ExsA and LcrF Recognize Similar Consensus Binding Sites, but Differences in Their Oligomeric State Influence Interactions with Promoter DNA

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ExsA activates type III secretion system (T3SS) gene expression in *Pseudomonas aeruginosa* and is a member of the AraC family of transcriptional regulators. AraC proteins contain two helix-turn-helix (HTH) DNA binding motifs. One helix from each HTH motif inserts into the major groove of the DNA to make base-specific contacts with the promoter region. The amino acids that comprise the HTH motifs of ExsA are nearly identical to those in LcrF/VirF, the activators of T3SS gene expression in the pathogenic yersiniae. In this study, we tested the hypothesis that ExsA/LcrF/VirF recognize a common nucleotide sequence. We report that *Yersinia pestis* LcrF binds to and activates transcription of ExsA-dependent promoters in *P. aeruginosa* and that plasmid-expressed ExsA complements a *Y. pestis lcrF* mutant for T3SS gene expression. Mutations that disrupt the ExsA consensus binding sites in both *P. aeruginosa* and *Y. pestis* T3SS promoters prevent activation by ExsA and LcrF. Our combined data demonstrate that ExsA and LcrF recognize a common nucleotide sequence. Nevertheless, the DNA binding properties of ExsA and LcrF are distinct. Whereas two ExsA monomers are sequentially recruited to the promoter region, LcrF binds to promoter DNA as a preformed dimer and has a higher capacity to bend DNA. An LcrF mutant defective for dimerization bound promoter DNA with properties similar to ExsA. Finally, we demonstrate that the activators of T3SS gene expression from *Photorhabdus luminescens*, *Aeromonas hydrophila*, and *Vibrio parahaemolyticus* are also sensitive to mutations that disrupt the ExsA consensus binding site.

The pathogenic lifestyles of *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Yersinia pestis*, and *Yersinia pseudotuberculosis* are each dependent upon a type III secretion system (T3SS) (1). The T3SS is thought to function like a molecular syringe to inject effector proteins into host cells (2). Although the T3SS structural components are highly conserved between *P. aeruginosa* and the yersiniae, the repertoires of translocated effectors are distinct. A primary role for the effector proteins from each of these organisms is to inhibit phagocytosis, thereby allowing the bacteria to replicate to high numbers and overwhelm the host (1, 3, 4). T3SSs have also been described as contact-dependent secretion systems because physical contact between the bacterium and the host cell serves as a signal to initiate translocation of the effectors and to induce high levels of T3SS gene expression (5). The host contact signal can be mimicked by growing *P. aeruginosa* or the yersiniae under calcium-limiting conditions (6). Host contact and calcium limitation also trigger the secretion/translocation of regulatory proteins that inhibit T3SS gene expression (7). This feature serves as an efficient mechanism to couple gene expression to secretory activity. Despite the many similarities between the *P. aeruginosa* and yersiniae T3SSs, however, the mechanisms involved in regulating T3SS gene expression are distinct.

The primary transcriptional activator of T3SS gene expression in *P. aeruginosa* is ExsA (8). ExsA-dependent transcription is activated in response to inducing conditions (i.e., low Ca^{2+} or host cell contact) by a partner-switching mechanism involving three additional proteins: ExsC, ExsD, and ExsE (7). ExsD functions as an antiactivator by directly binding to the amino-terminal domain (NTD) of ExsA and inhibiting ExsA-dependent transcription (9, 10). ExsC is an anti-antiactivator that binds to and antagonizes the inhibitory activity of ExsD (11–13). The final

component of the cascade, ExsE, is a secreted substrate of the type III export machinery. ExsE also binds to ExsC and prevents formation of the ExsC-ExsD complex (14, 15). The current model proposes that nonpermissive conditions (i.e., high Ca^{2+}) prevent T3SS gene expression through formation of the inhibitory ExsD-ExsA and ExsC-ExsE complexes (16). Conversely, inducing conditions trigger ExsE secretion and the partner-switching mechanism whereupon formation of the ExsD-ExsC complex is favored and free ExsA is available to activate transcription.

The yersiniae have an ExsA homolog (LcrF/VirF) that also activates T3SS gene expression but lack homologs of ExsE, ExsC, and ExsD and rely upon alternative mechanisms to regulate LcrF-dependent transcription. Expression of the yersiniae T3SS is induced in response to elevated temperature (i.e., 37°C). This induction is due, at least in part, to thermoregulation of LcrF expression and occurs at both the transcriptional and posttranscriptional level. LcrF is encoded as the last gene of the *yscW-lcrF* operon, with expression of the operon being directly repressed by YmoA, a small nucleoid-associated protein (17–19). YmoA is degraded by the Clp and Lon proteases at 37°C, thereby resulting in increased *yscW-lcrF* transcription (20). Posttranscriptional control of LcrF

expression involves an inhibitory secondary structure that forms in the 5′-untranslated leader region of the *lcrF* mRNA and prevents LcrF translation at moderate temperatures (19, 21). At 37°C, the mRNA undergoes a structural alteration that provides ribosomal access to the Shine-Dalgarno sequence, resulting in elevated LcrF translation. Neither transcriptional nor posttranscriptional regulation of LcrF expression, however, is directly linked to the activity of the secretion machinery. Instead, T3SS gene expression in the yersiniae is coupled to secretory activity via the combined action of two substrate chaperone complexes (YopD-SycD and LcrQ-SycH) that function, in part, to suppress T3SS gene expression prior to activation of the secretion process (22, 23). Although the mechanism by which these regulatory complexes suppress T3SS gene expression is not fully understood, recent studies suggest that these complexes directly interact with the 5′-untranslated regions of target mRNAs to block translation and enhance mRNA degradation (24). Upon contact with a eukaryotic cell, secretion of YopD and LcrQ is triggered, resulting in the disassembly of the YopD-SycD and LcrQ-SycH negative regulatory complexes and facilitating high-level T3SS gene expression. Secretion is further regulated by the YopN-SycN-YscB-TyeA complex that acts as a plug by blocking the Ysc secretion channel and preventing YopN transport in the presence of calcium and prior to contact with eukaryotic cells (25). Upon induction (i.e., low calcium/host cell contact), free YopN is subsequently secreted through an open channel, allowing Yop effectors to freely travel through the injectisome and into the host cell.

ExsA and LcrF are both members of the AraC/XylS family of transcriptional regulators. Prototypical AraC/XylS proteins consist of two distinct domains (amino- and carboxy-terminal domains [CTD]) separated by a flexible linker (26). The amino-terminal domain of AraC proteins is usually involved in self-association. Previous studies have established that ExsA is monomeric in solution but self-associates as a dimer when bound to DNA (27). In contrast, purified VirF/LcrF is thought to be dimeric in solution (28). In some cases, the NTD of AraC proteins can also serve as in input for regulatory signals. For instance, binding of arabinose to the NTD of AraC results in activation of the P_{BAD} promoter (29). Conversely, binding of ExsD to the NTD of ExsA inhibits DNA binding activity (9, 30). A regulatory role for the NTD of LcrF has not been described.

The carboxy-terminal domain of ExsA contains two helix-turn-helix (HTH) DNA binding motifs (26). Each HTH has a recognition helix that makes base-specific contacts with the target DNA (i.e., promoter region). Recent studies have characterized the interaction of ExsA with several *P. aeruginosa* T3SS promoters. Each ExsA-dependent promoter consists of two adjacent binding sites for monomeric ExsA (27). Binding site 1 is centered ~41 bp upstream of the transcription start, and binding site 2 is centered at the −65 position. An alignment of all 10 ExsA-dependent promoter sequences identified a consensus ExsA binding site, **AaAA A**nwmMy**grC**ynnnm**TG**ay**A**k, with the nucleotide positions indicated in bold typeface being required for maximal ExsA binding (27) and the uppercase letters representing more highly conserved positions than the lowercase letters. The conserved GnC and TGnnA sequences constitute binding site 1 and are recognized by the two HTH motifs of a single ExsA monomer (31). The adenine-rich sequence is positioned within binding site 2. The precise nature of the interaction of ExsA with binding site 2, however, is unclear and appears to differ between promoters (32). Occupation of the promoter region by ExsA occurs in an ordered manner

whereby one ExsA monomer binds to site 1 and then recruits a second monomer to site 2. Efficient occupation of site 2 requires self-association of the ExsA monomers that is mediated by the NTD (33). The ExsA monomers are positioned in a head-to-tail orientation when bound to sites 1 and 2 (31). Once bound to the promoter region, ExsA activates transcription by recruiting the RNA polymerase-σ^{70} complex to the promoter (34, 35).

We hypothesized that the detailed information available regarding ExsA binding activity might be applicable to the interaction of LcrF/VirF with promoter DNA. Using promoter fusions, mutagenesis, and *in vitro* DNA binding assays, we find that LcrF binds to and activates transcription of ExsA-dependent promoters in *P. aeruginosa* and that plasmid-expressed ExsA complements a *Y. pestis lcrF* mutant for T3SS gene expression. Promoter mutations that disrupt ExsA-dependent activation also inhibit activation by LcrF, suggesting that ExsA and LcrF recognize a common DNA sequence. In support of this conclusion, each of the core T3SS promoter regions from the yersiniae possesses well-defined ExsA consensus binding sites. Our findings with LcrF are also applicable to the activators of T3SS gene expression from *Photorhabdus luminescens*, *Aeromonas hydrophila*, and *Vibrio parahaemolyticus*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are provided in Table S1 in the supplemental material. *Escherichia coli* strains were cultured in LB-Miller (LB) broth or agar supplemented with ampicillin (100 μg/ml) or gentamicin (15 μg/ml) as required. *P. aeruginosa* strains were cultured on Vogel-Bonner minimal medium agar containing gentamicin (100 μg/ml) and/or carbenicillin (300 μg/ml) as required (36). To measure T3SS gene expression, *P. aeruginosa* strains were cultured at 30°C in Trypticase soy broth (TSB) supplemented with 100 mM monosodium glutamate, 1% glycerol, and 2 mM EGTA to an optical density at 600 nm (OD_{600}) of approximately 1.0. β-Galactosidase activity was measured using the 2-nitrophenyl-β-D-galactopyranoside substrate as previously described (12). All values reported within this study represent the average from at least three independent experiments.

