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Functional Analysis of *exsC* and *exsB* in Regulation of Exoenzyme S Production by *Pseudomonas aeruginosa*

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Expression of ExsC, ExsB, and ExsA (the exoenzyme S *trans*-regulatory locus) of *Pseudomonas aeruginosa* was analyzed by using complementation, RNase protection, translational fusion, and T7-directed protein expression analyses. T7 expression analyses in *E. coli* hosts demonstrated that ExsC, ExsA, and a truncated form of ExsD (a partial open reading frame located 3′ of ExsA) were translated; however, a product corresponding to ExsB was undetectable. T7-mediated transcription and translation of the antisense strand resulted in production of a 18.5-kDa product, termed ExsB′, which overlapped the predicted ExsB product. In complementation experiments, deletion of the region encoding ExsB and most of ExsB′ severely reduced exoenzyme S production. Site-specific mutagenesis of the start codons for ExsB and ExsB′, however, did not affect exoenzyme S production. RNase protection studies were initiated to examine the hypothesis that RNA encoded within the ExsB/ExsB′ region exerted a regulatory effect. RNA encoding ExsB′ was not detectable from chromosomal genes or complementation constructs, indicating that ExsB′ was not expressed in *P. aeruginosa*. To determine the pattern of translation, a chloramphenicol acetyltransferase gene (*cat*) reporter was fused in frame with ExsB and with ExsA in the context of the entire locus or in the absence of the *exsB* region. These experiments indicated that *exsB* was not translated but that deletion of the *exsB* region affected the translation of ExsA-CAT. RNase protection assays further suggested that deletion of *exsB* resulted in a processing of ExsA mRNA. Our data indicate that the untranslated *exsB* region of the *trans*-regulatory locus mRNA mediates either the stability or the translation of *exsA*. Complementation analysis further suggests that ExsC may play a role in the translation or stability of ExoS.

Exoenzyme S is an ADP-ribosyltransferase produced and secreted by the opportunistic pathogen *Pseudomonas aeruginosa* (3, 5, 22, 34, 42). Two immunologically related forms of exoenzyme S with molecular masses of 49 and 53 kDa have been identified (22). The two forms are encoded by separate but coordinately regulated genes (48). Exoenzyme S-mediated ADP-ribosyltransferase activity requires the participation of a eukaryotic protein termed FAS (Factor activating exoenzyme S) (5, 8). FAS is a member of the 14-3-3 family of proteins, which regulate the activity and interaction of several eukaryotic proteins (15). Exoenzyme S covalently modifies monomeric vimentin (5, 6) and a variety of small (21- to 25-kDa) GTP-binding proteins of the H- and K-Ras families (5, 7). In burn wound and lung infection models, exoenzyme S production correlates with the dissemination of *P. aeruginosa* from epithelial colonization sites to the bloodstream (34, 35, 36). An attractive model that fits these data suggests that exoenzyme S ADP-ribosyltransferase activity disrupts epithelial cell signal transduction pathways, resulting in a breakdown of the epithelial barrier.

Genetic studies and complementation analysis of the exoenzyme S-deficient mutant 388 *exs1*::Tn*1* (12) led to the identification of a *trans*-regulatory locus which is required for exoenzyme S synthesis. The locus contains several potential open reading frames: *exsC*, *exsB*, *exsA*, and *exsD*, all of which possess codon usage and translational signals characteristic of *P. aeruginosa* (10, 45). ExsA is homologous to the AraC family of transcriptional activators and has been shown to be a DNA-binding protein (21, 47). Genetic, promoter fusion, and transcriptional start site analyses indicate that ExsA is a positive regulator of several operons involved in exoenzyme S production (13, 21, 46). ExsA regulates transcription of the regulatory operon in which it resides (*exsC*, *-B*, and *-A*), an operon containing homologs to type III secretory proteins (*exsD* and *pscB* to *-L*), and the structural gene loci (*exoS*, *exoT*, and *orf1*) (21, 46–49). Since *exsC*, *exsB*, and *exsA* are transcribed as an operon, it was postulated that all three proteins may play a regulatory role in either exoenzyme S synthesis or secretion (46).

To determine the contributions of ExsC and ExsB in the regulation of exoenzyme S production, we performed complementation studies in which a *P. aeruginosa* strain not expressing the *trans*-regulatory locus gene products was provided with various versions of the locus in *trans*. Extracellular and cell-associated levels of exoenzyme S activity were measured. Expression analyses at the transcriptional and translational levels were performed. Our data indicate that ExsA is required but not sufficient for maximal exoenzyme S expression. ExsC was shown to have a posttranscriptional effect on ExoS production. The *exsB* region is not expressed as a protein but appears to contain an RNA region that facilitates the translation of ExsA or the stability of mRNA encoding *exsA*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani broth with ampicillin (100 μg/ml), kanamycin (50 μg/ml), or tetracycline (25 μg/ml) as needed. *P. aeruginosa* strains were grown on Vogel-Bonner minimal medium (43) plates with tetracycline (100 μg/ml) or carbenicillin (400 μg/ml). For maximal production of exoenzyme S, strains were grown in a deferrated

