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Characterization of ExsC and ExsD Self-Association and Heterocomplex Formation

Guinevere L. Lykken,[1] GuoZhou Chen,[2] Evan D. Brutinel,[1] Lingling Chen,[2] and Timothy L. Yahr[1]*

Department of Microbiology, University of Iowa, Iowa City, Iowa 52242,[1] and Department of Biology, Indiana University, Bloomington, Indiana 47401[2]

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Expression of the *Pseudomonas aeruginosa* type III secretion system (T3SS) is induced by calcium depletion and is positively regulated by the ExsA transcriptional activator and negatively regulated by the ExsD antiactivator. Under conditions permissive for expression of the T3SS, the negative regulatory activity of ExsD is antagonized by a direct binding interaction with ExsC. In the present study, the ExsC-ExsD binding interaction was characterized. Individually, both ExsC and ExsD form self-associated complexes, as judged by bacterial monohybrid and gel filtration experiments. A mixture of purified ExsC and ExsD readily formed a complex that elutes from gel filtration medium as a single included peak. The calculated molecular weight of the ExsC-ExsD complex is consistent with a complex containing multiple copies of ExsC and ExsD. Isothermic titration calorimetry experiments found formation of the ExsC-ExsD complex to be thermodynamically favorable, with a K_d of ~18 nM and a likely binding ratio of 1:1. To identify amino acid residues important for the regulatory activities of ExsC and ExsD, self-association, and complex formation, charged-cluster mutagenesis was performed. Two of the resulting ExsD charged-cluster mutants (DM2 and DM3) demonstrated a hyperrepressive phenotype for expression of the T3SS. By two-hybrid and copurification assays, the DM3 mutant was found to be impaired in its interaction with ExsC. This finding demonstrates that the binding of ExsC to ExsD is required for transcriptional induction of the T3SS under calcium-limiting growth conditions.

The flagellum and injectisome of gram-negative bacteria are large multiprotein organelles spanning the inner and outer membranes (12). Whereas flagella are involved in motility, injectisomes function by injecting toxins into eukaryotic host cells. The biogenesis of both organelles requires a dedicated protein export system referred to as a type III secretion system (T3SS). Many of the individual protein constituents of flagella and injectisomes are first secreted via the T3SS before assembly into the complete organelles (10). The flagellar T3SS is located at the base of the basal body in the inner membrane, where it secretes proteins required for assembly of the flagellar rod, hook, and filament (12). The T3SS associated with the injectisome is also located at the base of a basal body-like structure and secretes proteins required for injectisome assembly as well as the toxins destined for delivery to host cells (10).

An interesting regulatory feature of both the flagellum and injectisome systems is the direct coupling of transcription with secretion (16). The prototypical example of this regulatory mechanism is seen in *Salmonella* spp., where genes required for assembly of the flagellar basal body, hook, and filament are expressed in a temporal hierarchy (1). The late genes, required for the final stage of flagellar biogenesis, are not expressed until the basal body is completely assembled. Prior to completion of the basal body complex, the FlgM anti-σ factor accumulates in the cytoplasm, where it binds to and inhibits the FliA σ factor required for late gene transcription. Upon completion of the basal body complex, however, there is a switch in the substrate specificity of the T3SS, FlgM is secreted from cells, and FliA-dependent transcription of late genes ensues. The coupling of transcription to secretion provides an elegant means of coordinating gene expression with the different stages of the flagellar assembly process.

Secretion competence also serves as an inducing signal for transcription in the injectisome system (16). Unlike the case in the flagellar system, however, where FlgM secretion is linked to basal body assembly, secretion competence in many injectisome systems is controlled by environmental signals (2, 14, 15, 18, 19, 27). Through a poorly understood mechanism, these environmental signals convert the type III secretion machinery from a closed (secretion incompetent) conformation to an open (secretion competent) conformation. Conversion to the open state triggers a regulatory cascade resulting in transcriptional activation. Mechanistically, these regulatory cascades fall into one of the following three general categories: (i) secretion of a negative regulatory factor, seen in *Yersinia* spp.; (ii) sequestration of a coactivator, as reported for *Salmonella* and *Shigella* spp.; and (iii) sequestration of a negative regulatory factor, as recently described for *Pseudomonas aeruginosa* (6, 7, 13, 14, 19–21).

Transcription of the *P. aeruginosa* T3SS is induced under Ca^{2+}-limiting growth conditions or following contact of the bacterium with a host cell (9, 22). Although the mechanism of activation by host cell contact is unclear, the low-Ca^{2+} signal converts the T3SS machinery from a secretion-incompetent to a secretion-competent state (14). Recent studies have demonstrated that transcription of the T3SS is intimately linked to the activity of the type III secretion machinery. Transcription is repressed when the T3SS machinery is secretion incompetent and is derepressed when the machinery is secretion competent

* Corresponding author. Mailing address: University of Iowa, 540B Eckstein Medical Research Building, Iowa City, IA 52242-1101. Phone: (319) 335-9688. Fax: (319) 335-7949. E-mail: timothy-yahr@uiowa.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
P. aeruginosa		
PA103	Wild type	14
PA103 *exsC*	Repressed for type III transcription	7
PA103 *exsD*	Derepressed for type III transcription	14
E. coli		
SU101	Reporter strain for monohybrid assay	8
SU202	Reporter strain for two-hybrid assay	8
BL21(DE3)	Purification of ExsC and ExsD	Novagen
Plasmids		
pSR658	LexA expression vector, Tet^r	4
pSR659	$LexA_{408}$ expression vector, Amp^r	4
pDD506	LexA-CAT fusion protein,[a] Tet^r	4
pDP804	$LexA_{408}$-Jun fusion protein, Amp^r	8
pMS604	LexA-Fos fusion protein, Tet^r	8
pJN105	Arabinose-inducible expression vector, Gm^r	17
pJN-*exsC*	*exsC* cloned into pJN105	7
pJN-CM1-CM10	*exsC* charged-cluster mutants cloned into pJN105	This study
pJN-*exsD*	*exsD* cloned into pJN105	This study
pJN-DM1-DM10	*exsD* charged-cluster mutants cloned into pJN105	This study
pET25b	Hexahistidine fusion tag expression vector	Novagen
pET25-*exsC*	*exsC* cloned into pET25b	7
pET25-*exsD*	*exsD* cloned into pET25b	17

[a] CAT, chloramphenicol acetyltransferase.