The *Y. pestis* strains used in this study are Pgm⁻ and avirulent by peripheral routes of infection (37). *Y. pestis* KIM5-3001 and derivatives of these strains were routinely grown in heart infusion broth (HIB) liquid medium or on tryptose blood agar (TBA) plates at a temperature of 28°C. For secretion experiments, *Y. pestis* strains were grown in the presence or absence of 2.5 mM calcium chloride in thoroughly modified Higachi's (TMH) defined medium as previously described (25).

SDS-PAGE and immunoblotting. Whole-cell lysates of *P. aeruginosa* were prepared by harvesting 1.25 ml of cell culture (OD_{600} of 1.0) by centrifugation (16,000 × *g*, 5 min, 23°C), suspending in 0.25 ml 2× SDS-PAGE sample buffer, and sonicating for 5 s. Secreted protein samples were prepared from 1 ml of cell-free culture supernatant fluid by adding 350 μl of 50% trichloroacetic acid and incubating overnight at 4°C. Precipitated protein was collected by centrifugation (16,000 × *g*, 15 min, 23°C), washed with acetone, dried, and suspended in 15 μl 2× SDS-PAGE sample buffer. Samples were analyzed by 15% SDS-PAGE and subjected to immunoblotting or silver staining.

Construction of *lcrF* deletion mutants. Deletion of the *lcrF* coding sequence and insertion of a *kan* (KIM5-3233-F2) or *dhfr* (KIM5-3001-F1) gene was accomplished using lambda red-mediated recombination essentially as described by Datsenko and Wanner (38). PCR products used to construct gene replacements were generated from template plasmid pKD4 (*kan*) or by using the EZ::TN<DHFR> transposon (Epicentre, Madison, WI) as a template for PCR. Primers P1 and P2 (see Table S2 in the supplemental material) were used to amplify the *kan* and *dhfr* PCR products. *Y. pestis* KIM5-3001 and KIM5-3233 were electroporated with

pKD46, encoding the red recombinase. *Y. pestis* strains carrying pKD46 were grown in HIB at 28°C to an OD_{620} of 0.5 and then for 2 h with 0.2% L-arabinose. Electrocompetent cells were prepared as previously described and electroporated with purified PCR products (39). Gene replacements were confirmed by PCR using oligonucleotides LcrF-F1 and LcrF-R1. The FLP recombination target (FRT)-flanked *kan* cassette was removed via FRT-mediated recombination using plasmid pCP20 as previously described (40). Plasmids pKD46 and pCP20 were cured from the *Y. pestis* deletion mutants by overnight growth at 39°C.

Plasmid construction and site-directed mutagenesis. All of the reporter and plasmid constructs used in this study are provided in Tables S3 and S4, respectively, in the supplemental material. The pBAD30-LcrF expression vector was constructed by amplifying a 0.9-kb LcrF-encoding DNA fragment amplified from plasmid pCD1 (41) using PCR primers LcrF-KpnI (TTTGGTACCTTTAGATTTTTAGGACAGTAT) and LcrF-HindIII (TTTAAGCTTACTTTATAGTCCAAAAGTGTC) (see Table S2 in the supplemental material). The PCR product was cloned into the KpnI/HindIII restriction sites of pBAD30 (42), generating plasmid pBAD30-LcrF. The LcrF expression vector (pJK32) was constructed by first performing site-directed mutagenesis on pBAD30-LcrF (QuikChange system) using primer pair 81126787-81126788 (see Table S2) to destroy the NdeI restriction site (through introduction of a silent mutation) within *lcrF*. The resulting plasmid (pJK29) was used in a subsequent PCR with primer pair 81559809-81126789 (see Table S4) to amplify *lcrF*. The PCR product was cloned into the NdeI/SacI restriction sites of pEB131, which replaces the *exsA* coding sequence with *lcrF*, resulting in pJK32. To generate the pET16b*lcrF* expression plasmid, primer pair 81559809-82001905 (see Table S4) was used to amplify *lcrF*, and the resulting PCR product was cloned into NdeI/BamHI restriction sites in pET16b. To generate the pVxsA plasmid, *vxsA* was PCR amplified from *V. parahaemolyticus* genomic DNA using primer pair 85928866-86519886 (see Table S4), and the PCR product was cloned into pEB131 vector so that translation was controlled by the native ExsA ribosomal binding site.

The *Y. pestis* transcriptional reporters were made by PCR amplifying the P_{yopN}, P_{lcrG}, and P_{yscN} promoter regions using the primer sets indicated in Table S3 in the supplemental material. PCR products were cloned into mini-CTX-lacZ as EcoRI/HindIII restriction fragments and integrated onto the PA103 chromosome as previously described (43). P_{yscN} promoter mutations were introduced using a two-step PCR method. The P_{yscN} 5′ Hind primer (88203767) and the specific primers indicated in Table S2 in the supplemental material (minictxJK503-511) were used to generate megaprimers. The megaprimers were used in a second PCR with the P_{yscN} 3′ Eco primer. The resulting PCR products were cloned as HindIII/EcoRI restriction fragments into mini-CTX-lacZ and integrated onto the PA103 chromosome as described above.

Protein expression and purification. *E. coli* Tuner(DE3) carrying either the pET16b*exsA* or pET16b*lcrF* expression vector was grown at 30°C in LB supplemented with ampicillin (200 μg/ml). When the culture OD_{600} reached 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM final concentration) was added to induce ExsA or LcrF expression. Cultures were incubated an additional 2 to 4 h at 30°C. Cells were harvested by centrifugation (6,000 × *g*, 10 min, 4°C) and suspended in 30 ml ExsA buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 20 mM imidazole, 0.5% Tween 20) supplemented with two protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN). Cells were lysed using a Microfluidizer (Microfluidics, Newton, MA), and the suspension was centrifuged (20,000 × *g*, 20 min, 4°C) to remove cell debris. ExsA and LcrF were purified from the cleared lysates using Ni^{2+}-affinity chromatography and dialyzed overnight at 4°C in ExsA buffer lacking imidazole and supplemented with 1 mM dithiothreitol (DTT) as previously described (27). Protein concentration was determined using a BCA protein assay (Thermo Scientific, Rockford, IL).

Circular permutation and EMSAs. Probes for the circular permutation assays and the methodology were performed as previously described (27). DNA probes for electrophoretic mobility shift assays (EMSAs) were generated by standard PCR using the primer pairs listed in Table S5 in the supplemental material. Specific promoter probes containing the ExsA or LcrF binding sites were ~200 bp, and the nonspecific probe (160 bp) was derived from the *algD* promoter region. The nonspecific portions of the probes described in Fig. 5B were also derived from the *algD* promoter region. PCR products were gel purified using the QIAquick gel extraction method (Qiagen, Valencia, CA). Short promoter probes (~50 bp) were generated by annealing complementary oligonucleotides (25 pmol each) diluted in duplex buffer (30 mM HEPES [pH 7.5], 100 mM potassium acetate; 50 μl final volume). The primer mixture was heated to 95°C for 5 min and gradually cooled to 25°C at a rate of 1°C/min. Reactions were purified using the Qiagen nucleotide removal kit. Promoter probes were end labeled with 10 μCi of [γ-^{32}P]ATP as previously described (27). EMSA reactions (20 μl) contained 0.06 nM of the nonspecific and/or specific probes, 10 μl 2× DNA binding buffer (20 mM Tris [pH 7.5], 100 mM KCl, 2 mM EDTA, 2 mM DTT, and 10% glycerol), 25 ng/μl poly(2′-deoxyinosinic 2′-deoxycytidylic acid), and 100 μg/ml bovine serum albumin. Reaction mixtures were incubated at room temperature for 5 min, and then ExsA or LcrF was added at the specified concentrations. Reaction mixtures were incubated at room temperature for 15 min and analyzed on 5% polyacrylamide glycine gels. Gels were analyzed with an FLA-7000 phosphorimager (Fujifilm) and Multigage version 3.0 software (Fujifilm).

Cross-linking experiments. Purified LcrF was exchanged into cross-linking buffer (20 mM HEPES [pH 7.9], 500 mM NaCl, 0.5% Tween 20, 1 mM DTT) using Micro Bio-Spin 6 columns (Bio-Rad). Cross-linking reactions were performed in cross-linking buffer by incubating LcrF (800 nM) with the indicated concentration of Sulfo-EGS [ethylene glycol bis(sulfosuccinimidylsuccinate)], DSS (disuccinimidyl suberate), or DMP (dimethyl pimelimidate) for 60 min. Samples were immediately loaded onto a 12% SDS-polyacrylamide gel and subjected to immunoblot analyses using LcrF polyclonal antiserum.