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
P. aeruginosa strains		
PAO1	Wild type	20
PAO1*exsC*::Ω	Ω-inactivated *trans*-regulatory locus	13
E. coli strains		
TB1	JM83 *hsdR* (r_K^- m_K^+)	2
K38	Strain harboring pGP1-2 used in labeling studies	39
BL21(DE3)	Strain harboring IPTG-inducible T7 RNA polymerase as a lysogen	41
Plasmids		
pGP1-2	Plasmid containing heat-inducible T7 RNA polymerase, Km^r	41
pUC18CBA	Subclone containing 2,854-bp fragment, Ap^r	This study
pDF120	Subclone containing 3,055-bp fragment in pUC18, Ap^r	11
pT7-5, pT7-6	Expression vectors utilizing the T7 gene 10 promoter, Ap^r	41
pT7-5A1A2	*exsA* cloned under the control of the T7 promoter	12
pT7-6CBA	*Hin*dIII-*Eco*RI 2,856-bp fragment of pUC18CBA cloned in pT7-6	This study
pT7-5ABC	Reverse orientation of the CBA fragment in pT7-5	This study
pT7-5CB	*Hin*dIII-*Xho*I 1,555-bp fragment of pUC18CBA in pT7-5	This study
pT7-6BC	Reverse orientation clone of pT7-5CB in pT7-6	This study
pT7-5B	*exsB* on a 560-bp *Stu*I fragment in pT7-5	This study
pT7-5CΔn	A 907-bp *Hin*cII fragment in pT7-5	This study
pT7-5CΔnR	CΔn fragment in reverse orientation in pT7-5	This study
pT7-5H1.2R	1,194-bp *Hin*cII fragment in pT7-5	This study
pT7-5*exoS*	*exoS* cloned in reverse orientation of the T7 promoter in pT7-5	This study
pLAFR	Broad-host-range cosmid cloning vector, Tc^r mob^+	14
pLAFRCBA	pLAFR containing the *trans*-regulatory locus	This study
pLAFRC-STOPBA	pLAFRCBA containing translational stops in *exsC*	This study
pLAFRCA	pLAFRCBA with a *Stu*I deletion of *exsB*	This study
pLAFRA	pLAFRCBA with an *Eco*NI-*Bgl*II deletion of *exsC* and *exsB*	This study
pLAFRAΔNI-StuI	pLAFRCBA with an *Eco*NI-*Stu*I deletion of *exsC* and *exsB*	This study
pLAFRCBA*exsB**	pLAFRCBA with a start site mutation in *exsB*	This study
pLAFRCBA*exsB′**	pLAFRCBA with a start site mutation in *exsB′*	This study
pUCP18	pUC-derived cloning vector able to replicate in *Pseudomonas*	38
pUCP18CBA	pUCP18 containing the *trans*-regulatory locus	This study
pUCP18CBA-CAT	pUCP18CBA containing an *exsA*-CAT fusion	This study
pUCP18CA-CAT	pUCP18CA containing an *exsA*-CAT fusion	This study
pUCP18CB-CAT	pUCP18CBA containing an *exsB*-CAT fusion and *exsA* deletion	This study
pT7-5CBA-CAT	pT7-5CBA containing an *exsA*-CAT fusion	This study
pT7-5CAT-ABC	pT7-5 containing CBA-CAT in the opposite orientation	This study
pT7-5CA-CAT	pT7-5 containing CA-CAT	This study
pT7-5A1A2-CAT	pT7-5A1A2 containing an *exsA*-CAT fusion	This study
pT7-5CB-CAT	pT7-5 containing CB-CAT	This study
pETBam2	T7, gene 10 epitope tag, 10-histidine tag expression derivative of pET expression vectors	Novagen (4)
pETBamExsC	pETBam2 containing *exsC* in frame with the gene 10 and histidine tags	This study

dialysate of Trypticase soy broth supplemented with 10 mM nitrilotriacetic acid (NTA; Sigma Chemical Co., St. Louis, Mo.), 1% glycerol, and 100 mM monosodium glutamate at 32°C (12, 34).

Construction of recombinant plasmids. (i) T7 expression. The source of DNA for T7 constructs was a subclone of the *trans*-regulatory locus, pUC18CBA (Fig. 1A). Clones pT7-6CBA and pT7-5ABC (reverse orientation) were constructed from the ligation of the 2,854-bp *Hin*dIII-*Eco*RI fragment of pUC18CBA into the *Hin*dIII-*Eco*RI sites of pT7-6 and pT7-5, respectively. To construct pT7-5CB and pT7-6BC (reverse orientation), a 1,555-bp *Hin*dIII-*Xho*I fragment of pUC18CBA was treated with the Klenow fragment of DNA polymerase I and ligated into the *Sma*I site of pT7-5 or pT7-6. A clone containing only the *exsB* open reading frame (pT7-5B) was constructed by ligating a 560-bp *Stu*I fragment from pDF120 (11) into pT7-5 cleaved with *Sma*I. To determine the start site for ExsC translation, a 907-bp *Hin*cII fragment from pDF120 was cloned into the *Sma*I site of pT7-5. Additionally, a 1,194-bp *Hin*cII fragment extending from the center of *exsB* to near the 3′ end of *exsA* was cloned into the *Sma*I site of pT7-5 to yield pT7-5H1.2R. All constructs (Fig. 1A) were confirmed by restriction endonuclease cleavage and subsequently transformed into *E. coli* K38 harboring the temperature-inducible T7 RNA polymerase gene on the pGP1-2 plasmid (17, 41).

(ii) Complementation. The *trans*-regulatory locus subclones (Fig. 1B) were constructed in pUC18 and transferred as *Eco*RI fragments into the low-copy-number, broad-host-range cosmid pLAFR (14). All constructs contained *exsA* and were under the transcriptional control of pC (Fig. 1B). To construct pLAFRC-STOPBA, an Ω insertion in the *Eco*NI site of *exsC* was removed by digestion with *Hin*dIII, leaving stop codons in all forward open reading frames. *exsB* was deleted by removing a 560-bp *Stu*I fragment from pUC18CBA and subcloned as an *Eco*RI fragment in pLAFR, resulting in pLAFRCA. *exsC* and *exsB* were deleted by digestion with *Eco*NI and *Bgl*II (pLAFRA) or *Eco*NI and *Stu*I (pLAFRAΔNI-StuI). Each construct was transferred into *P. aeruginosa* PAO1*exsC*::Ω by conjugation.

