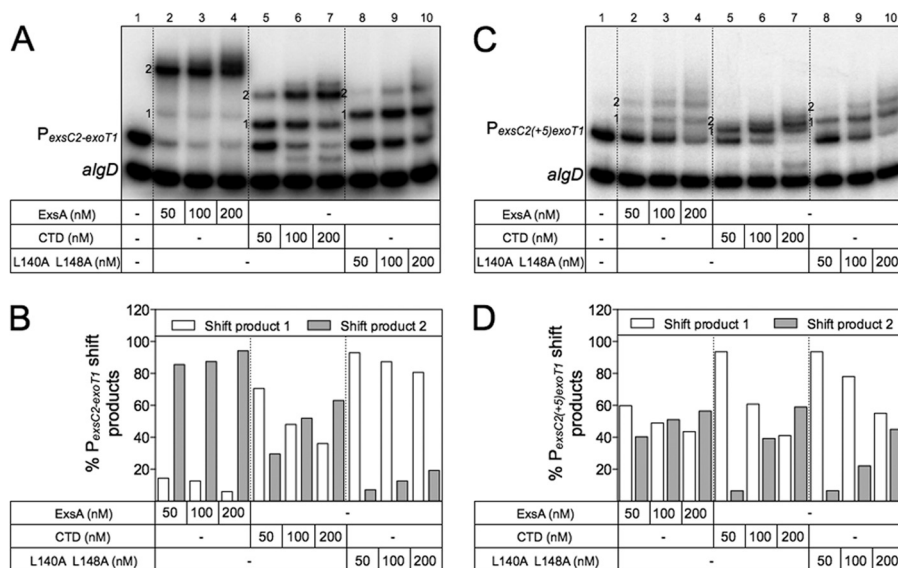
FIG 9 Binding properties of ExsA, CTD, and ExsA$_{L140,L148A}$ to P$_{exsC2-exoT1}$ promoter probes. The indicated concentrations of ExsA, CTD, and ExsA$_{L140,L148A}$ were incubated with the P$_{exsC2-exoT1}$ (A) or P$_{exsC2(+5)exoT1}$ (C) promoter probe (0.05 nM each) for 15 min at 25°C. Binding reactions 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 P$_{exsC2-exoT1}$ (B) and P$_{exsC2(+5)exoT1}$ (D) promoter regions.

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
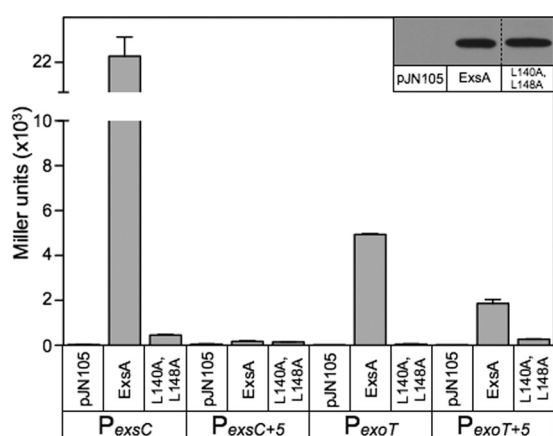
FIG 10 PA103 *exsA*::Ω mutant carrying the indicated transcriptional reporters was transformed with a vector control (pJN105) or with ExsA or ExsA$_{L140,L148A}$ (pAM79) expression vectors. The resulting strains were grown under inducing conditions (+EGTA) for T3SS gene expression, and protein expression was induced by addition of 0.01% arabinose. Reporter activity was measured by β-galactosidase activity and is reported in Miller units. The reported values are averages from three independent experiments. Protein expression levels were measured by immunoblotting of whole-cell lysates with ExsA antiserum.

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 α-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 α-helices and β-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 α-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

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 ExsA$_{L140A,L148A}$, the corresponding mutations recently were introduced into *Yersinia pestis* LcrF, a close homolog of ExsA that exists as a dimer. As expected, the LcrF$_{L136A,L144A}$ 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$_{exsC}$ 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$_{exoT}$ site 2 with a high-affinity site (site 2 from P$_{exsC}$) 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$_{exsC}$ 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.