RESULTS

LcrF activates T3SS gene expression in *P. aeruginosa*. The AraC family of transcriptional regulators is characterized by the presence of a DNA binding domain consisting of two helix-turn-helix (HTH) motifs (26). The first helices of each HTH motif (termed the recognition helix) serve as the primary determinants of binding specificity and function by inserting into adjacent major grooves of the DNA to make base-specific contacts with the promoter region (44). We observed that the amino acid sequences comprising the recognition helices of ExsA are virtually identical to the activators of T3SS gene expression (LcrF/VirF) in the pathogenic yersiniae (see Fig. S1 in the supplemental material). Based on this observation, we hypothesized that LcrF/VirF recognizes a nucleotide sequence similar to the ExsA consensus binding site. To test this idea, we performed a complementation experiment by introducing an LcrF expression plasmid (pLcrF) into a PA103 *exsA*::Ω mutant carrying the ExsA-dependent $P_{exsC-lacZ}$, $P_{exsD-lacZ}$, or $P_{exoT-lacZ}$ transcriptional reporter. The resulting strains were cultured under noninducing (high Ca^{2+}/−EGTA) and inducing (low Ca^{2+}/+EGTA) conditions for T3SS gene expression and assayed for β-galactosidase activity. As shown in Fig. 1A to C, plasmid-expressed ExsA and LcrF both complemented the *exsA*::Ω mutant for expression of the $P_{exsC-lacZ}$, $P_{exsD-lacZ}$, and $P_{exoT-lacZ}$ reporters with two notable differences. First, LcrF-dependent reporter activity was significantly elevated compared to ExsA-dependent activity. Second, whereas activation of the reporters by ExsA was further enhanced when cells were grown under low Ca^{2+} conditions (3-, 7-, and 7-fold for $P_{exsC-lacZ}$, $P_{exsD-lacZ}$, and $P_{exoT-lacZ}$, respectively), activation of the same reporters by LcrF was only modestly elevated in the absence of Ca^{2+} (1.3-, 1.4-, and 1.4-fold, respectively). A trivial explanation for the LcrF-depen-
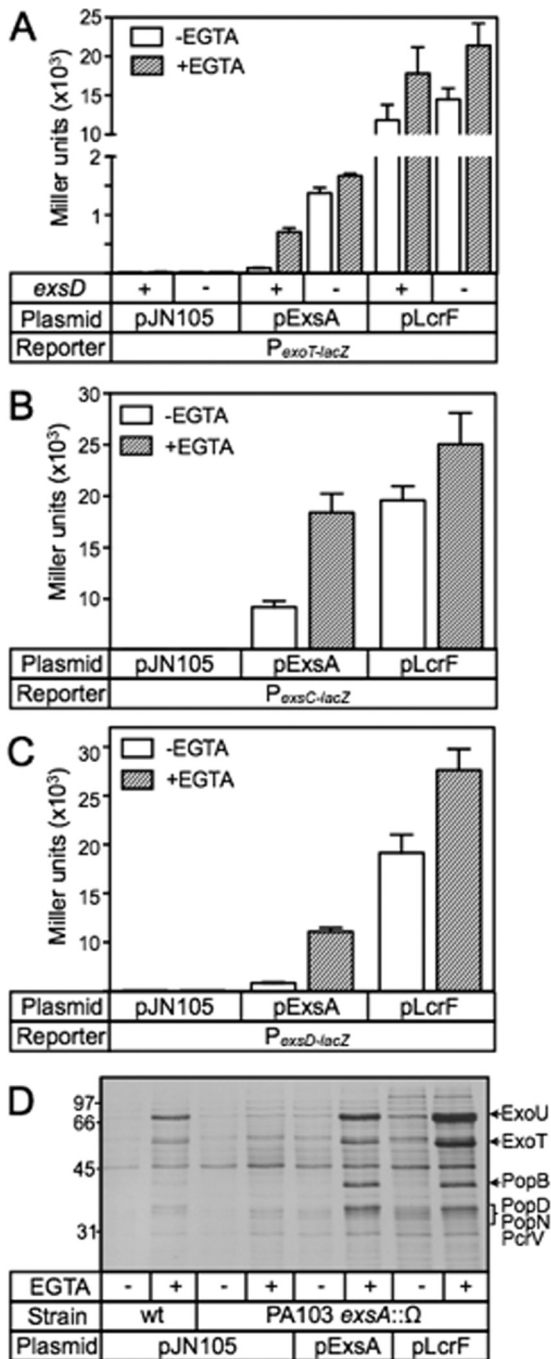
FIG 1 LcrF complements an *exsA* mutant for T3SS gene expression. (A to C) The PA103 *exsA*::Ω strain carrying either the P$_{exoT\text{-}lacZ}$ (A), P$_{exsC\text{-}lacZ}$ (B), or P$_{exsD\text{-}lacZ}$ (C) transcriptional reporter was transformed with a vector control (pJN105), an ExsA expression vector (pExsA), or an LcrF expression vector (pLcrF). The resulting strains were cultured under noninducing (−EGTA, open bars) or inducing (+EGTA, hatched bars) conditions for T3SS gene expression and assayed for β-galactosidase activity as reported in Miller units. *, $P < 0.001$; **, $P < 0.01$. (D) Silver-stained gel of concentrated culture supernatant fluid prepared from wild-type PA103 or the PA103 *exsA*::Ω strain carrying the indicated plasmids following growth under noninducing (−EGTA) or inducing (+EGTA) conditions for T3SS gene expression. The positions of molecular mass standards are indicated on the left side of the gel, and the type III secreted proteins ExoU, ExoT, PopB, PopD, PopN, and PcrV are labeled on the right side of the gel (49).

dent increase in reporter activity is that the steady-state expression levels of LcrF are elevated relative to ExsA. Immunoblotting of whole-cell extracts using purified LcrF and ExsA as standards, however, indicated that the amounts of LcrF and ExsA are similar in *P. aeruginosa* (see Fig. S2 in the supplemental material).

Since the increase in LcrF-dependent reporter activity could not be linked to elevated LcrF levels, we next addressed the possibility that LcrF is insensitive to the ExsD antiactivator. ExsD inhibits ExsA-dependent activation under high Ca^{2+} conditions but is largely inactive under low Ca^{2+} conditions, owing to the action of the ExsC/ExsE regulatory cascade (16). In the absence of *exsD*, therefore, ExsA-dependent activation of the P$_{exoT\text{-}lacZ}$ reporter is derepressed and largely insensitive to Ca^{2+} levels (Fig. 1A). LcrF-dependent activation of the P$_{exoT\text{-}lacZ}$ reporter, however, was similar in both the presence and absence of *exsD* (Fig. 1A). Based on this finding, we conclude that LcrF is not regulated by ExsD, thereby accounting for the insensitivity of LcrF to Ca^{2+} levels.

The *P. aeruginosa* T3SS regulon consists of 10 ExsA-dependent promoters that control the expression of the secretion machinery, effectors, chaperones, and translocator proteins (45). To determine whether plasmid-expressed LcrF activates the entire T3SS regulon, culture supernatant samples were collected from cells grown under noninducing and inducing conditions for T3SS gene expression and subjected to SDS-PAGE and silver staining. As shown in Fig. 1D, expression of either ExsA or LcrF in the *exsA* mutant resulted in elevated secretion of the ExoU/ExoT effectors, the PopB/PopD/PcrV translocators, and the PopN regulator relative to the vector control (pJN105). The finding that LcrF supported a higher level of secretion than ExsA is consistent with the reporter data presented in Fig. 1A to C and indicates that LcrF activates all 10 of the ExsA-dependent promoters.

LcrF DNA binding properties. We next compared the DNA binding properties of ExsA and LcrF using electrophoretic mobility shift assays (EMSAs). ExsA and LcrF were expressed as amino-terminal histidine-tagged fusion proteins in *E. coli* and purified by Ni^{2+}-affinity chromatography (see Fig. S3A in the supplemental material). To independently confirm a previous report that purified LcrF is dimeric (28), we performed cross-linking studies using a panel of amine-reactive cross-linkers of various spacer arm lengths (DSS, DMP, and Sulfo-EGS). Purified LcrF (with a molecular mass of 30 kDa) was incubated with the cross-linkers for 60 min, electrophoresed on denaturing polyacrylamide gels, and immunoblotted for LcrF. Whereas incubation of LcrF with either Sulfo-EGS (see Fig. S3B) or DMP (data not shown) resulted in a large amount of an ~60-kDa cross-linked species, the cross-linked species was absent from the sample containing the vehicle (dimethyl sulfoxide [DMSO]) alone. In contrast, cross-linking experiments with LcrF$_m$, a monomeric variant described below, resulted in only small amounts of the ~60-kDa cross-linked species, thereby confirming that purified LcrF is dimeric in solution, while LcrF$_m$ is primarily monomeric.