(7, 14, 21). The mechanism of coupling transcription to secretion involves a cascade of four interacting regulatory proteins (ExsA, ExsD, ExsC, and ExsE). ExsA is a positive activator of T3SS transcription, while ExsD functions as an antiactivator by binding to and inhibiting ExsA activity (11, 14, 25, 26). ExsC functions as an anti-antiactivator by binding to and inhibiting the negative regulatory activity of ExsD and also as a type III-specific chaperone for ExsE (7, 21). Finally, ExsE binds to ExsC and inhibits its activity (20, 21). A key feature of the system is that ExsE is secreted from cells under low-Ca^{2+} conditions. Based on these findings, the following model has been proposed to account for the coupling of transcription with secretion (7, 21). Under high-Ca^{2+} conditions, intracellular ExsE binds to and sequesters ExsC, and ExsD is preferentially bound to ExsA, resulting in the inhibition of ExsA-dependent transcription. Under low-Ca^{2+} conditions, however, ExsE is secreted from cells. This results in a shift in the binding equilibrium whereby ExsD is preferentially bound to ExsC, and liberated ExsA is made available to activate transcription of the T3SS.

In the present study, we characterize the ExsC-ExsD binding interaction. Both ExsC and ExsD were found to form self-associated multimeric complexes. ExsC and ExsD readily form a complex when coexpressed in vivo or when individually purified and mixed in vitro. Biochemical analyses suggest that formation of the complex is thermodynamically favorable and consists of multiple copies of ExsC and ExsD. Finally, an ExsD mutant unable to interact with ExsC was isolated and found to have a hyperrepressive phenotype.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Pseudomonas aeruginosa* strains (Table 1) were maintained on Vogel Bonner minimal medium with antibiotics, as required (gentamicin [100 μg/ml] and carbenicillin [300 μg/ml]) (23). For expression of the T3SS, strains were grown at 30°C with vigorous aeration in Trypticase soy broth supplemented with 1% glycerol, 100 mM monosodium glutamate, 2 mM EGTA, and antibiotics, as required (gentamicin [80 μg/ml] and carbenicillin [300 μg/ml]). *Escherichia coli* strains (Table 1) were maintained on Luria-Bertani (LB) medium with antibiotics, as required (10 μg/ml tetracycline and 100 μg/ml ampicillin). Expression of LexA fusion proteins from pSR658, pSR659, and pDD506 was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Expression from the pBAD promoter (pJN105) was induced by the addition of arabinose, as indicated.

Expression constructs. Oligonucleotide primers incorporating XbaI and SacI restriction sites were used to amplify *exsC* and *exsD* by PCR. The PCR products were cloned into the corresponding sites of pJN105 (17), resulting in plasmids pJN-*exsC* and pJN-*exsD*, respectively. Site-directed mutagenesis of pJN-*exsC* and pJN-*exsD* was performed by Bio S&T (Montreal, Quebec, Canada), and all mutations were confirmed by nucleotide sequence analysis. The exact nature of each mutation is described in Table 2. To construct the LexA expression vectors, an XbaI restriction site was introduced into the multiple cloning sites of pSR658 and pSR659 (4) by oligonucleotide (5′GATGACGATAAGGATCGATCTAG ATCCGAGCTCGAGATCTGC)-directed mutagenesis. Wild-type and mutant alleles of *exsC* and *exsD* were excised from pJN105 as XbaI and SacI restriction fragments and cloned into the corresponding sites of pSR658(XbaI) and pSR659(XbaI). For coexpression studies, *exsC* and *exsD* were amplified by PCR and cloned into the pCOLADuet-1 expression vector as NdeI-XhoI and NcoI-NotI restriction fragments, respectively. The hexahistidine-tagged ExsC and ExsD expression constructs were described previously (7, 14).

β-Galactosidase assays. For monohybrid assays, *E. coli* reporter strain SU101 was transformed with the pSR658 derivatives, while strain SU202 was transformed with the pSR658 and pSR659 derivatives for two-hybrid assays (4). To assay for β-galactosidase activity, overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in LB containing 1 mM IPTG and the required antibiotics. Cultures were incubated at 30°C with vigorous aeration until the OD_{600} reached 1.0. *E. coli* and *P. aeruginosa* cells were harvested and assayed for β-galactosidase activity as previously described (7).

ExsC and ExsD purification and biochemical methods. Carboxy-terminally hexahistidine-tagged proteins (ExsC-His_6 or ExsD-His_6) were expressed in *E. coli* BL21(DE3) cells and purified by Ni^{2+}-affinity, ion-exchange, and Superdex 200 16/60 gel filtration (Amersham Biosciences) chromatography. For complex formation, purified ExsD-His_6 was mixed with an excess of purified ExsC-His_6 and incubated overnight at 4°C in buffer A (50 mM sodium phosphate, pH 7.5, 200 mM NaCl, 0.5 mM EDTA, and 1 mM dithiothreitol). The complex was isolated using Superdex 200 gel filtration chromatography. The gel filtration column was calibrated with a set of protein standards (ferritin, catalase, aldolase, albumin, ovalbumin, chymotrypsinogen A, and RNase A [Amersham Biosciences]) whose elution volumes (V_e) and molecular weights (MW) were fitted linearly as follows: $(V_e - V_0)/(V_t - V_0)$ versus log MW, where V_0 and V_t are the void volume and total bed volume of the column, respectively. For copurification studies, ExsC was coexpressed with ExsD, DM2, or DM3 in *E. coli* Tuner(DE3), and cell extracts were subjected to Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) affinity chromatography as previously described (7).

Two independent isothermal titration calorimetry (ITC) experiments were performed in a VP-ITC (MicroCal) calorimeter at 25°C, using buffer A. In the first experiment, ExsC (150 μM) was injected 30 times, at 10 μl per injection with a 4-minute interval, into 1.4 ml ExsD (15 μM). In a second experiment (data not shown), ExsD (150 μM) was injected into ExsC (15 μM). To derive the binding parameters, data were fitted with a single-site binding model using Origin ITC software (Origin Lab).

SDS-PAGE, immunoblots, and quantitative analyses. For analysis of cell-associated fractions, cells were grown in either Trypticase soy broth (*P. aeruginosa*) or LB (*E. coli*), as described above. Samples were prepared by sedimenting cells from a 1.25-ml culture (OD_{600} = 1.0), suspending them in 250 μl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and sonicating them for 10 s. Samples were heated for 5 min at 100°C and analyzed by SDS-PAGE (Hi-Tris gel to visualize ExsC or 15% polyacrylamide gel to visualize ExsD). Immunoblots were probed with ExsC or ExsD antiserum and developed using ECL reagents (Amersham Pharmacia Biotech) and anti-rabbit–horseradish peroxidase conjugate (Chemicon). Immunoblots were analyzed by densitometry using IPLAB gel software (Signal Analytics).