(iii) Site-specific mutagenesis. Translational start sites were changed to either ACG or ATC by using the Sculptor mutagenesis system (Amersham, Arlington Heights, Ill.).

(iv) Translational fusions. Translational fusions using a chloramphenicol acetyltransferase gene (*cat*) reporter were constructed by inserting PCR-amplified *cat* flanked (lacking the start codon) with appropriate restriction sites into the *Bgl*II and *Xho*I sites of *exsB* and *exsA*, respectively. CAT fusions were made in the complementation constructs either in pUCP18 for expression in *P. aeruginosa* (38) or in pT7-5 and -6 for expression in *E. coli.*

T7 expression studies. The expression procedure was essentially that of Tabor and Richardson (41). *E. coli* K38 containing constructs under the control of the T7 promoter and the T7 RNA polymerase-containing plasmid pGP1-2 were induced by incubation at 42°C. Host cell RNA synthesis was inhibited by the addition of 200 μg of rifampin per ml, and proteins were metabolically labeled by incorporation of [^{35}S]methionine (Tran^{35}S-Label; 1,017 Ci/mmol; ICN Pharmaceuticals, Inc., Costa Mesa, Calif.). After labeling, cells were suspended in loading buffer and boiled, and an aliquot was analyzed by sodium dodecyl sulfate

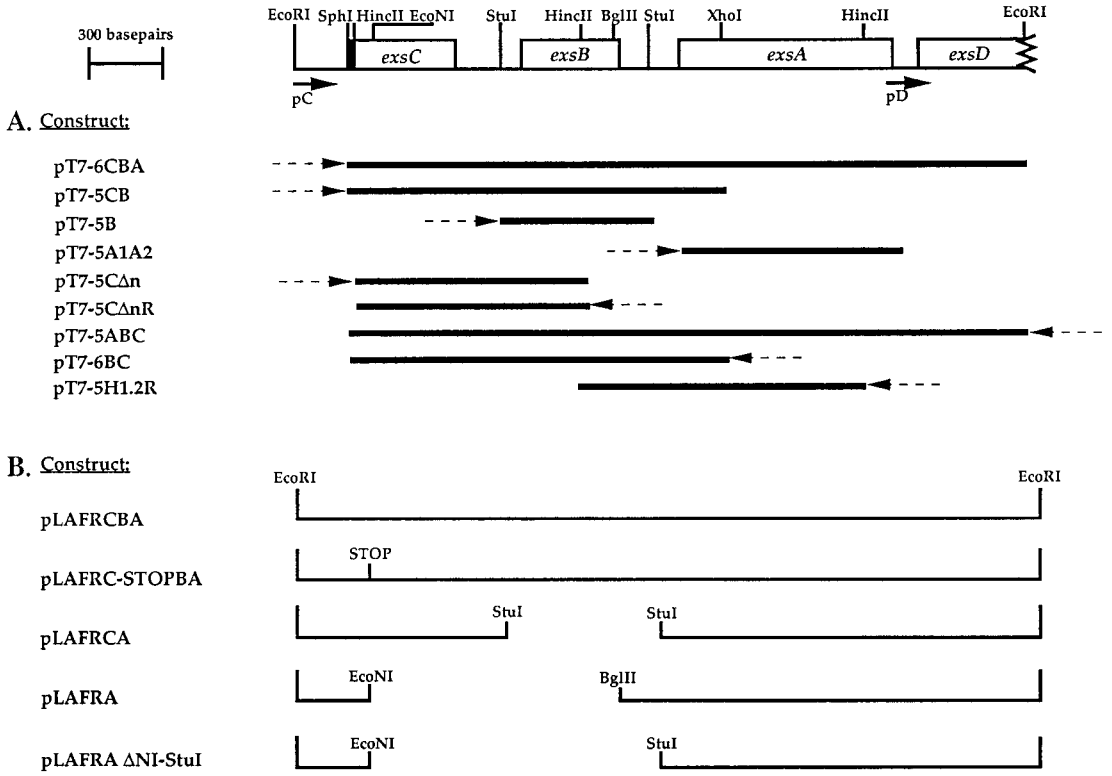

FIG. 1. (A) Map of the *trans*-regulatory locus clones constructed with the pT7-5/-6 expression system. The *trans*-regulatory locus for exoenzyme S synthesis is a 3,055-bp segment of *P. aeruginosa* 388 chromosomal DNA containing the complete *exsC*, *-B*, and *-A* genes and a truncated *exsD* gene. Complete open reading frames are represented by open boxes, and the incomplete open reading frame is indicated by an open box followed by a jagged line. Restriction endonuclease cleavage sites are labeled. Fragments of DNA cloned into T7 expression vectors are shown below the map, with arrows representing orientation with respect to the T7 promoter (dashed arrow). (B) Map of the *trans*-regulatory locus constructs in pLAFR for expression in *Pseudomonas*. Restriction endonuclease cleavage sites for the generation of deletions are labeled. pLAFRC-STOPBA contains translational stops of Ω cloned into the *Eco*NI site.

(SDS)-polyacrylamide gel electrophoresis (PAGE) (11% gel) (26). Following electrophoresis, gels were fixed, dried, and subjected to autoradiography.