EMSAs were performed by incubating ExsA or LcrF with a nonspecific (160-bp) control probe derived from the *algD* promoter region and a specific (~200-bp) probe derived from the ExsA-dependent P$_{exsC}$, P$_{exoT}$, and P$_{exsD}$ promoters. Samples were subjected to electrophoresis on nondenaturing polyacrylamide gels and phosphorimaging. Previous studies with ExsA found that the P$_{exsC}$, P$_{exoT}$, and P$_{exsD}$ promoter regions each contain two binding sites for monomeric ExsA (27, 31). Binding to the P$_{exoT}$ and P$_{exsD}$ promoters occurs through a monomer assembly pathway
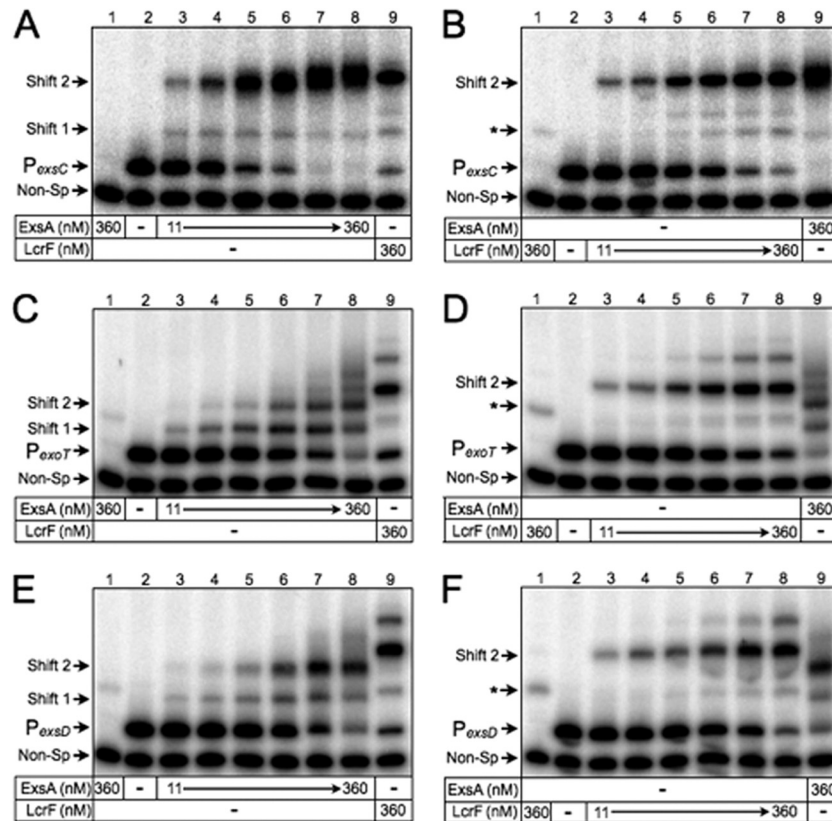
FIG 2 DNA binding properties of purified ExsA and LcrF. EMSAs were performed using radiolabeled probes derived from the ExsA-dependent P_{exsC} (A and B), P_{exoT} (C and D), and P_{exsD} (E and F) promoters. A nonspecific probe (Non-Sp) derived from the *algD* promoter region was included in all binding reactions as a negative control. Probes (0.05 nM each) were incubated in the absence or presence of 11, 23, 45, 90, 180, or 360 nM ExsA (A, C, and E) or LcrF (B, D, and F) for 15 min at 25°C. Samples were analyzed by native polyacrylamide gel electrophoresis and phosphorimaging. The positions of shift products 1 and 2 are indicated. The asterisk indicates background shifting of the nonspecific probe by LcrF.

whereby one ExsA monomer binds to site 1 (resulting in shift product 1 in Fig. 2C and E) and then recruits another ExsA monomer to a second binding site (site 2) that is positioned just upstream of site 1 (represented as shift product 2). ExsA likely binds to the P_{exsC} promoter via monomer assembly as well but occurs in a highly cooperative manner such that the binding kinetics are very rapid, thereby resulting in the formation of an abundance of shift product 2 and only fleeting amounts of shift product 1 (Fig. 2A).

Whereas ExsA binding resulted in the formation of shift products 1 and 2, LcrF binding resulted in a single predominant shift product when bound to the P_{exoT}, P_{exsD}, and P_{exsC} promoter probes (Fig. 2B, D, and F). Lower mobility complexes were also evident with LcrF, but the precise nature of those complexes was not examined. For reasons described below, the predominant shift products formed by LcrF were designated shift product 2. The mobilities of shift product 2 formed by ExsA and LcrF were indistinguishable from one another when using the P_{exsC} promoter probe (Fig. 2A and B, lanes 8 and 9). Since both the isoelectric points (8.6 and 9.1) and molecular masses (31.6 and 30.8 kDa) of ExsA and LcrF are similar, this finding suggested that shift product 2 represents two molecules bound to the P_{exsC} promoter probe. The manner in which shift product 2 is formed, however, differs in that formation of shift product 2 by ExsA results from the sequential binding of two ExsA monomers, whereas occupation by LcrF results from the binding of a single LcrF dimer.

In contrast to our findings for the P_{exsC} promoter probe, the primary LcrF promoter probe complexes observed for P_{exoT} and P_{exsD} (shift product 2) had reduced mobility compared to that of shift product 2 generated by ExsA (Fig. 2C to F, lanes 8 and 9). In a previous study, we found that the P_{exsC}, P_{exsD}, and P_{exoT} promoter probes, while not inherently bent, do bend when bound by ExsA (27). While ExsA and LcrF appear to bend the P_{exsC} promoter probe to a similar level (Fig. 2A and B), we hypothesized that LcrF bends the P_{exoT} and P_{exsD} promoter probes to a higher degree than ExsA and that this increase in bending accounted for the difference in the mobility of shift product 2. To test this idea, we performed EMSAs using smaller 50-bp promoter probes that were previously shown to negate the effect that ExsA-dependent bending has on mobility owing to their smaller size (3). When using the smaller P_{exsC}, P_{exsD}, and P_{exoT} promoter probes, the mobilities of shift product 2 formed by ExsA and LcrF were similar in each case (Fig. 3A to C, lanes 3 and 7). This finding suggested that the reduced mobility of the LcrF promoter probe complexes observed in Fig. 2 results from increased DNA bending by LcrF relative to ExsA.

To further confirm the differential effect that ExsA and LcrF have on bending, we performed circular permutation assays using the P_{exsD} promoter probe. Circular permutation is based on the observation that the electrophoretic mobility of bent DNA is more severely retarded when the bend is located in the center of a DNA fragment (46). To test for differential bending, we used a panel of
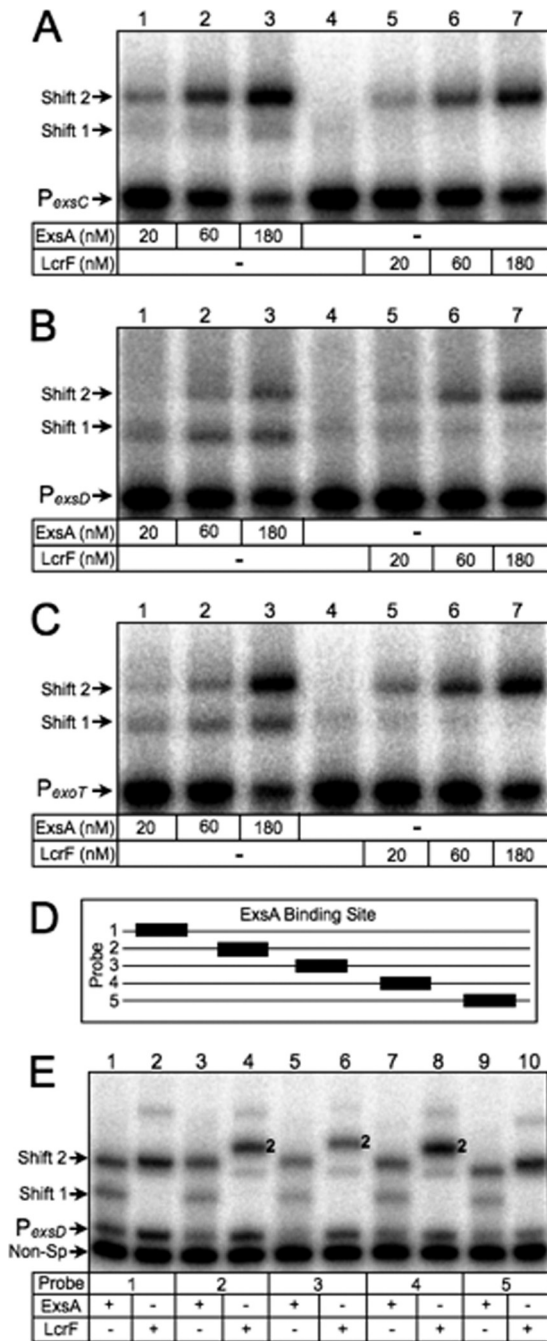
FIG 3 DNA bending properties of ExsA and LcrF. (A to C) EMSAs using 50-bp radiolabeled probes derived from the ExsA-dependent P_{exsC} (A), P_{exsD} (B), and P_{exoT} (C) promoters. Probes (0.05 nM each) were incubated in the presence of 20, 60, or 180 nM ExsA (lanes 1 to 3 in each panel) or LcrF (lanes 5 to 7 in each panel) for 15 min at 25°C. Samples were analyzed by native polyacrylamide gel electrophoresis and phosphorimaging. (D) Diagram depicting the position of the ExsA binding site (black box) derived from the P_{exsD} promoter within probes 1 to 5 (solid line). (E) Circular permutation experiment performed using probes 1 to 5 (0.05 nM each) incubated in the presence of 180 nM ExsA (odd-numbered lanes) or LcrF (even-numbered lanes) for 15 min at 25°C. Samples were analyzed by native polyacrylamide gel electrophoresis and phosphorimaging. The positions of shift products 1 and 2 are indicated.

five P_{exsD} promoter probes in which the ExsA binding site was positioned at evenly spaced intervals across an ~200-bp DNA fragment (Fig. 3D). As expected, the mobility of shift product 2 formed by LcrF was most highly retarded when the ExsA binding site was positioned toward the center of the EMSA probes (Fig. 3E, probes 2 to 4) and retarded to a higher extent than seen with ExsA. These combined data demonstrate that ExsA and LcrF have differential effects on DNA bending and show that these effects are promoter dependent.