RESULTS

Genetic and biochemical analyses of ExsC and ExsD self-associations. ExsC and ExsD were previously shown to interact

TABLE 2. Properties of charged-cluster mutants

Mutant[a]	Amino acid position[b]	Amino acid sequence		Nucleotide sequence[d]
		Wild type[c]	Mutant	
CM1	6	SKVNRL	SAVNGL	AGCgcGGTCAACgGACTG
CM2	26	LDEEG	LAAAG	CTCGcCGcGGcGGGC
CM3	36	FDEQ	FAAQ	TTCGcCGcACAG
CM4	48	AERERL	AAAAGL	GCCGcGgcCGcGgGTCTG
CM5	55	LEADV	LAAAV	CTGGcGGCCGcTGTG
CM6	81	HRFDL	HAFAL	CACgcTTTCGcTCTG
CM7	89	FEDL	FAAL	TTCGcCGcGCTG
CM8	109	LECFEA	LACFAA	CTCGcATGCTTCGcGGCG
CM9	120	LDHAEF	LAHAAF	CTCGcTCACGCCGcGTTC
CM10	134	SDREA	SAAAA	AGTGcTgcCGcGGCG
DM1	4	QEDDKQ	QAAAAQ	CAGGcAGcCGcTgcGCAG
DM2	11	SREA	SAAA	TCCgcAGcAGCG
DM3	18	GRRV	GAAV	GGCgcagcGGTA
DM4	26	SDARS	SAAAS	TCGGcaGCCgcaTCG
DM5	30	SRGRV	SAGAV	TCGgcaGGTgctGTG
DM6	43	YRES	YAAS	TATgcTGcGTCC
DM7	69	FRCEW	FACAW	TTCgctTGCGcATGG
DM8	77	QRLARG	QALAGG	CAGgcaCTGGCGgGCGGC
DM9	85	GREEV	GAAAV	GGGgcaGcgGctGTG
DM10	98	QDDDG	QAAAG	CAGGcaGcCGctGGC
DM11	109	GDRV	GAAV	GGCGcagcGGTC
DM12	148	VELET	VALAT	GTGGcaCTGGctACC
DM13	154	SRQLRV	SAQLGV	TCCgctCAACTGgGAGTC
DM14	159	VKSEF	VASAF	GTCgcaTCCGcATTC
DM15	168	AELEP	AALAP	GCCGcaCTGGcaCCG
DM16	174	AREEL	AAAAL	GCCgcaGctGccCTG
DM17	197	GKLET	GALAT	GGCgcaCTGGctACG
DM18	234	PEVLEC	PAVLAC	CCCGcaGTCCTCGcATGC
DM19	247	LDDDV	LAAAV	CTGGcaGctGcCGTC
DM20	262	HERN	HAAN	CACGcAgcCAAC

[a] The ExsC and ExsD charged-cluster mutants are designated CM1 to CM10 and DM1 to DM20, respectively.
[b] Refers to the location of the amino acid in the full-length protein corresponding to the first amino acid listed in the wild-type sequence column.
[c] The wild-type sequence consists of the residue amino-terminal to the charged cluster, the charged-cluster sequence, and the residue carboxy-terminal to the charged cluster.
[d] Sequence used in the construction of the charged-cluster mutants by site-directed mutagenesis. Mutations are shown in lowercase.

in bacterial two-hybrid and copurification assays (7). To better understand the binding interaction between these proteins, we first examined the oligomeric state of ExsC and ExsD, using the LexA monohybrid system. In this system, the dimeric LexA repressor binds to an operator sequence and represses transcription of a chromosomally encoded *lacZ* reporter (4, 8). To test for self-associations, the dimerization domain of plasmid-encoded LexA was replaced with either ExsC or ExsD and introduced into an *E. coli* reporter strain (SU101) carrying a LexA-repressible *lacZ* reporter. A positive control consisting of a LexA fusion to chloramphenicol acetyltransferase (known to form trimers in vivo) resulted in strong repression of the *lacZ* reporter (Fig. 1A) (4). Likewise, LexA fusions to either ExsC or ExsD strongly repressed expression of the *lacZ* reporter, indicating that both ExsC and ExsD are capable of self-association. Repression of *lacZ* expression was not seen with LexA lacking a dimerization domain or with fusions of ExsC or ExsD to $LexA_{408}$. $LexA_{408}$, used in the two-hybrid assay, is a mutant with altered DNA-binding specificity and is unable to bind the wild-type operator sequence in the SU101 reporter strain (8).

To verify that ExsC and ExsD form self-associated complexes, the proteins were expressed as hexahistidine-tagged fusion proteins in *E. coli* and purified sequentially by Ni^{2+}-affinity, ion-exchange, and gel filtration chromatography (Fig. 1B). The predicted molecular masses of monomeric ExsC-His_6 and ExsD-His_6 are 19 kDa and 34 kDa, respectively. When purified by Superdex 200 gel filtration chromatography, ExsC-His_6 eluted as a single included peak with an estimated molecular mass of 79 kDa, whereas ExsD-His_6 was purified as a single included peak with an estimated molecular mass of 128 kDa, both of which are suggestive of self-association into higher aggregation states (dimer, trimer, etc.). The combined monohybrid and gel filtration data demonstrate that both ExsC and ExsD have a strong tendency to form self-associated oligomeric complexes both in vivo and in vitro.

Biochemical analyses of the ExsC-ExsD complex. ExsC and ExsD are also known to form a heteromeric complex. To characterize the ExsC-ExsD complex, purified ExsD-His_6 was incubated with an excess of ExsC-His_6, and the sample was analyzed by gel filtration chromatography. The predicted molecular mass of a heteromeric complex consisting of single molecules of ExsC and ExsD is 53 kDa. In gel filtration chromatography, however, the ExsC-ExsD complex eluted as a single included peak of 197 kDa, suggesting that the complex contains multiple copies of ExsC and ExsD (Fig. 1B).