Measurement of exoenzyme S ADP-ribosyltransferase activity. Exoenzyme S ADP-ribosyltransferase activity was measured in supernatant and cell lysate samples from *P. aeruginosa* strains as the incorporation of radiolabel from [adenylate phosphate-^{32}P]NAD (New England Nuclear Research Products, Wilmington, Del.) into soybean trypsin inhibitor (SBTI) as previously described (6–8, 24, 25). Briefly, each reaction mixture contained, in a final volume of 40 μl, 0.2 M sodium acetate (pH 6.0), 100 μM SBTI, 250 μM [adenylate phosphate-^{32}P]NAD (specific activity, 0.4 Ci/mmol), wheat germ extract as a source of FAS, and a source of exoenzyme S. Reaction mixtures were incubated at 25°C for 30 min, and reactions was stopped by the addition of SDS-PAGE loading buffer. Radiolabeled SBTI was quantitated by SDS-PAGE and scintillation counting of isolated bands. Enzyme activities were reported as femtomoles of ADP-ribosylated SBTI per minute per microliter of sample. Each sample was titrated such that reported enzyme activity fell within the linear range of the assay. *P. aeruginosa* lysates were prepared as previously described (12).

Exoenzyme S and CAT antigen analysis. The production of exoenzyme S antigen was analyzed by Western blot analysis using antisera that recognize the 53- and 49-kDa proteins (12, 13, 35). Cell lysate samples of *P. aeruginosa* and *E. coli* BL21 strains containing CAT translational fusions were subjected to SDS-PAGE (10% gel) (26) and Western blot analysis using anti-CAT immunoglobulin G (5 Prime-3 Prime, Inc., Boulder, Colo.). Bound immunoglobulin G was detected with ^{125}I-protein A and autoradiography. Radioanalytical analysis was performed on Western blots to quantitate the amount of exoenzyme S antigen (Ambis, San Diego, Calif.).

CAT assays. Translational fusion strains were grown under inducing and noninducing conditions for exoenzyme S synthesis and assayed for CAT as previously described (34, 46, 47). Reaction mixtures (250 μl) contained *P. aeruginosa* lysate as a source of CAT, 1 mM chloramphenicol, 10 μl of [*butyryl*-1-^{14}C] butyryl coenzyme A (4.0 mCi/mmol; New England Nuclear), 100 mM Tris-HCl (pH 7.8), and 5 ml of Econoflour II (New England Nuclear). Units of CAT were determined from a standard curve generated with purified CAT (Boehringer Mannheim Corporation, Indianapolis, Ind.). Reported CAT activity is normalized to cell number.

RNA isolation and RNase protection analyses. Total RNA was extracted from *P. aeruginosa* strains grown to a final optical density at 540 nm of approximately 1.0 (44, 46). For RNase protection analysis, riboprobes were synthesized and hybridized to total RNA as described previously (30, 31). The riboprobes were transcribed from linearized DNA templates derived from T7 expression constructs as shown in Fig. 4A. Hybrids were treated with RNase T_1 (10 U/ml; Calbiochem, San Diego, Calif.) and RNase A (5 μg/ml; Boerhinger Mannheim) for 60 min at 23°C, phenol-chloroform (1:1) extracted, and precipitated with ethanol in the presence of carrier tRNA. Samples were resuspended in loading buffer, heated to 85°C for 5 min, and loaded onto 4% polyacrylamide–8 M urea sequencing gels (28, 30).

RNA-ExsC cross-linking studies. The open reading frame of ExsC was fused in frame to an amino-terminal gene 10 epitope tag–10-histidine tag in a derivative of a pET vector (4). The fusion protein was expressed and isolated by nickel affinity chromatography. Purified, tagged ExsC (0.5 μg) was incubated with in vitro-transcribed, ^{32}P-labeled (10^5 cpm) RNA corresponding to *exsC*, *exsB*, *exsA*, and *exoS* as test RNAs. *cat* antisense RNA was used as a negative control. As a positive control, the eukaryotic splicing factor ASFΔRS and its corresponding target RNA (the negative regulator of splicing portion of the Rous sarcoma virus genome) were cross-linked in the same experiments (29). Labeled RNA and protein were incubated for 20 min at 25°C in 25 mM Tris-HCl–40 mM KCl–0.08 mM EDTA–10% glycerol in 25-μl reaction volumes. Reaction mixtures were irradiated with UV light (254 nm) at a distance of 4.5 cm for 15 min at 4°C. Samples were incubated at 37°C for 15 min with RNase A (1 mg/ml) and were analyzed by SDS-PAGE. After staining with Coomassie blue to localize size standards, the gels were dried and autoradiography was performed.

RESULTS

Expression of the *trans*-regulatory locus proteins in *E. coli*. To map the expressed products of the *P. aeruginosa trans*-regulatory locus, various clones were constructed in pT7 vectors (Fig. 1A). When the entire locus was under the control of T7 RNA polymerase, three products with apparent molecular masses of 33.8, 19.3, and 14.3 kDa were labeled (Fig. 2A, lane 2). Subcloning and expressing each open reading frame (Fig.