The EMSA data generated using the shorter, 50-bp probes (Fig. 3A to C) and circular permutation probes (Fig. 3E) further support the conclusion that ExsA and LcrF bind as monomers and dimers, respectively. The primary basis for this conclusion is the absence of shift product 1 when using LcrF for the EMSA reactions presented in Fig. 3A to C and E. While some faint bands in the area of shift product 1 can be seen for LcrF in Fig. 3B and C, the same bands are also present in the lane that contains the promoter probes alone (lane 4 compared to lane 5).

Genetic determinants for LcrF binding. ExsA-dependent activation of the $P_{exoT-lacZ}$ reporter requires two highly conserved sequences (GnC and TGnnA) that constitute binding site 1 (Fig. 4A). To determine whether LcrF is sensitive to the same mutations, a panel of mutant $P_{exoT-lacZ}$ reporter strains was transformed with the pLcrF expression plasmid, cultured under inducing (+EGTA) conditions for T3SS gene expression, and assayed for β-galactosidase activity. Similar to our previous findings with ExsA (27), activation of the $P_{exoT-lacZ}$ reporter by LcrF was highly sensitive to mutations that disrupted the core GnC and TGnnA sequences (Fig. 4B). The C-39A substitution, which represents a weakly conserved position in the ExsA consensus binding sequence, had an intermediate effect on activation by LcrF. Finally, the C-50G substitution, which was included as a negative control, had no effect on activation by ExsA or LcrF.

To confirm that the activation defects observed in Fig. 4B correlated with reduced DNA binding, we used promoter probes derived from the mutant $P_{exoT-lacZ}$ reporters in EMSAs. Because occupation of binding site 2 by ExsA is dependent upon prior occupation of site 1, mutations in site 1 result in a significant decrease in the formation of shift products 1 and 2 (Fig. 4C) (27). Binding of LcrF to the same promoter probes, however, was largely unaffected by substitutions that disrupt the ExsA consensus site (Fig. 4D). We speculated that LcrF, being dimeric in solution, might be less sensitive to substitutions in site 1, because additional interactions at site 2 compensate for binding defects at site 1. To test this hypothesis, we generated a monomeric variant of LcrF based upon prior knowledge of AraC dimerization. AraC dimerizes through an antiparallel, coiled-coil region that is stabilized by leucine triads located at each end of the interface (47). Two of the predicted leucine triad residues in LcrF (L136 and L144) were changed to alanine, and the resulting protein was designated $LcrF_m$ (monomeric). $LcrF_m$ was significantly impaired for activation of the $P_{exoT-lacZ}$ reporter in an exsA mutant but was stably expressed (see Fig. S3C in the supplemental material). $LcrF_m$ was purified by Ni^{2+}-affinity chromatography and found to be largely monomeric in cross-linking studies (see Fig. S3A and B). In EMSAs, the binding properties of monomeric ExsA and $LcrF_m$ were similar, resulting in the formation of shift products 1 and 2 at the P_{exoT} promoter probe (Fig. 2C and Fig. 5A, lanes 6 to 10). In contrast, LcrF formed only shift product 2 (Fig. 5A, lanes 1 to 5).
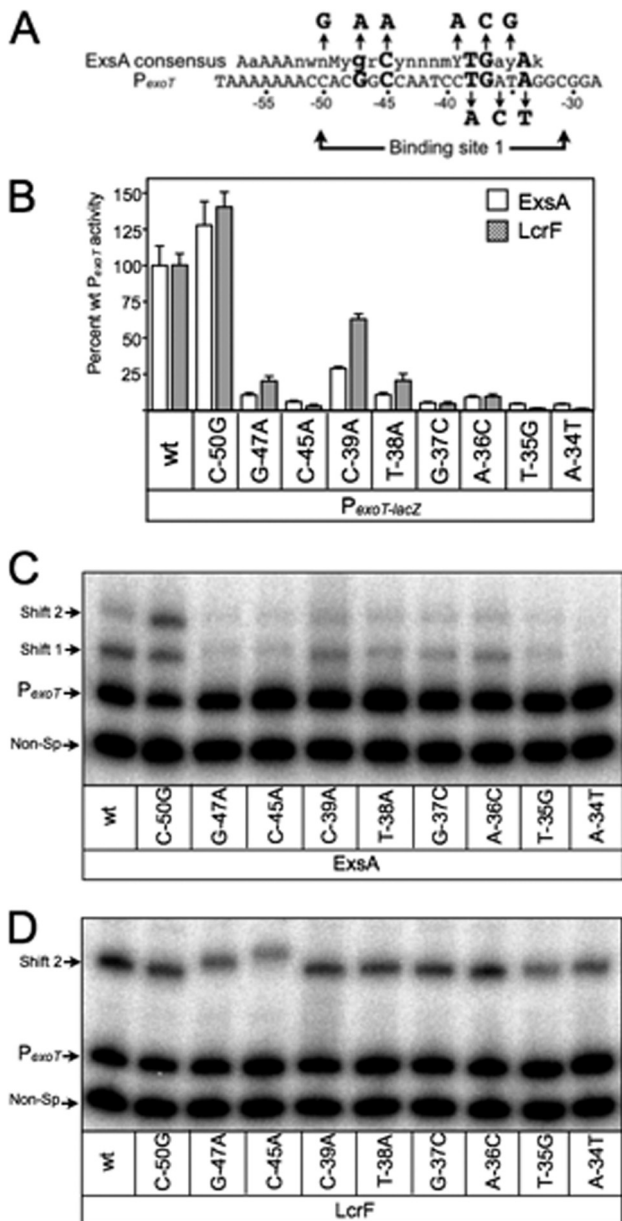
FIG 4 ExsA- and LcrF-dependent activation is sensitive to nucleotide substitutions in the ExsA consensus site. (A) Sequence of the P_{exoT} promoter showing the conserved GnC and TGnnA sequences (highlighted in bold) and the nucleotide substitutions indicated with an arrow. (B) The PA103 $exsA::\Omega$ strain carrying the indicated $P_{exoT-lacZ}$ reporters was transformed with pExsA or pLcrF. The resulting strains were cultured in the presence of EGTA and assayed for β-galactosidase activity. Activation by ExsA (open bars) and LcrF (hatched bars) is reported as the percent activity normalized to the activity of the wild-type $P_{exoT-lacZ}$ reporter. (C and D) EMSAs using radiolabeled probes derived from the mutant P_{exoT} promoters. The nonspecific P_{algD} probe (Non-Sp) was included as a negative control. Probes (0.05 nM each) were incubated in the presence of 45 nM ExsA (C) or LcrF (D) for 15 min at 25°C. Samples were analyzed by native polyacrylamide gel electrophoresis and phosphorimaging. The positions of shift products 1 and 2 are indicated.

Although $LcrF_m$ preferentially formed shift product 1 at the P_{exoT} promoter, it was unclear whether occupation was occurring at binding site 1 or 2. To examine this further, we used a panel of P_{exoT} promoter probes with truncations that disrupt binding sites

1 and/or 2 (Fig. 5C). The probe lengths were maintained at 60 bp by replacing the deleted regions with nonspecific DNA. The minimal promoter probe consisted of the adenine-rich, GnC, and TGnnA sequences and supported formation of shift products 1 and 2 by ExsA and $LcrF_m$ (Fig. 5C, lanes 1 and 3). The same probes were previously used to show that occupation of site 2 by ExsA is dependent upon the presence of binding site 1 (27), and we demonstrate similar findings here for $LcrF_m$. Probe 2, which lacks a functional site 2, resulted in a complete lack of shift product 2 formation by ExsA and $LcrF_m$ but still supported formation of shift product 1. In contrast, ExsA and $LcrF_m$ binding was significantly impaired using probes lacking either site 1 (probe 3) or sites 1 and 2 (probe 4). In contrast to our findings for ExsA and $LcrF_m$, removal of either binding site 1 or 2 (probe 2 and 3) had only modest effects on formation of shift product 2 by wild-type LcrF (Fig. 5C, lanes 2, 5, 8), and it was not until both sites were removed (probe 4) that a significant reduction in LcrF binding was observed (Fig. 5C, lane 11). These findings demonstrate that dimeric LcrF is more tolerant of substitutions that disrupt the ExsA consensus binding site and that $LcrF_m$ preferentially binds to site 1. Having established the latter point, we next asked whether $LcrF_m$ was sensitive to nucleotide substitutions that disrupt the ExsA consensus binding site using the panel of mutant P_{exoT} promoter probes described in Fig. 4A. Whereas native LcrF binding was largely unaffected by substitutions in the ExsA consensus region (Fig. 4D), binding by $LcrF_m$ was significantly reduced (Fig. 5D), demonstrating that the ExsA consensus binding site is required for maximal occupation of site 1.