To further characterize the ExsC-ExsD complex, ITC was employed. This technique measures the enthalpy exchange during a binding reaction and can be used to calculate binding constants and the reaction stoichiometry (3). Purified ExsC (150 μM) was injected (10-μl injections every 4 min for a total

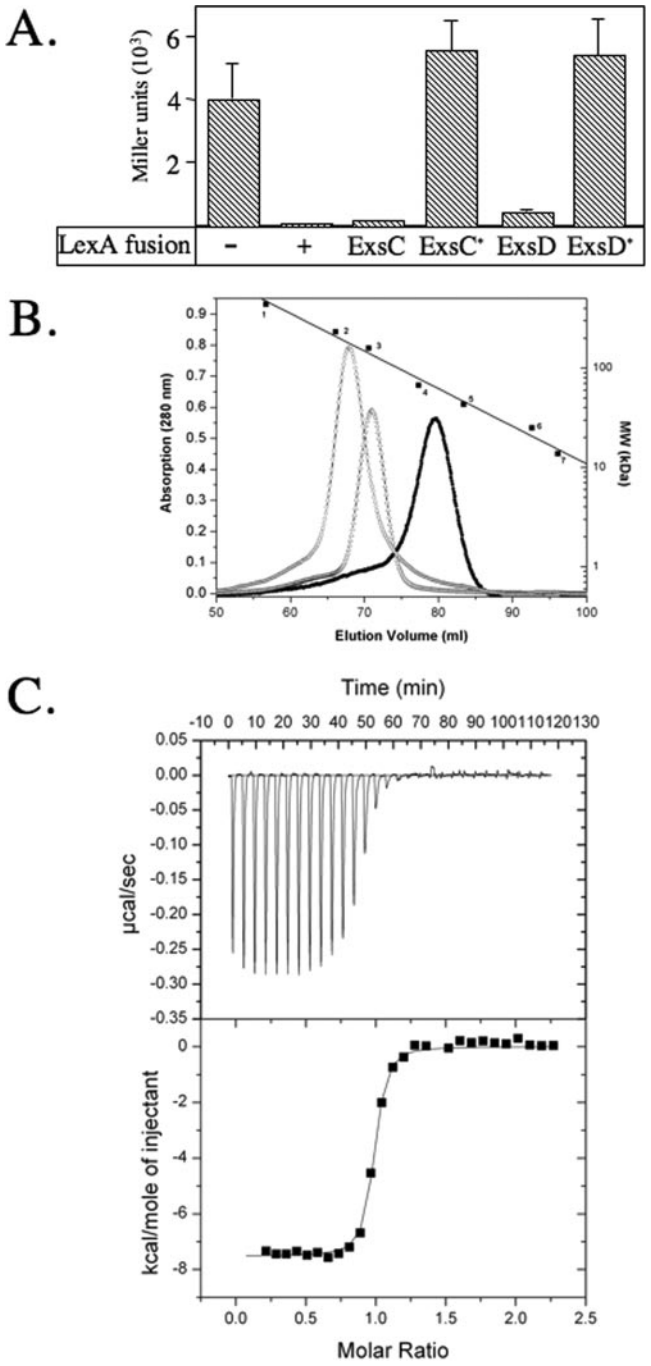

FIG. 1. Characterization of ExsC and ExsD oligomers. (A) *E. coli* SU101 strains expressing the LexA or $LexA_{408}$ (indicated by an asterisk) DNA-binding domain alone (−) or fused to chloramphenicol acetyltransferase (+), ExsC, or ExsD were assayed for β-galactosidase activity as described in the text. β-Galactosidase activity is expressed in Miller units, and the data represent the averages for at least three independent experiments. (B) Gel filtration chromatography of ExsC-His_6 (right peak), ExsD-His_6 (middle peak), and the ExsC-His_6left peakExsD-His_6 complex (left peak). Also shown are the elution volumes of a set of calibration proteins with known molecular weights (1, ferritin; 2, catalase; 3, Aldolase; 4, albumin; 5, ovalbumin; 6, chymotrypsinogen A; 7, ribonuclease A). The solid line approximates the elution volume versus the molecular weight. (C) Titration of ExsD-His_6 with ExsC-His_6, monitored by ITC. Heat exchange during each injection is presented in the upper panel, and the corresponding integrated enthalpies (after background correction) are shown in the lower panel. The solid line in the lower panel represents a fit of the data to a single-site binding model.

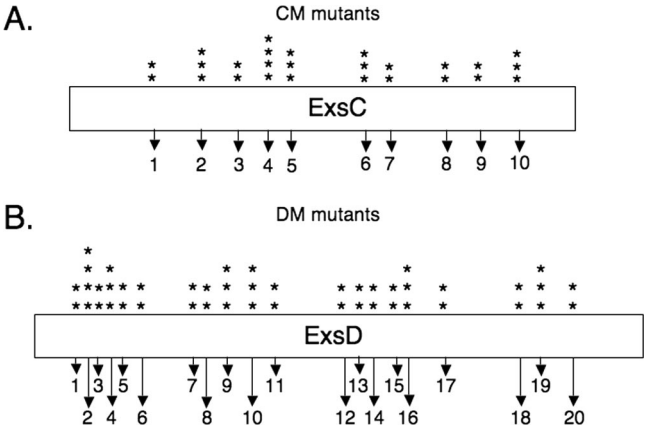

FIG. 2. Diagram of *exsC* and *exsD* charged-cluster mutants. The *exsC* mutants (A) are labeled CM1-10, and the *exsD* mutants (B) are labeled DM1-20. Asterisks indicate the locations of charged-cluster mutations, labeled by position. The number of asterisks corresponds to the number of charged residues within the 4-amino-acid window for each mutant.

of 30 injections) into a reaction cell containing 15 μM ExsD. Analysis of the ITC data found formation of the ExsC-ExsD complex to be exothermic and thermodynamically favorable (Fig. 1C). Similar results were seen when the experiment was repeated by injecting ExsD into a reaction cell containing ExsC. The stable complex has an average dissociation constant (K_d) of ~18 nM and an average binding stoichiometry of 0.87, consistent with a complex containing a 1:1 molar ratio of ExsC and ExsD. These data indicate that ExsC and ExsD form a stable complex with a K_d in the 18-nM range and a molecular mass of ~197 kDa.