TABLE 2. ADP-ribosyltransferase activities of complemented strains

Strain	fmol of ADP-ribosylated SBTI/min/μl ± SD[a]	
	+NTA[b]	−NTA[c]
PAO1/pLAFR	26.3 ± 6.3	0.3 ± 0.3
PAO1*exsC*::Ω/pLAFR	0.4 ± 0.7	0
PAO1*exsC*::Ω/pLAFRCBA	135.0 ± 28.5	0.6 ± 0.4
PAO1*exsC*::Ω/pLAFR C-STOPBA	59.6 ± 16.3	1.3 ± 1.2
PAO1*exsC*::Ω/pLAFRCA	0.6 ± 0.6	0
PAO1*exsC*::Ω/pLAFRA	16.5 ± 11.1	0.2 ± 0.3
PAO1*exsC*::Ω/pLAFRCBA*exsB**	177.0 ± 60.9	2.5 ± 1.2
PAO1*exsC*::Ω/pLAFRCBA*exsB′**	217.7 ± 37.8	2.2 ± 3.2

[a] Standard deviations are indicated for experiments performed in triplicate. Zeros represent reactions containing levels of radioactivity equal to or below the levels detected for control reactions not containing exoenzyme S.

[b] Growth in the presence of the chelator NTA induces the production of exoenzyme S.

[c] Noninducing growth conditions for exoenzyme S production.

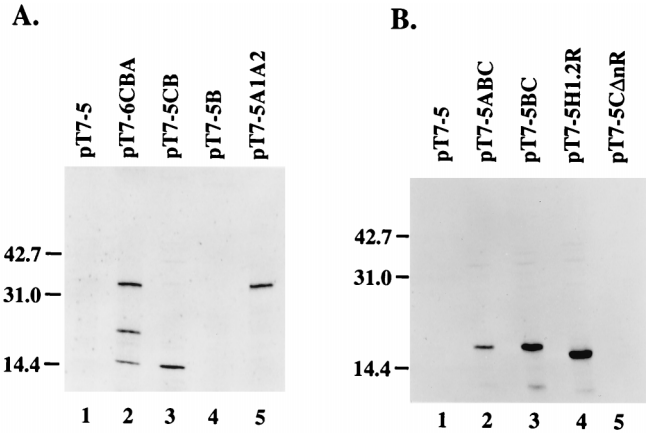

FIG. 2. Autoradiogram of ^{35}S-labeled protein products of subclones of the *trans*-regulatory locus expressed under T7 promoter control in *E. coli* K38/pGP1-2. (A) Expression of labeled proteins from sense-strand constructs. (B) T7-mediated expression of proteins encoded on the antisense strand of the *trans*-regulatory locus. Constructs subjected to the labeling procedure are indicated above the lanes. pT7-5 was used as a vector control. Nucleotide sequence analysis and translation of each expressed and nonexpressed open reading frame indicates that both cysteine and methionine amino acids are present. Sizes are indicated in kilodaltons.

1A) demonstrated that *exsC* encoded the 14.3-kDa product (Fig. 2A, lane 3) and *exsA* encoded the 33.8-kDa protein (Fig. 2A, lane 5). The 19.3-kDa protein appeared to be a truncated product of *exsD*. A second potential start site for ExsC was eliminated when a construct utilizing the secondary ribosomal binding and start site did not express a protein product (pT7-5CΔn [data not shown]). *exsB* was not expressed (Fig. 2A, lanes 3 and 4). The same subcloning and T7 expression strategy identified an 18.5-kDa product encoded by the antisense strand (Fig. 2B, lanes 2 and 3). Production of a truncated protein product from clone pT7-5H1.2R (Fig. 2B, lane 4) and no product from pT7-5CΔnR (Fig. 2B, lane 5) localized an additional open reading frame, termed *exsB′*. *exsB′* is predicted to encode a protein product of 148 amino acids with a molecular mass of 16,791 Da and a pI of 11.91. Use of the FASTA algorithm indicated that ExsB′ sequences are unique (10).

Contribution of the *trans*-regulatory locus gene products to exoenzyme S production. A complementation approach was used to determine the relative contribution of each gene product to exoenzyme S synthesis from *P. aeruginosa*. The design of this strategy was based on previous data which indicated that the *trans*-regulatory locus was transcribed as an operon from the pC promoter and that a functional *exsA* gene was required for exoenzyme S production (21, 46, 47). The wild-type locus and various versions of the locus (Fig. 1B) were constructed in the low-copy-number plasmid pLAFR (14). Constructs were transferred into strain PAO1*exsC*::Ω (13), supernatants were collected from strains grown under inducing (with NTA) or noninducing (without NTA) conditions, and exoenzyme S ADP-ribosyltransferase activity was assayed (Table 2). The wild-type PAO1 vector control strain expressed a moderate amount of extracellular exoenzyme S activity. An Ω insertion in *exsC* reduced activity levels to background. When the *trans*-regulatory locus from strain 388 was provided in *trans*, exoenzyme S enzyme activity increased approximately fivefold. This increase in exoenzyme S production could be due to the increase in copy number of the plasmid-borne locus (from two to four) relative to the single chromosomal copy, or it could be due to differences in the locus from strain 388 relative to strain PAO1. Since all complementation clones were constructed in the same vector, fold changes are reported in comparison to the fully complemented strain (PAO1*exsC*::Ω/pLAFRCBA).

Translational stop codons inserted within the *exsC* open reading frame resulted in a two- to threefold reduction in extracellular exoenzyme S activity ($P < 0.001$). These data suggest that ExsC modulates the final yield of exoenzyme S but is not absolutely required for expression. The deletion of the *exsB* region (*exsB* and part of *exsB′*) resulted in a severe reduction in exoenzyme S production. Deletion of both *exsC* and the *exsB* region seemed to restore exoenzyme S production relative to the deletion of the *exsB* region. Start site mutations were introduced into the *exsB* and *exsB′* predicted open reading frames to identify the gene responsible for changes in exoenzyme S activity. As shown in Table 2, the start site mutations in the context of wild-type ExsC and ExsA did not alter or caused a slight increase in exoenzyme S production. Assays on lysate samples showed the same pattern of activity, indicating that deletion of either the *exsB* region or *exsC* and the *exsB* region did not result in an intracellular accumulation of exoenzyme S (data not shown).