ACKNOWLEDGMENTS

REFERENCES

1. **Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, Kallen A, Limbago B, Fridkin S.** 2013. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. Infect. Control Hosp. Epidemiol. **34:**1–14. http://dx.doi.org/10.1086/668770.
2. **Anonymous.** 2004. National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992 through June 2004, issued October 2004. Am. J. Infect. Control **32:**470–485. http://dx.doi.org/10.1016/j.ajic.2004.10.001.
3. **Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK.** 2008. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect. Control Hosp. Epidemiol. **29:**996–1011. http://dx.doi.org/10.1086/591861.
4. **Cornelis GR.** 2006. The type III secretion injectisome. Nat. Rev. Microbiol. **4:**811–825. http://dx.doi.org/10.1038/nrmicro1526.
5. **Holder IA, Neely AN, Frank DW.** 2001. Type III secretion/intoxication system important in virulence of Pseudomonas aeruginosa infections in burns. Burns **27:**129–130. http://dx.doi.org/10.1016/S0305-4179(00)00142-X.
6. **Holder IA, Neely AN, Frank DW.** 2001. PcrV immunization enhances survival of burned Pseudomonas aeruginosa-infected mice. Infect. Immun. **69:**5908–5910. http://dx.doi.org/10.1128/IAI.69.9.5908-5910.2001.
7. **Kurahashi K, Kajikawa O, Sawa T, Ohara M, Gropper MA, Frank DW, Martin TR, Wiener-Kronish JP.** 1999. Pathogenesis of septic shock in

Pseudomonas aeruginosa pneumonia. J. Clin. Investig. **104:**743–750. http://dx.doi.org/10.1172/JCI7124.

8. **Sawa T, Yahr TL, Ohara M, Kurahashi K, Gropper MA, Wiener-Kronish JP, Frank DW.** 1999. Active and passive immunization with the Pseudomonas V antigen protects against type III intoxication and lung injury. Nat. Med. **5:**392–398. http://dx.doi.org/10.1038/7391.

9. **Alibaud L, Kohler T, Coudray A, Prigent-Combaret C, Bergeret E, Perrin J, Benghezal M, Reimmann C, Gauthier Y, van Delden C, Attree I, Fauvarque MO, Cosson P.** 2008. Pseudomonas aeruginosa virulence genes identified in a Dictyostelium host model. Cell. Microbiol. **10:**729–740. http://dx.doi.org/10.1111/j.1462-5822.2007.01080.x.

10. **Abd H, Wretlind B, Saeed A, Idsund E, Hultenby K, Sandstrom G.** 2008. Pseudomonas aeruginosa utilises its type III secretion system to kill the free-living amoeba Acanthamoeba castellanii. J. Eukaryot. Microbiol. **55:**235–243. http://dx.doi.org/10.1111/j.1550-7408.2008.00311.x.

11. **Matz C, Moreno AM, Alhede M, Manefield M, Hauser AR, Givskov M, Kjelleberg S.** 2008. Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae. ISME J. **2:**843–852. http://dx.doi.org/10.1038/ismej.2008.47.

12. **Brannon MK, Davis JM, Mathias JR, Hall CJ, Emerson JC, Crosier PS, Huttenlocher A, Ramakrishnan L, Moskowitz SM.** 2009. Pseudomonas aeruginosa type III secretion system interacts with phagocytes to modulate systemic infection of zebrafish embryos. Cell. Microbiol. **11:**755–768. http://dx.doi.org/10.1111/j.1462-5822.2009.01288.x.

13. **Hauser AR.** 2009. The type III secretion system of Pseudomonas aeruginosa: infection by injection. Nat. Rev. Microbiol. **7:**654–665. http://dx.doi.org/10.1038/nrmicro2199.

14. **Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, Sawa T, Frank DW, Wiener-Kronish JP.** 2001. Type III protein secretion is associated with death in lower respiratory and systemic Pseudomonas aeruginosa infections. J. Infect. Dis. **183:**1767–1774. http://dx.doi.org/10.1086/320737.

15. **El-Solh AA, Hattemer A, Hauser AR, Alhajhusain A, Vora H.** 2012. Clinical outcomes of type III Pseudomonas aeruginosa bacteremia. Crit. Care Med. **40:**1157–1163. http://dx.doi.org/10.1097/CCM.0b013e3182377906.

16. **Hauser AR, Cobb E, Bodi M, Mariscal D, Valles J, Engel JN, Rello J.** 2002. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by Pseudomonas aeruginosa. Crit. Care Med. **30:**521–528. http://dx.doi.org/10.1097/00003246-200203000-00005.

17. **Frank DW.** 1997. The exoenzyme S regulon of Pseudomonas aeruginosa. Mol. Microbiol. **26:**621–629. http://dx.doi.org/10.1046/j.1365-2958.1997.6251991.x.