The ExsA consensus binding site is present in *Y. pestis* LcrF-dependent promoters. The finding that LcrF is sensitive to mutations that disrupt the ExsA consensus binding site suggested that the same recognition sequence exists in the yersiniae. To examine this further, we searched the P_{yopN}, P_{lcrG}, P_{yscN}, and P_{yscB} promoter regions from *Y. pestis* and identified a nearly perfect match to the ExsA consensus binding sequence in each promoter (Fig. 6). As previously observed for ExsA-dependent promoters (27), the consensus TGnnA sequence in each *Y. pestis* promoter was separated from the putative Pribnow (TATAAT) boxes by ~21 to 22 bp. This prompted us to test whether $P_{yopN-lacZ}$, $P_{lcrG-lacZ}$, and $P_{yscN-lacZ}$ transcriptional reporters were responsive to ExsA. For these experiments, the ExsA and LcrF expression plasmids were introduced into an $exsA::\Omega$ $\Delta exsD$ double-mutant background to avoid feedback regulation on ExsA activity by ExsD. Although both ExsA and LcrF resulted in significant activation of the $P_{yopN-lacZ}$, $P_{lcrG-lacZ}$, and $P_{yscN-lacZ}$ reporters, LcrF-dependent activity at each of the promoters was significantly elevated compared to ExsA-dependent activity (Fig. 7A to C).

To further examine the interaction of ExsA and LcrF with the *Y. pestis* P_{yopN}, P_{lcrG}, and P_{yscN} promoter regions, we performed EMSAs. Binding of ExsA to each promoter probe resulted in the generation of shift products 1 and 2 (Fig. 7D to F, lanes 3 to 6). Lower mobility promoter probe complexes were also apparent with the P_{yopN} and the P_{yscN} promoter probes at the highest concentrations of ExsA (Fig. 7D to F, lane 6). These products likely represent nonspecific interactions with ExsA. Binding of LcrF to the *Y. pestis* promoter probes resulted in only a single predominant species (shift product 2) that exhibited reduced mobility relative to the ExsA-promoter probe complexes (Fig. 7D to F, lanes 5 and 8). These findings further support the conclusion that ExsA binds as a monomer, whereas
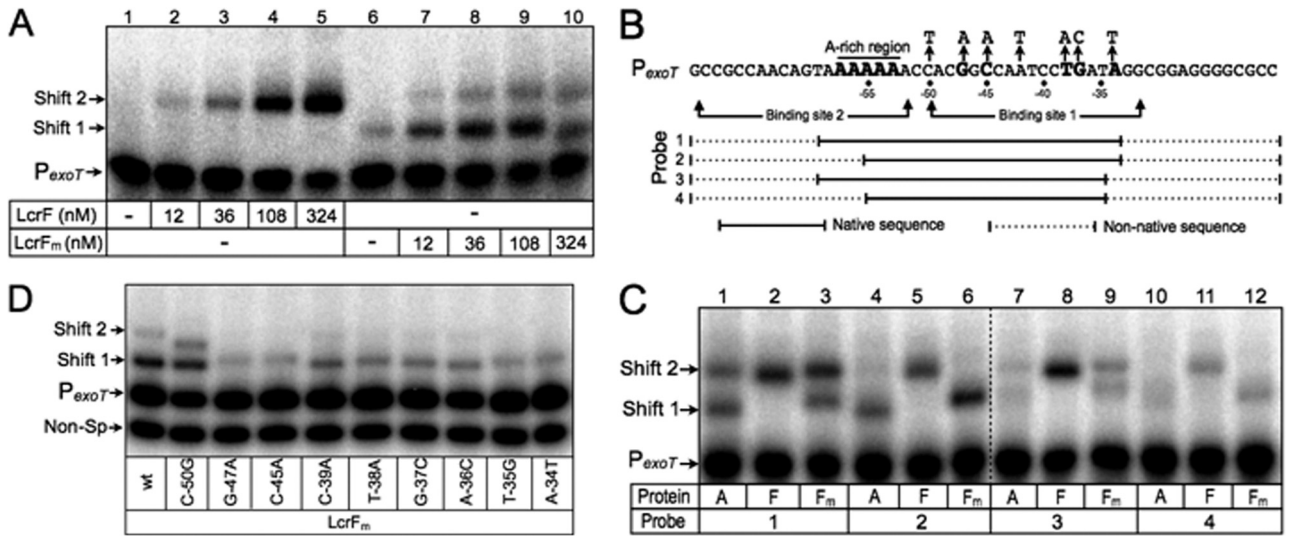
FIG 5 LcrF binds to the *P. aeruginosa* P$_{exoT}$ promoter probe as a dimer. (A) EMSA using 50-bp radiolabeled probes derived from the ExsA-dependent P$_{exoT}$ promoter. Probes (0.05 nM each) were incubated in the presence of 12, 36, 108, 324 nM LcrF (lanes 2 to 5) or LcrF$_m$ (lanes 6 to 10) for 15 min at 25°C. Samples were analyzed by native polyacrylamide gel electrophoresis and phosphorimaging. (B) Diagram depicting P$_{exoT}$ promoter probes with truncations that destroy binding sites 1 and/or 2. Solid and dotted lines represent native and nonnative P$_{exoT}$ sequences, respectively. The adenine-rich region and conserved GnC and TGnnA sequences are indicated in bold. (C) EMSA using 60-bp radiolabeled probes derived from the ExsA-dependent P$_{exoT}$ promoter. Probes (0.05 nM each) were incubated in the presence of 90 nM ExsA(A), LcrF(F), or LcrF$_m$ for 15 min at 25°C. Samples were analyzed and imaged as described above.

LcrF binds as a dimer, and that LcrF has increased DNA bending activity relative to that of ExsA.
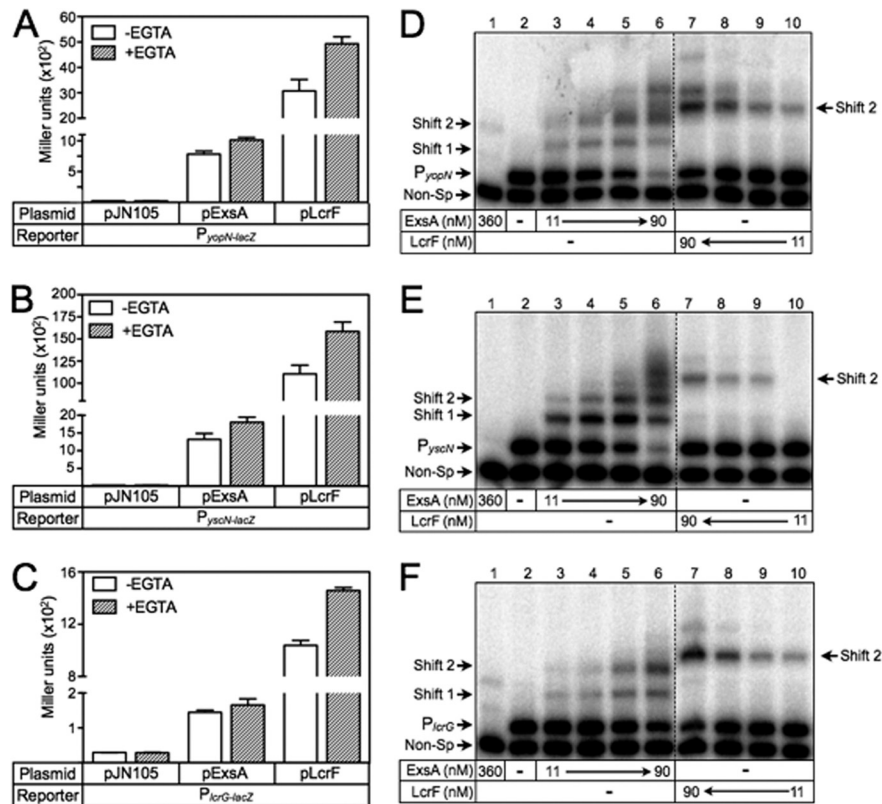
Since ExsA activates transcription of LcrF-dependent promoters, we next tested whether the nucleotides that comprise the ExsA binding consensus are required for activation of the *Y. pestis* P$_{yscN}$ promoter. Similar to our findings with the mutant P$_{exoT-lacZ}$ reporters in Fig. 4B, mutations that disrupted the core GnC and TGnnA sites in the P$_{yscN-lacZ}$ transcriptional reporter also resulted
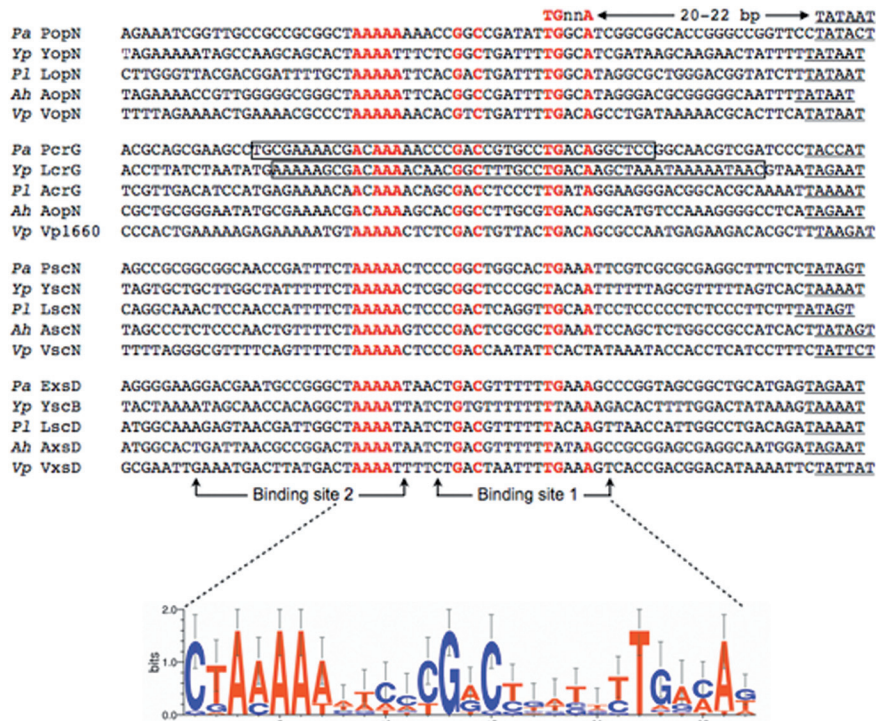
FIG 6 Sequence alignment of the ExsA consensus binding sequence in ExsA (*Pseudomonas aeruginosa*)-, LcrF (*Yersinia pestis*)-, PxsA (*Photorhabdus luminescens*)-, AxsA (*Aeromonas hydrophila*)-, and VxsA (*Vibrio parahaemolyticus*)-dependent promoters. ExsA binding sites 1 and 2 are indicated with arrows. The consensus sequence is indicated in red, and −10 regions are underlined. The boxed regions in the *P. aeruginosa* P$_{pcrG}$ and *Y. pestis* P$_{lcrG}$ promoters correspond to regions protected by ExsA and LcrF/VirF from DNase I cleavage, respectively (see Fig. S5 in the supplemental material) (31, 48).
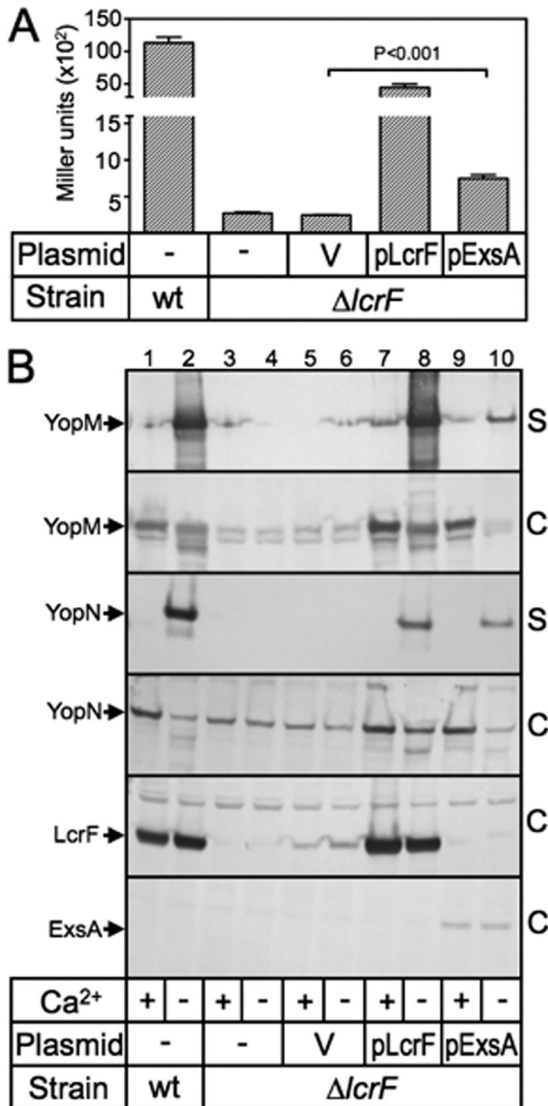
FIG 7 ExsA activates transcription of *Y. pestis* transcriptional reporters. (A to C) The PA103 *exsA*::Ω strain carrying either the P*yopN-lacZ* (A), P*yscN-lacZ* (B), or P*lcrG-lacZ* (C) reporters was transformed with pJN105, pExsA, or pLcrF. The resulting strains were cultured under noninducing (−EGTA, open bars) or inducing (+EGTA, hatched bars) conditions for T3SS gene expression and assayed for β-galactosidase activity. Values were reported in Miller units. (D to F) EMSAs using radiolabeled probes derived from the LcrF-dependent P*yopN* (D), P*yscN* (E), and P*lcrG* (F) promoters. The P*algD* probe was included in all binding reactions as a nonspecific (Non-Sp) control. Probes (0.05 nM each) were incubated in the absence (lane 2) or presence of 11, 23, 45, or 90 nM ExsA (lanes 3 to 6 in each panel) or LcrF (lanes 7 to 10 in each panel) for 15 min at 25°C. Samples were analyzed by native polyacrylamide gel electrophoresis and phosphorimaging. The positions of shift products 1 and 2 are indicated.