To control for possible posttranslational modifications affecting exoenzyme S activity, Western blot analyses of supernatant (Fig. 3) and lysate (data not shown) fractions were performed. Radioanalytical analysis of exoenzyme S antigen

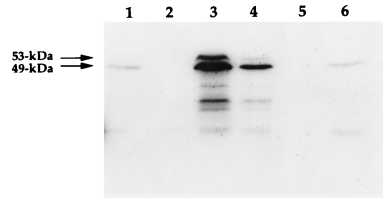

FIG. 3. Western blot analysis of exoenzyme S extracellular protein levels from the expression of complementation constructs in *P. aeruginosa*. Supernatants from the cultures grown under inducing conditions for exoenzyme S production were concentrated 10-fold. Ten-microliter samples were loaded per lane on an SDS–10% polyacrylamide gel. Exoenzyme S antigen was detected by using an antibody that recognizes both the 49- and 53-kDa forms of exoenzyme S. Lane 1, PAO1/pLAFR; lane 2, PAO1*exsC*::Ω/pLAFR; lane 3, PAO1*exsC*::Ω/pLAFRCBA; lane 4, PAO1*exsC*::Ω/pLAFRC-STOPBA; lane 5, PAO1*exsC*::Ω/pLAFRCA; lane 6, PAO1*exsC*::Ω/pLAFRA. Similar analyses were performed for cell lysate samples (data not shown), and exoenzyme S was not detectable except for low levels observed in PAO1*exsC*::Ω/pLAFRCBA extracts. Positions of the 49- and 53-kDa forms of exoenzyme S are indicated by arrows.

bands corresponded to the changes in enzyme activity when different constructs were tested. Combined, our results suggest that (i) ExsC may function to modulate the yield of exoenzyme S, (ii) the *exsB* region regulates exoenzyme S production rather than affecting exoenzyme S activity or export, (iii) introduction of start codon mutations in the predicted *exsB* or *exsB′* open reading frame did not affect exoenzyme S production, and (iv) deletion of both *exsC* and the *exsB* region has less deleterious effects on exoenzyme S production than deletion of the *exsB* region alone. We interpret this last observation as indicating that ExsC and ExsB or ExsB′ may interact in some fashion to regulate exoenzyme S production.

The complementation analysis was subject to a number of limitations. The pLAFRC-STOPBA mutation may be able to restart translation to result in a partially functional form of ExsC. This possibility appears unlikely, as a control T7 construct that removed the native ribosomal binding site and start codon (pT7-5CΔn) did not express a labeled protein in *E. coli*. Translation of the nucleotide sequence of the *exsC* region indicated that cysteine and methionine residues were present in all three frames, indicating that the failure to label a product was not due to the absence of these amino acids in potential proteins. Alternatively, the placement of the stop codons in *exsC* would allow translation of the first 25 amino acids and perhaps partial activity. We attempted to specifically delete *exsC* in a construct retaining the native promoter (pC), *exsB*, and *exsA*. Although several strategies resulted in stable *exsC* minus constructs in *E. coli*, the pLAFR derivatives appeared to rearrange or suffer deletions when transferred to *P. aeruginosa*. Lastly, the start site mutation in *exsB′* may be ineffective since a functional chromosomal copy of *exsB′* could be expressed independently of the *trans*-regulatory locus in PAO1*exsC*::Ω.

Analysis of the *trans*-regulatory locus RNA. To address the expression of *exsB′* in *P. aeruginosa* and to determine if antisense RNA (16) or RNA processing played a role in the regulation of exoenzyme S production, RNase protection assays were performed. Riboprobes were designed to examine regions of the *trans*-regulatory locus mRNA and RNA transcribed from the opposing strand of DNA (Fig. 4A). Riboprobes hybridizing to the *trans*-regulatory locus mRNA from a wild-type strain (grown with NTA) were protected (Fig. 4B, lanes 3, 7, and 9). A partially protected fragment of approximately 198 bp was observed in assays using the B′ probe (Fig. 4B, lane 5). In contrast, probes designed to hybridize to antisense RNA were not protected. Protection of probes for antisense RNA (*exsB′* mRNA) was not detectable when RNA was isolated from wild-type PAO1 grown under either inducing or noninducing conditions for exoenzyme S production (Fig. 4B, lanes 11 and 12). Additionally, probes for antisense RNA were not protected when the complementation construct pLAFRCBA was provided in *trans* (Fig. 4B, lanes 14, 15, 17, 18, 20, and 21). These results indicate that *exsB′* mRNA is not transcribed in *P. aeruginosa* under the conditions tested. We concluded that ExsB′ was an artifact of T7 expression in *E. coli* and was not a regulatory protein expressed in *P. aeruginosa*. Our analysis also suggests that antisense RNA is not detectable from the *trans*-regulatory locus. Combined with the absence of ExsB expression in *E. coli*, these data suggested that the regulatory effect of the *exsB* deletion may reside at the level of mRNA.