18. **Brutinel ED, Vakulskas CA, Brady KM, 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:**657–671. http://dx.doi.org/10.1111/j.1365-2958.2008.06179.x.

19. **King JM, Brutinel ED, Marsden AE, Schubot FD, Yahr TL.** 2012. Orientation of Pseudomonas aeruginosa ExsA monomers bound to promoter DNA and base-specific contacts with the P(exoT) promoter. J. Bacteriol. **194:**2573–2585. http://dx.doi.org/10.1128/JB.00107-12.

20. **Brutinel ED, King JM, Marsden AE, Yahr TL.** 2012. The distal ExsA-binding site in Pseudomonas aeruginosa type III secretion system promoters is the primary determinant for promoter-specific properties. J. Bacteriol. **194:**2564–2572. http://dx.doi.org/10.1128/JB.00106-12.

21. **Brutinel ED, Vakulskas CA, Yahr TL.** 2010. ExsD inhibits expression of the Pseudomonas aeruginosa type III secretion system by disrupting ExsA self-association and DNA binding activity. J. Bacteriol. **192:**1479–1486. http://dx.doi.org/10.1128/JB.01457-09.

22. **Brutinel ED, Vakulskas CA, Yahr TL.** 2009. Functional domains of ExsA, the transcriptional activator of the Pseudomonas aeruginosa type III secretion system. J. Bacteriol. **191:**3811–3821. http://dx.doi.org/10.1128/JB.00002-09.

23. **Bustos SA, Schleif RF.** 1993. Functional domains of the AraC protein. Proc. Natl. Acad. Sci. U. S. A. **90:**5638–5642. http://dx.doi.org/10.1073/pnas.90.12.5638.

24. **LaRonde-LeBlanc N, Wolberger C.** 2000. Characterization of the oligomeric states of wild type and mutant AraC. Biochemistry **39:**11593–11601. http://dx.doi.org/10.1021/bi001262g.

25. **Soisson SM, MacDougall-Shackleton B, Schleif R, Wolberger C.** 1997. Structural basis for ligand-regulated oligomerization of AraC. Science **276:**421–425. http://dx.doi.org/10.1126/science.276.5311.421.

26. **Ruiz R, Marques S, Ramos JL.** 2003. Leucines 193 and 194 at the N-terminal domain of the XylS protein, the positive transcriptional regulator of the TOL meta-cleavage pathway, are involved in dimerization. J. Bacteriol. **185:**3036–3041. http://dx.doi.org/10.1128/JB.185.10.3036-3041.2003.

27. **Yang J, Dogovski C, Hocking D, Tauschek M, Perugini M, Robins-Browne RM.** 2009. Bicarbonate-mediated stimulation of RegA, the global virulence regulator from Citrobacter rodentium. J. Mol. Biol. **394:**591–599. http://dx.doi.org/10.1016/j.jmb.2009.10.033.

28. **Poore CA, Coker C, Dattelbaum JD, Mobley HL.** 2001. Identification of the domains of UreR, an AraC-like transcriptional regulator of the urease gene cluster in Proteus mirabilis. J. Bacteriol. **183:**4526–4535. http://dx.doi.org/10.1128/JB.183.15.4526-4535.2001.

29. **Dasgupta N, Lykken GL, Wolfgang MC, Yahr TL.** 2004. A novel anti-anti-activator mechanism regulates expression of the Pseudomonas aeruginosa type III secretion system. Mol. Microbiol. **53:**297–308. http://dx.doi.org/10.1111/j.1365-2958.2004.04128.x.

30. **Butala M, Zgur-Bertok D, Busby SJ.** 2009. The bacterial LexA transcriptional repressor. Cell. Mol. Life Sci. **66:**82–93. http://dx.doi.org/10.1007/s00018-008-8378-6.

31. **Childers BM, Cao X, Weber GG, Demeler B, Hart PJ, Klose KE.** 2011. N-terminal residues of the Vibrio cholerae virulence regulatory protein ToxT involved in dimerization and modulation by fatty acids. J. Biol. Chem. **286:**28644–28655. http://dx.doi.org/10.1074/jbc.M111.258780.

32. **King JM, Schesser Bartra S, Plano G, Yahr TL.** 2013. ExsA and LcrF recognize similar consensus binding sites, but differences in their oligomeric state influence interactions with promoter DNA. J. Bacteriol. **195:**5639–5650. http://dx.doi.org/10.1128/JB.00990-13.