in a significant decrease in ExsA- and LcrF-dependent activity (Fig. 8A and B). Conversely, mutations that altered the nonconserved −50 and poorly conserved −39 positions had less severe effects on ExsA- and LcrF-dependent activity.

ExsA complements a *Y. pestis* lcrF mutant for T3SS gene expression. The finding that LcrF complements a *P. aeruginosa exsA* mutant for T3SS gene expression led us to examine whether ExsA complements a *Y. pestis lcrF* mutant. An *lcrF* deletion mutant was generated by gene replacement in a strain bearing a *yopM*::*lacZYA* transcriptional reporter. The wild-type and Δ*lcrF yopM*::*lacZYA* reporter strains were transformed with the pExsA and pLcrF expression plasmids and assayed for reporter activity following growth in the absence of Ca²⁺. Similar to our finding that LcrF complements an *exsA* mutant, ExsA was able to restore *yopM*::*lacZYA* reporter activity in the *Y. pestis* Δ*lcrF* mutant (Fig. 9A), albeit to a lesser extent than LcrF. To determine if ExsA could fully activate the *Y. pestis* T3SS, strains were cultured in the presence or absence of Ca²⁺, separated into cell lysate and supernatant fractions, and analyzed by immunoblotting for YopM and YopN. As shown in Fig. 9B, LcrF and ExsA both complemented the Δ*lcrF* mutant for expression and low Ca²⁺-dependent secretion of YopM and YopN. Based on these data, we conclude that ExsA complements a *Y. pestis* Δ*lcrF* mutant for T3SS gene expression albeit at reduced levels relative to LcrF, likely owing to poor ExsA expression in *Y. pestis* (Fig. 9B).
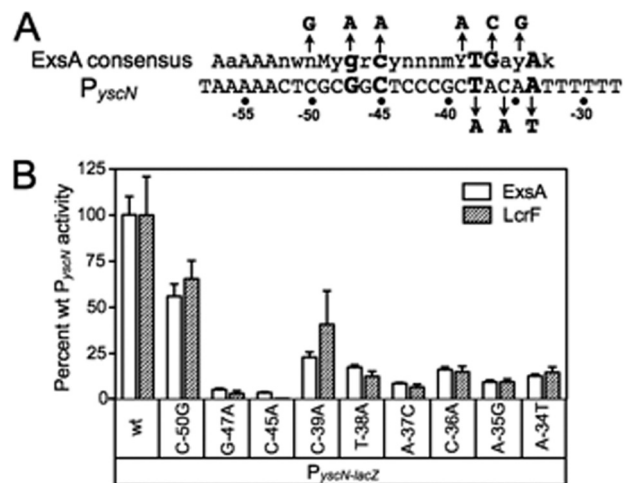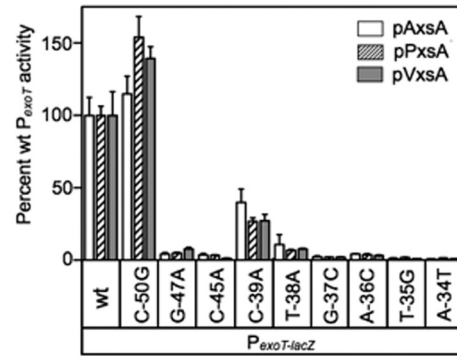

FIG 8 *Y. pestis* P*yscN* reporter activity is sensitive to substitutions that disrupt the ExsA binding site. (A) The P*yscN* promoter sequence showing the ExsA consensus sequence in binding site 1. The GnC and TGnnA sequences are highlighted in bold, and each promoter nucleotide substitution is indicated with an arrow. (B) The PA103 *exsA*::Ω strain carrying the indicated mutant P*yscN-lacZ* transcriptional reporters was transformed with either pExsA or pLcrF. The resulting strains were cultured in the presence of EGTA and assayed for β-galactosidase activity. Activation by pExsA (open bars) and pLcrF (hatched bars) is reported as the percent activity of the mutant promoters normalized to the activity at the wild-type P*yscN* promoter.

FIG 10 The PA103 *exsA*::Ω strain carrying the indicated P*exoT-lacZ* transcriptional reporters was transformed with AxsA (pAxsA), PxsA (pPxsA), or VxsA (pVxsA) expression vectors. The resulting strains were cultured in the presence of EGTA and assayed for β-galactosidase activity. Activation by pAxsA (open bars), pPxsA (hatched bars), and pVxsA (gray bars) is reported as the percent activity of the mutant promoters normalized to the activity of the wild-type P*exoT* promoter.

FIG 9 ExsA activates expression of the *Y. pestis* T3SS. (A) *Y. pestis* KIM5-3001 (*yopM*::*lacZYA*) and KIM5-3233-F2 (Δ*lcrF yopM*::*lacZYA*) carrying a vector control (V), pLcrF, or pExsA were cultured under inducing conditions for T3SS gene expression and then assayed for β-galactosidase activity as reported in Miller units. (B) Wild-type *Y. pestis* KIM5-3001 (lanes 1 and 2) or KIM5-3001-F1 (Δ*lcrF*) (lanes 3 to 10) lacking a vector (lanes 1 to 4) or carrying a vector control (lanes 5 and 6), pLcrF (lanes 7 and 8), or pExsA (lanes 9 and 10) were grown under noninducing conditions (+calcium) or inducing (−calcium; odd-numbered lanes) conditions for T3SS gene expression. Concentrated supernatant fluid (S) and cell-associated fractions (C) were prepared and subjected to SDS-PAGE and immunoblot analysis. Arrows indicate protein products.