Effect of the *exsB* region mRNA on *exsA* and *exoS* transcripts. To identify the effect of the *exsB* region, RNA was isolated from strains complemented with various *trans*-regulatory locus constructs. RNase protection analyses were performed with a riboprobe specific for *exsA* or *exoS* (Fig. 5). Complementation with pLAFRCBA (Fig. 5, lanes 4), pLAFRC-STOPBA (Fig. 5, lanes 8), pLAFRA (Fig. 5, lanes 6), or pLAFRAΔNI-StuI (Fig. 5, lanes 7), resulted in protection of both the *exsA*- and *exoS*-specific riboprobes. A similar pattern of protected fragments that were smaller than the fully pro-

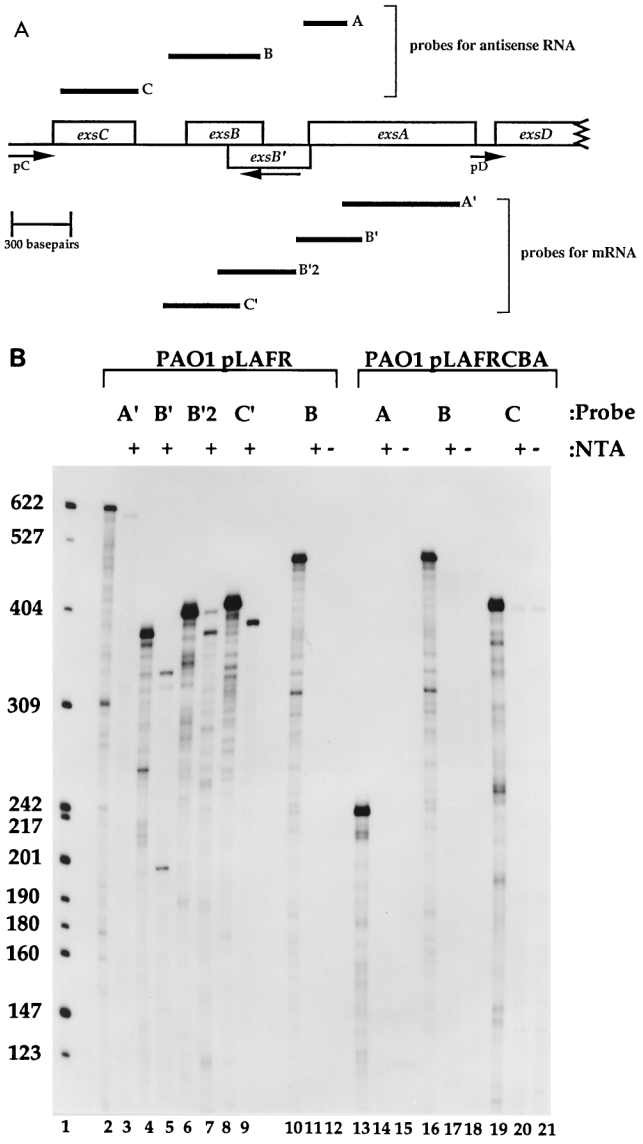

FIG. 4. RNase protection analysis of RNAs produced from the *trans*-regulatory locus in *P. aeruginosa* PAO1. (A) Map of the *trans*-regulatory locus and the locations of riboprobes relative to coding sequences. Riboprobes are labeled for the in vitro transcripts that they represent. Probes A, B, and C correspond to sense-strand RNA and hybridize to antisense RNA. Probes A′, B′, B′2, and C′ correspond to the antisense-strand RNA and hybridize to *trans*-regulatory locus mRNA. (B) Autoradiogram of protection assays using ^{32}P-labeled riboprobes depicted in panel A. Lane 1, molecular size markers reported in base pair lengths; lanes 2, 4, 6, and 8, the A′, B′, B′2, and C′ riboprobes, respectively; lanes 3, 5, 7, and 9, RNase protection reactions in which total RNA isolated from strain PAO1/pLAFR grown under inducing conditions for exoenzyme S production (with NTA) was hybridized with the indicated riboprobes; lane 10, B riboprobe; lanes 11 and 12, RNase protection reactions in which total RNA isolated from PAO1/pLAFR grown under inducing or noninducing conditions for exoenzyme S production (with or without NTA, respectively) was hybridized with probe B; lanes 13, 16, and 19, riboprobes A, B, and C, respectively; lanes 14, 15, 17, 18, 20, and 21, RNase protection reactions in which total RNA isolated from PAO1/pLAFRCBA grown under inducing or noninducing conditions for exoenzyme S production (with or without NTA) was hybridized with the indicated probes. Each riboprobe is designed to be approximately 30 nucleotides longer than the protected fragment.