Charged-cluster analysis of ExsC and ExsD. Having examined the nature of the ExsC-ExsD complex, we wished to identify amino acid residues important for the regulatory activities of ExsC and ExsD, for self-association, and for formation of the ExsC-ExsD complex. The first strategy employed was a truncation analysis of ExsD. Truncations in ExsD as short as 10 amino acids from either the amino or carboxy terminus, however, rendered the protein completely unstable when expressed in *P. aeruginosa* (data not shown). To circumvent this problem, a charged-cluster analysis was performed (24). A charged cluster was defined by the presence of at least two charged amino acids within a four-residue window. One advantage of this approach is that charged clusters are often surface exposed. For this reason, substitutions within the charged clusters are less likely to perturb the overall protein structure and/or stability (5). Analysis of the primary amino acid sequence identified 10 charged clusters in ExsC and 20 in ExsD (Fig. 2). For each cluster, the charged residues were replaced with either alanine or glycine (Table 2). The resulting mutants, designated CM1-10 and DM1-20, were assayed for (i) complementation of an *exsC* or *exsD* mutant, (ii) self-association in the LexA monohybrid assay, and (iii) ExsC-ExsD complex formation in the LexA two-hybrid assay.

Complementation by ExsC and ExsD charged-cluster mutants. For complementation studies, wild-type *exsC* and *exsD* and each of the charged-cluster mutants were cloned into an arabinose-inducible expression vector. The resulting plasmids

were used to transform an *exsC* or *exsD* mutant with a chromosomally carried *lacZ* transcriptional reporter (P_{exsD}-*lacZ*), and strains were assayed for β-galactosidase activity. We previously established that P_{exsD}-*lacZ* transcriptional activity serves as a reliable surrogate for the transcriptional activity of all T3SS genes in strain PA103 (7, 14). The expression of P_{exsD}-*lacZ* is repressed in an *exsC* mutant carrying a vector control and is derepressed in an *exsD* mutant carrying a vector control (7, 14). To determine the optimal inducer concentration for the complementation analyses, arabinose titrations were performed. ExsC-dependent derepression was not seen in the absence of arabinose and was maximal at the highest concentration of arabinose tested. Significant ExsD-dependent repression was seen in the absence of arabinose and was maximal at 0.1% arabinose. To achieve maximal biological activity without saturating the system with a large excess of regulatory proteins, expression of the ExsC and ExsD charged-cluster mutants was induced with 0.5% and 0.1% arabinose, respectively.

Similar to wild-type ExsC, charged-cluster mutants CM1 and CM10 complemented a chromosomal *exsC* deletion mutant for expression of P_{exsD}-*lacZ*, indicating that these charged clusters are not important for ExsC activity (Fig. 3A). In contrast, charged-cluster mutants CM2-9 were significantly impaired in the ability to complement the *exsC* mutant. A trivial explanation for the lack of complementation by CM2-9 is that the charged-cluster mutations rendered the proteins unstable. This appears to be the case for mutants CM2-4 and CM8-9, since anti-ExsC immunoblots show the steady-state expression levels of CM2 and CM8-9 to be 15% and those of CM3-4 to be 1% of wild-type ExsC levels (Fig. 3A). We concluded that the lack of complementation by the CM2-4 and CM8-9 charged-cluster mutants most likely results from the instability of these proteins in *P. aeruginosa*. The most interesting of the *exsC* charged-cluster mutants are CM5-7. The steady-state expression levels for these mutants were 50%, 90%, and 80% of the level of the wild type, respectively, indicating that the stabilities of these mutants are not drastically altered in vivo. In the complementation analysis, however, the CM5-7 mutants retained only 20%, 25%, and 6% of the activity of wild-type *exsC*. These data suggest that the CM5-7 charged clusters are required for full ExsC activity.

Complementation by the *exsD* charged-cluster mutants was examined next. Plasmid-encoded wild-type ExsD complemented the *exsD* chromosomal mutant, resulting in repression of P_{exsD}-*lacZ* expression (Fig. 4A). The *exsD* charged-cluster mutants fell into four classes based upon their complementation patterns. The first class of mutants (DM1, DM8-10, and DM19) did not complement an *exsD* mutant and, with the exception of DM8, were not stably expressed in *P. aeruginosa* (Fig. 4A). The second class of charged-cluster mutants (DM4-7, DM11-12, DM14-15, and DM17-18) repressed P_{exsD}-*lacZ* expression to within 10% of the level seen with wild-type *exsD*. We concluded that these charged clusters are not required for the negative regulatory activity of ExsD. The third class of mutants, consisting of DM13, -16, and -20, repressed the expression of P_{exsD}-*lacZ* to within 25% of the level seen with wild-type *exsD*, suggesting that these residues are required for full ExsD activity (Fig. 4A). Finally, the last class of charged-cluster mutants (DM2-3) resulted in a hyperrepressive phenotype compared to wild-type *exsD* (discussed in more detail below).