ExsA homologs from other pathogens are also sensitive to substitutions in the P*exoT* promoter. The core structural components of the *P. aeruginosa*, yersiniae, *A. hydrophila*, *P. luminescens*, and *V. parahaemolyticus* T3SSs are encoded within 4 highly conserved operons. Based on the *P. aeruginosa* designations, those operons consist of *popN-pcrR*, *pcrG-popD*, *pscN-pscU*, and *exsD-pscM* (see Fig. S4 in the supplemental material). The only apparent variance in genetic organization among the five organisms is the yersiniae *yscB-yscM* operon, which lacks *exsD*. An alignment of the promoter regions located upstream of each operon revealed

nearly perfect ExsA consensus binding sites in each organism (Fig. 6). Bolstering our confidence in the alignment is that the spacing seen between the TGnnA sequence and the putative −10 Pribnow boxes is 20 to 22 bp. As mentioned earlier, a similar spacing arrangement is seen in all 10 ExsA-dependent promoters. Finally, the derived consensus from an alignment of all 20 promoter regions shown in Fig. 6 is essentially identical to the ExsA consensus derived using only the *P. aeruginosa* promoters. Based on these observations, we tested whether the activators from *A. hydrophila*, *P. luminescens*, and *V. parahaemolyticus* are sensitive to the same P*exoT-lacZ* reporter mutations that disrupt ExsA- and LcrF-dependent activation. Like ExsA and LcrF, AxsA (*A. hydrophila*), PxsA (*P. luminescens*), and VxsA (*V. parahaemolyticus*) were sensitive to substitutions in the GnC and TGnnA sequences of the P*exoT-lacZ* reporter, while the poorly conserved −39 and nonconserved −50 positions had intermediate and no effects on activity, respectively (Fig. 10).

DISCUSSION

Several regions in the *yopE*, *lcrG*, *virC*, and *yopH* promoter regions were previously shown to be protected from DNase I cleavage by *Y. enterocolitica* VirF [28, 48]. The protected regions contained a putative consensus binding sequence (TTTTaGYcTgTat, where uppercase letters indicate more highly conserved positions than lowercase letters) that was present in at least three copies per promoter and organized as either single sites or inverted repeats. The authors proposed that each monomer of the VirF dimer bound to a consensus site (half-site) and that high-affinity binding occurred at inverted repeats bearing the strongest match to consensus [48]. That study also noted, however, that the consensus binding sequences were highly degenerate and variably spaced from the transcription starts sites and acknowledged that the consensus site assignment remained questionable. The high degree of similarity between the HTH motifs of LcrF/VirF and ExsA prompted us to test the hypothesis that each protein recognizes a similar DNA sequence. Herein, we demonstrate that the ExsA consensus binding site (AaAAAnwmMyGrCynnnmTGayAk) is present in *Y. pestis* promoter regions and is required for LcrF-dependent activation of transcription.

Purified ExsA generates two promoter probe complexes in EMSAs, representing monomeric ExsA bound to site 1 (product 1) and both sites 1 and 2 (product 2) (27). In contrast, purified LcrF resulted in the appearance of one primary promoter probe complex which we designated shift product 2 (Fig. 2 and 7). A previous study suggested that purified LcrF is dimeric in solution (28). That finding is consistent with our own cross-linking studies with purified LcrF (see Fig. S3B in the supplemental material). The simplest interpretation of our binding data, therefore, is that shift product 2 results from the binding of one LcrF dimer. In support of this conclusion, the binding properties of a monomeric LcrF$_m$ variant resembled those of ExsA in that both shift products 1 and 2 were readily formed (Fig. 5A). Furthermore, the mobilities of the LcrF-P$_{exsC}$ and ExsA-P$_{exsC}$ shift product 2 complexes were similar (Fig. 2A and B), indicating binding of two molecules of LcrF or ExsA. Binding of LcrF to the P$_{exoT}$, P$_{exsD}$, P$_{yopN}$, P$_{yscN}$, and P$_{lcrG}$ promoter probes, however, resulted in the formation of complexes with reduced mobility relative to those formed by ExsA. Although the lower mobility complexes generated by LcrF could reflect binding of multiple LcrF dimers, our data indicate that the basis for reduced mobility is differential DNA bending. The significance of ExsA- and LcrF-induced bending is unclear, but it is interesting to note that LcrF was better than ExsA in activation of each transcriptional reporter examined in this study. ExsA and LcrF induced P$_{exsC}$ promoter bending to a similar extent (Fig. 2A and B), and there was only a 30% difference in P$_{exsC-lacZ}$ reporter activity (Fig. 1B). With the other promoter probes (P$_{exoT}$, P$_{exsD}$, P$_{yopN}$, P$_{yscN}$, and P$_{lcrG}$), however, LcrF resulted in a higher degree of bending and a significant increase in activity relative to ExsA (ranging from 2.5-fold higher for the P$_{exsD-lacZ}$ reporter to nearly 20-fold higher for the P$_{exoT-lacZ}$ reporter). Increased bending, therefore, positively correlates with promoter activity and may partially account for the observation that LcrF activates transcription better than ExsA.

DNase I footprints of LcrF/VirF and ExsA bound to the *P. aeruginosa* P$_{exsC}$ and yersiniae P$_{lcrG}$ promoter regions identified a similar region of protection (Fig. 6; see also Fig. S5 in the supplemental material) (28, 48). In each case, the protected area was centered on the ExsA consensus site. Fe-Babe footprinting studies with ExsA found that the protected region consists of two adjacent binding sites for monomeric ExsA and that both monomers are bound in the same orientation (i.e., head to tail) (31). The ExsA monomer bound to site 1 makes base-specific contacts with the conserved GnC and TGnnA sequences through amino acids located in the first (L198, T199, and K202) and second HTH (Y250) motifs, respectively (31). Since the highest degree of sequence conservation between ExsA- and LcrF-dependent promoters occurs at binding site 1, we focused on the interaction of LcrF with site 1 and conclude that ExsA and LcrF bind to site 1 in the same manner. Supportive of this, *in vivo* data using the *P. aeruginosa* P$_{exoT}$ and yersiniae P$_{yscN}$ transcriptional reporters found that disruption of the GnC and TGnnA determinants resulted in a significant reduction in activation by both ExsA and LcrF (Fig. 4B and 8B). Paradoxically, however, single nucleotide substitutions in the P$_{exoT}$ GnC and TGnnA determinants had no obvious effect on LcrF binding (Fig. 4D), presumably owing to the stabilizing effect of binding as a dimer. Nevertheless, the finding that LcrF$_m$ binding is sensitive to GnC and TGnA substitutions in site 1 suggests that the same substitutions perturb the interaction of dimeric LcrF at site 1. One model to account for the discrepancy, therefore, is

that the GnC and TGnnA substitutions alter the binding of the LcrF molecule at site 1 in a manner that prevents recruitment of RNAP but overall have no observable effect on binding of the LcrF dimer. Alternatively, it is possible that the *in vitro* binding data are not reflective of the *in vivo* situation (i.e., the GnC and TGnnA substitutions actually do disrupt wild-type LcrF binding *in vivo*).

ExsA-dependent activation is controlled through a direct interaction with the ExsD antiactivator (10). *Y. pestis* lacks an obvious ExsD homolog. Nevertheless, we tested LcrF sensitivity to ExsD by examining P$_{exoT-lacZ}$ reporter activity in an *exsD* deletion strain. In the absence of ExsD, there was virtually no increase in LcrF-dependent reporter activity (Fig. 1A). Second, we tested LcrF-dependent activation while overexpressing ExsD from a second plasmid and found that P$_{exoT-lacZ}$ reporter activity was only modestly reduced (data not shown). Taken together, these data suggest that ExsD is a poor inhibitor of LcrF activity, a property that likely contributes to increased LcrF activity in *P. aeruginosa*. The dimeric state of LcrF in solution may also partially explain increased transcriptional activation by LcrF. Whereas ExsA-dependent activation requires ordered binding of two monomers, dimeric LcrF binds to promoter DNA in a single step that may be kinetically favored. It is curious that purified ExsA is monomeric in solution when most other characterized members of the AraC/XylS family are dimeric. ExsA is also unusual in that it forms a 1:1 complex with the ExsD antiactivator. Because self-association and ExsA-ExsD complex formation are mutually exclusive interactions (30, 33), high-affinity self-association of ExsA might be incompatible with ExsA-ExsD complex formation. The weaker self-association properties of ExsA, therefore, may strike a balance between the requirements to both self-associate and interact with ExsD.

Photorhabdus, *Aeromonas*, and *Vibrio parahaemolyticus* all have ExsD homologs, and we have previously shown that ExsA from *Photorhabdus* and *Aeromonas* can complement a *P. aeruginosa exsA* mutant for T3SS gene expression (27). Our *in vivo* findings demonstrated that the ExsA consensus binding sequence is a primary determinant for promoter recognition by each activator (Fig. 10). Although biochemical assays will be necessary to characterize the binding characteristics of these activators, we speculate that each is monomeric in solution and will exhibit DNA binding properties similar to ExsA, because the activity of each is controlled by an ExsD homolog.

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