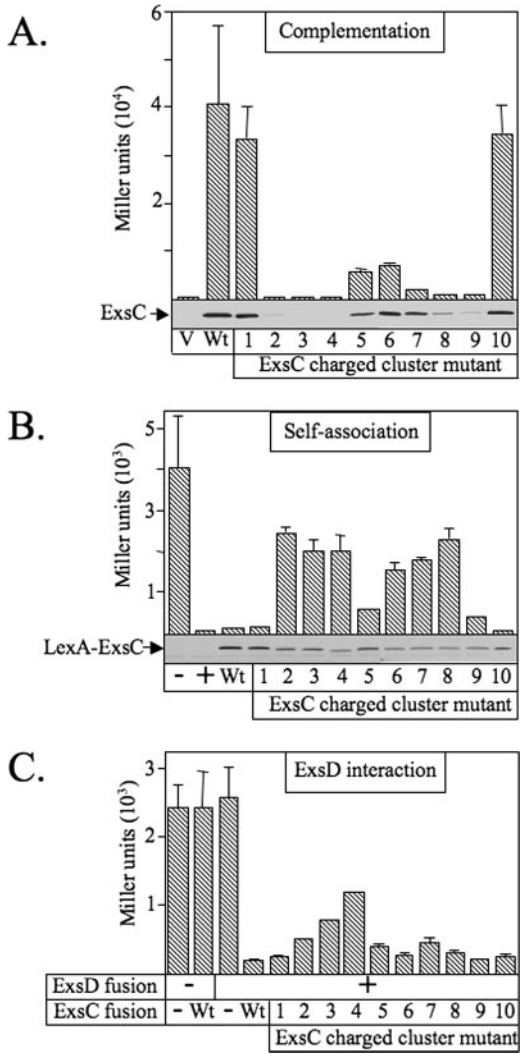

FIG. 3. Characterization of ExsC charged-cluster mutants. (A) Complementation of an *exsC* mutant. PA103 *exsC* P_{exsD}-*lacZ* carrying either a vector control (V), a wild-type ExsC expression vector (Wt), or vectors expressing each of the ExsC charged-cluster mutants (CM1 to CM10) was grown under inducing conditions for expression of the T3SS and assayed for β-galactosidase activity. (B) *E. coli* SU101 strains expressing the LexA DNA-binding domain alone (−) or fused to chloramphenicol acetyltransferase (+), wild-type ExsC, or each of the ExsC charged-cluster mutants were assayed for β-galactosidase activity as described in the text. (C) Interaction of ExsC with ExsD by the LexA two-hybrid assay in *E. coli* reporter strain SU202. Strains expressing the LexA DNA-binding domain alone (−) or LexA fusions to wild-type ExsD and the DNA-binding domain of $LexA_{408}$ alone (−) or $LexA_{408}$ fusions to wild-type ExsC or ExsC charged-cluster mutants were grown in LB and assayed for β-galactosidase activity. The values represent the averages for at least three independent experiments. Panels at the bottom of the graphs represent ExsC immunoblots using anti-ExsC antisera. The positions of ExsC (A) and the LexA-ExsC fusion proteins (B) are indicated by arrows.

Self-association of ExsC and ExsD charged-cluster mutants. The second assay for analyses of the ExsC and ExsD charged-cluster mutants was a self-association assay using the LexA monohybrid system described above. LexA fusions to each of

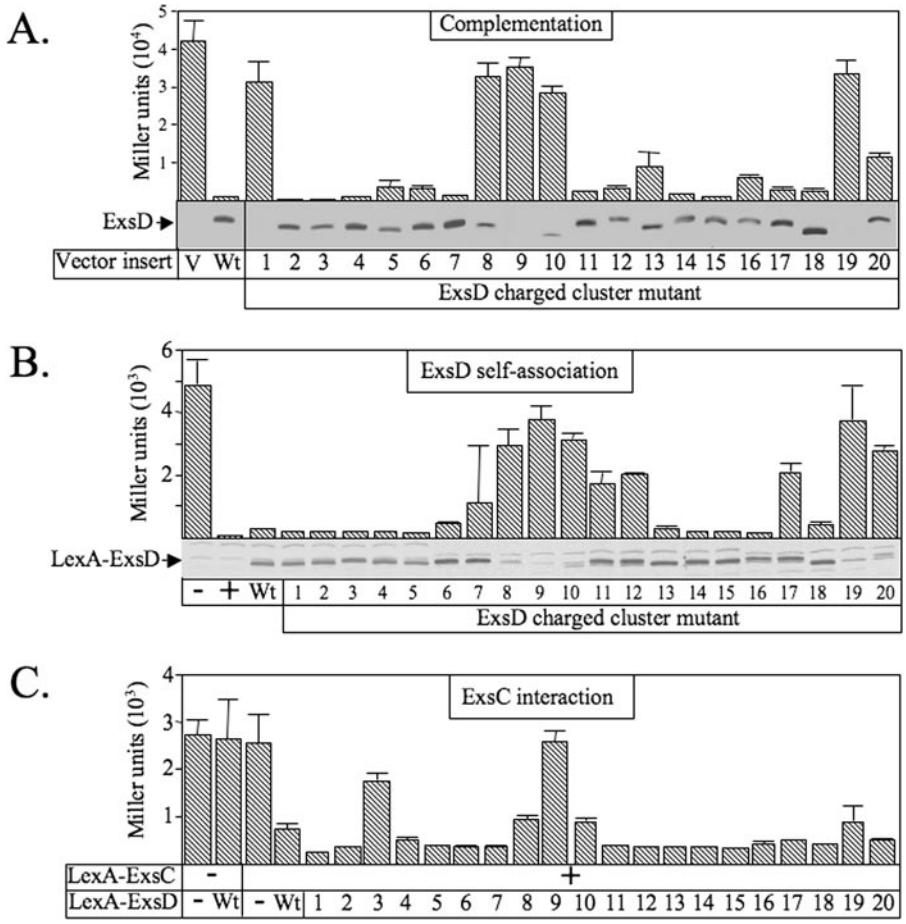

FIG. 4. Characterization of ExsD charged-cluster mutants. (A) Complementation of an *exsD* mutant. PA103 *exsD* P_{exsD}-*lacZ* was transformed with a vector control (V), pJ*exsD* (Wt), or *exsD* charged-cluster mutants DM1 to DM20 and assayed for β-galactosidase activity. (B) *E. coli* SU101 strains expressing the DNA-binding domain of LexA alone (−) or the indicated fusions of LexA to either chloramphenicol acetyltransferase (+), wild-type ExsD (Wt), or ExsD charged-cluster mutants were assayed for self-association using the monohybrid assay. (C) Strains expressing the indicated fusions of LexA to ExsC and $LexA_{408}$ to either wild-type ExsD (Wt) or ExsD charged-cluster mutants were grown in LB and assayed for β-galactosidase activity in the LexA two-hybrid assay. The values represent the averages for at least three independent experiments. Panels at the bottom of graphs A and B represent ExsD immunoblots. The positions of ExsD (A) and LexA-ExsD fusion proteins (B) are indicated by arrows.

the ExsC charged-cluster mutants were constructed, and expression levels were measured by anti-ExsC immunoblot analysis. Although the CM2-4 and CM8-9 charged-cluster mutants were unstable when expressed in *P. aeruginosa* (Fig. 3A), all of the LexA-ExsC charged-cluster fusions were stably expressed in *E. coli* (Fig. 3B). When tested for self-association, the LexA-CM1 and -CM10 fusions demonstrated strong repression of the *E. coli* SU101 *lacZ* reporter, indicating that these charged clusters are not important for ExsC self-association (Fig. 3B). The remaining LexA fusions (CM2-9), however, were impaired in self-association to various degrees. The ability of the charged-cluster mutants to self-associate correlates nicely with the complementation data (i.e., charged-cluster mutants that complemented an *exsC* mutant also demonstrated strong self-association in the monohybrid assay) (Fig. 3A versus Fig. 3B). These data suggest that the regulatory activity and/or stability of ExsC in *P. aeruginosa* may depend upon self-association.

Likewise, the steady-state expression level for each of the LexA-ExsD charged-cluster fusions was examined by anti-ExsD immunoblotting. With the exception of DM8-10 and DM19-20, the remaining charged-cluster fusions were stably expressed in *E. coli* (Fig. 4B). When assayed for self-association, charged-cluster fusions DM1-6, DM13-16, and DM18 repressed *lacZ* expression to a similar extent as the wild-type LexA-ExsD fusion, indicating that the affected charged clusters are not required for self-association (Fig. 4B). The lack of self-association for DM8-10 and DM19-20 is a reflection of their poor stability in *E. coli*. Charged-cluster fusions DM7, DM11-12, and DM17, however, are impaired in self-association and yet stably expressed in *E. coli* and *P. aeruginosa*. Furthermore, all four of these charged-cluster mutants complemented an *exsD* mutant, suggesting that self-association is not necessarily required for ExsD regulatory activity.

Two-hybrid analysis of charged-cluster mutants. Finally, each of the charged-cluster mutants was examined for formation of an ExsC-ExsD complex in the LexA two-hybrid system. This system makes use of wild-type LexA, a LexA mutant ($LexA_{408}$) with altered DNA-binding specificity, and a hybrid

operator with half-sites specific for LexA and $LexA_{408}$ (8). Only heterodimers of LexA and $LexA_{408}$ can bind to the hybrid operator and repress transcription of the *lacZ* reporter. For our analyses, the dimerization domains of LexA and $LexA_{408}$ were replaced with wild-type ExsC or ExsD and with each of the charged-cluster mutants, respectively. As previously reported, the combination of LexA-ExsD and $LexA_{408}$-ExsC fusions strongly repressed expression of the *lacZ* reporter, whereas repression was not seen with the vector controls (Fig. 3C) (7). Charged-cluster mutants CM1, CM5-6, and CM8-10 resulted in strong repression of the *lacZ* reporter, indicating that these charged-cluster substitutions have little impact on ExsC-ExsD interactions. Charged-cluster mutants CM2-4 and CM7, however, were impaired for ExsD interaction to various degrees.

Most of the ExsD charged-cluster mutants that were stably expressed in *E. coli* retained interactions with ExsC (Fig. 4B and C). The only exception, DM3, though stably expressed as a LexA fusion, was unable to repress expression of the *lacZ* reporter to the full extent seen with the wild-type LexA-ExsD fusion. These data suggest that most ExsD charged-cluster substitutions do not significantly influence the ExsC-ExsD interaction.

Characterization of DM2 and DM3 charged-cluster mutants. The most interesting mutants to arise from the charged-cluster analyses were DM2 and DM3. Both mutants demonstrated a hyperrepressive phenotype in the complementation analysis compared to wild-type ExsD (Fig. 4A). In that analysis, expression of plasmid-encoded ExsD, DM2, and DM3 was induced by the addition of 0.1% arabinose to the growth medium. To further characterize the DM2 and DM3 mutants, the complementation experiment was repeated in the presence or absence of 0.1% arabinose. At this concentration of inducer, the steady-state expression levels of wild-type ExsD, DM2, and DM3 were comparable, and all three proteins strongly repressed expression of the P_{exsD}-*lacZ* reporter (Fig. 5A). In the absence of inducer, the steady-state expression levels were also comparable for all three proteins (Fig. 5A). Despite the similarity in expression levels, however, only DM2 and DM3 strongly repressed expression of the P_{exsD}-*lacZ* reporter when cells were grown in the absence of inducer. These data demonstrate that the hyperrepressive phenotype does not result from an increase in the steady-state expression levels of DM2 and DM3.

In our model for induction of the T3SS by calcium-limiting growth conditions, ExsD is proposed to form complexes with both ExsA and ExsC (7, 14). The ExsD-ExsA complex prevents transcription of the T3SS under high-calcium conditions, whereas formation of the ExsD-ExsC complex under calcium-limiting conditions relieves the block on transcription. This model predicts that a hyperrepressive phenotype could result from charged-cluster substitutions that prevent ExsD from binding ExsC. In the two-hybrid analysis, however, only DM2 demonstrated an unimpaired interaction with ExsC (Fig. 4C). To independently verify this result, a copurification assay was developed in which hexahistidine-tagged ExsC was coexpressed with either wild-type ExsD, DM2, or DM3 in *E. coli*. Cell extracts were then applied to a Ni-NTA affinity column and washed to remove unbound material. Bound material was then eluted with an excess of imidazole and analyzed by immunoblot analysis. As expected, wild-type ExsD was only present in the eluate when coexpressed with hexahistidine-tagged ExsC (Fig. 5B). Likewise, copurification of DM2 was dependent on the presence of ExsC, confirming that DM2 interacts with ExsC. In contrast, DM3, though present in a comparable amount to those of ExsD and DM2 in the initial cell extract, did not copurify with hexahistidine-tagged ExsC. These findings are consistent with the two-hybrid data for both DM2 and DM3 and confirm that DM3 is impaired in its ability to interact with ExsC.

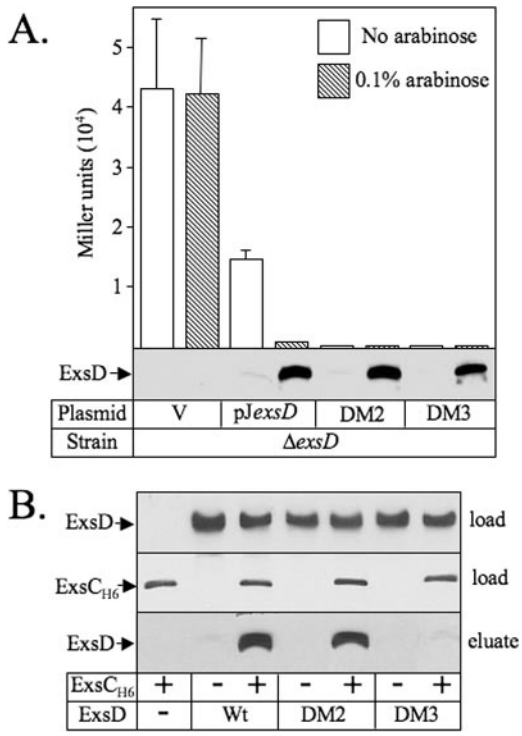

FIG. 5. Characterization of charged-cluster mutants DM1 and DM2. (A) Strain PA103 *exsD* P_{exsD}-*lacZ* carrying a vector expressing wild-type ExsD, DM2, or DM3 was grown under inducing conditions for expression of the T3SS in the absence or presence of 0.1% arabinose and assayed for β-galactosidase activity. β-Galactosidase activity is expressed in Miller units, and the data represent the averages for at least three independent experiments. (B) ExsD, DM2, and DM3 were expressed from the pCOLADuet-1 vector in the absence (−) or presence (+) of hexahistidine-tagged ExsC ($ExsC_{H6}$) in *E. coli*. Cell extracts (load) were applied to a Ni^{2+}-NTA spin column and washed to remove unbound material, and the column eluate was collected. The load and eluate were subjected to anti-ExsC or anti-ExsD immunoblot analyses. The positions of ExsD and ExsC-His_6 are indicated with arrows.

DISCUSSION

Induction of the *P. aeruginosa* T3SS under calcium-limiting growth conditions requires the binding of ExsC to ExsD. In the present study, purified ExsC and ExsD were shown individually to form self-associated complexes. The fact that both self-associated complexes remained intact throughout Ni^{2+}-NTA, anion-exchange, and gel filtration chromatography suggests that the complexes are relatively stable, with subunit interactions of high affinity. We estimate that there are ~25 copies of

ExsC, ExsD, and ExsA per low-calcium-induced cell, corresponding to a cellular concentration of ~40 nM for each protein. Our data do not address whether this value is above or below the threshold for ExsC and ExsD oligomerization. A previous study found that ExsC and ExsD steady-state expression levels increase three- to fourfold in response to calcium-limiting growth conditions (7). Although it is possible that dynamic changes in the oligomeric state contribute to the regulatory activities of ExsC and ExsD, we favor a model in which ExsC and ExsD are always present as self-associated complexes.

A heteromeric ExsC-ExsD complex was readily formed by mixing ExsC with ExsD. By gel filtration chromatography, the ExsC-ExsD complex has an estimated molecular mass of 197 kDa. This is much larger than the predicted molecular mass of a heterodimeric complex consisting of ExsC and ExsD (53 kDa). Isothermic titration calorimetry experiments found the ExsC-ExsD binding interaction to have a high affinity, with a K_d of ~18 nM and a binding stoichiometry of 0.87, which suggests a 1:1 molar ratio of ExsC and ExsD in the complex. Based on the calculated molecular weight of the ExsC-ExsD complex and the strong tendency for ExsC and ExsD to self-associate, it seems most likely that the ExsC-ExsD complex consists of one self-associated ExsC complex and one self-associated ExsD complex. Verification of this binding configuration and efforts to solve the crystal structures of ExsC, ExsD, and the ExsC-ExsD complex are currently under way.

Due to the small size of ExsC and the finding that ExsD truncations were unstable when expressed in *P. aeruginosa*, charged-cluster mutagenesis of ExsC and ExsD was performed to identify amino acid residues important for biological activity, self-association, and formation of the ExsC-ExsD heteromeric complex. Whereas only 5 of the 10 ExsC charged-cluster mutants were stably expressed in *P. aeruginosa*, all of the ExsC mutants were more stably expressed in *E. coli* as LexA fusions. It is unclear whether the increased stability of the ExsC mutants in *E. coli* results from the absence of some proteolytic activity or from stabilizing effects of the LexA fusion partner. Nevertheless, the increased stability in *E. coli* allowed us to determine the relative contribution of each ExsC charged-cluster substitution to self-association and complex formation with ExsD by using the LexA system. Charged-cluster mutants CM2-9 were impaired for self-association in the monohybrid assay and also failed to complement an *exsC* mutant. These data suggest that self-association may be important for both ExsC stability (in the case of charged-cluster mutants CM2-4 and CM8-9) and biological activity (CM5-7). Surprisingly, self-association was not required for formation of the ExsC-ExsD complex in the case of charged-cluster mutant CM6. Since this charged-cluster mutant cannot complement an *exsC* mutant, formation of the ExsC-ExsD complex may be necessary but not sufficient for ExsC regulatory activity. Similar results were seen with charged-cluster mutant CM5, which is mildly impaired in self-association and interactions with ExsD yet fails to complement an *exsC* mutant. In light of these observations, it is important to note that ExsD inhibits transcription of the T3SS by binding to the transcription factor ExsA (14). It is unclear whether the binding of ExsD to ExsC and ExsA is mutually exclusive or involves two distinct binding sites. It is possible that ExsC binds the ExsD-ExsA complex and induces a conformational change that results in the release of ExsA. The lack of complementation by the CM5 and CM6 mutants may reflect an inability to disrupt the ExsD-ExsA complex upon binding to ExsD. An alternative possibility is that these mutants bind ExsD in a stoichiometry different from that seen for wild-type ExsC and that this binding configuration lacks biological activity.

Several of the ExsD charged-cluster substitutions resulted in significant decreases in steady-state expression levels in *P. aeruginosa* (DM1, DM8-10, and DM19) or *E. coli* (DM8-10 and DM19-20). All of those mutants, with the exception of DM1, were also impaired in self-association. There were also four charged-cluster mutants impaired in self-association (DM7, DM11-12, and DM17) that were stably expressed in both *P. aeruginosa* and *E. coli*. All four of these mutants were capable of interacting with ExsC and retained full biological activity. These data suggest that ExsD self-association is not necessarily required for stability, heterocomplex formation, or biological activity.

The most interesting ExsD charged-cluster substitutions were the hyperrepressive DM2 and DM3 mutants. We hypothesized that a hyperrepressive phenotype would result if a charged-cluster substitution in ExsD disrupted interactions with ExsC. This was indeed the case for the DM3 mutant, which failed to associate with ExsC in two-hybrid and coprecipitation assays. We concluded that the hyperrepressive phenotype of DM3 most likely resulted from the inability of the DM3-ExsA complex to be recognized or disrupted by ExsC. In contrast, the DM2 mutant retained full interaction with ExsC in the two-hybrid and copurification assays, suggesting that the mechanism of hyperrepression by DM2 is distinct from that of the DM3 mutant. Hyperrepression might result from an increased affinity of DM2 for ExsA. In the LexA two-hybrid assay, however, there was no evidence that DM2 had an increased association with ExsA compared to that of wild-type ExsD (data not shown). Alternatively, DM2 may still bind ExsC but may be refractile to ExsC-dependent disruption of the DM2-ExsA complex. Understanding the mechanism of hyperrepression by the DM2 mutant will be a focus of future studies.

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