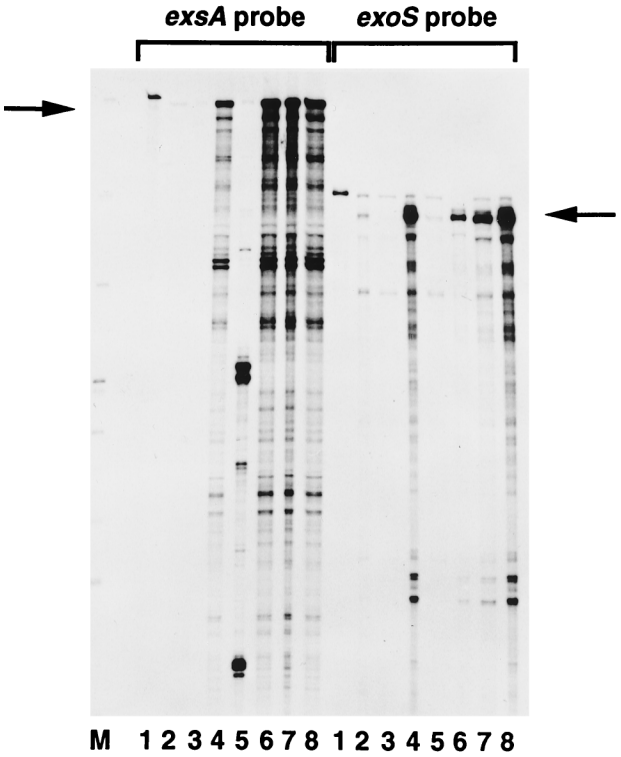

FIG. 5. RNase protection analysis of *exsA* and *exoS* transcripts from *P. aeruginosa* strains containing subclones of the *trans*-regulatory locus. The riboprobe used to detect specific mRNA is indicated above each set of lanes. RNA was isolated from strains grown under inducing conditions for exoenzyme S production. Arrows indicate the sizes of full-length protected probes. *exsA* probe: lane 1, riboprobe for *exsA*; lane 2, PAO1/pLAFR; lane 3, PAO1*exsC*::Ω/pLAFR; lane 4, PAO1*exsC*::Ω/pLAFRCBA; lane 5, PAO1*exsC*::Ω/pLAFRCA; lane 6, PAO1 *exsC*::Ω/pLAFRA; lane 7, PAO1*exsC*::Ω/pLAFRAΔNI-StuI; lane 8, PAO1*exsC*::Ω/pLAFRC-STOPBA. *exoS* probe: lane 1, riboprobe for *exoS*; lanes 2 to 8, identical RNA samples as shown for the *exsA* probe.

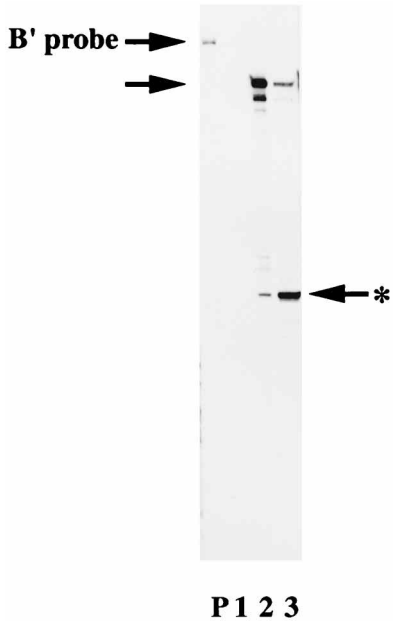

FIG. 6. RNase protection analysis of the 5′ region of *exsA* mRNA in the presence and absence of *exsB*. The B′ riboprobe (Fig. 4A and labeled arrow) was used to examine the processing of mRNA corresponding to coding and noncoding regions of the *exsA* part of the message. RNAs used in these assays were isolated from strains grown under inducing conditions for exoenzyme S production. Lane P, B′ probe; lane 1, PAO1*exsC*::Ω/pLAFR; lane 2, PAO1*exsC*::Ω/pLAFRCBA; lane 3, PAO1*exsC*::Ω/pLAFRCA. The full-length protected fragment is indicated by an unmarked arrow on the left. A smaller protected fragment is marked by an asterisk on the right.

tected probe were also observed. As these products seem to be the same in each construct, they may reflect normal degradation products. Complementation with the pLAFRCA construct had two detectable effects. Under these conditions, the *exoS* riboprobe was not protected (Fig. 5, *exoS* probe, lane 5), indicating that *exoS* was not transcribed. In addition, the *exsA* mRNA appeared to be processed as a unique pattern of smaller fragments was observed in this reaction (Fig. 5, *exsA* probe, lane 5). These results suggested that the *exsB* region affected *exsA* mRNA stability. Alternatively, in the absence of *exsA* translation, the *exsA* mRNA may be unstable, resulting in the detection of processed products and the absence of *exoS* transcript.

To determine if processing of *exsA* mRNA occurred 5′ of the A′ riboprobe, RNAs were isolated from PAO1*exsC*::Ω bearing pLAFR, pLAFRCBA, and pLAFRCA and subjected to RNase protection with the B′ probe (Fig. 4A and 6). Protection was not observed in the negative control reaction (Fig. 6, lane 1). RNA from PAO1*exsC*::Ω/pLAFRCBA showed primarily full-length protection of the B′ probe, with two prominent smaller products (Fig. 6, lane 2). The strain containing pLAFRCA showed a pattern of protection similar to that of the fully complemented strain except that the full-length product appeared reduced and a smaller fragment was more prominent (Fig. 6, lane 3). These results suggest that the *exsA* mRNA is susceptible to either degradation or processing events that appear to increase when the *exsB* region is deleted from the operon message.

Analysis of ExsA and ExsB production in *Pseudomonas*. A start site mutation suggested that ExsB was not involved in exoenzyme S production. The possibility has been raised, however, that the predicted ATG start site of *exsB* (12) may be incorrect and that a GTG located 12 bp upstream may serve as the start site for ExsB translation (1). To determine if ExsB was translated in *P. aeruginosa* and to verify that the *exsB* RNA was necessary for ExsA expression, translational fusions were constructed and transformed into a wild-type strain of *P. aeruginosa*. A vector control (pUCP18), the *exsB*-CAT fusion (pUCP18CB-CAT), and an *exsB* deletion construct (pUCP18CA-CAT) showed no detectable levels of CAT activity. A full-length construct with CAT fused in frame with *exsA* demonstrated high levels of CAT activity (4.86 U per 10^8 cells). RNase protection analyses using a riboprobe specific for CAT

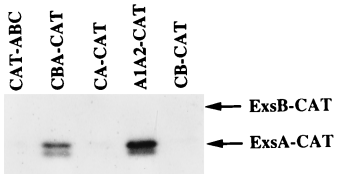

FIG. 7. Western blot analysis of T7 controlled CAT translational fusions of ExsA and ExsB in *E. coli* BL21(DE3). The expression constructs tested in this experiment are indicated above the lanes. CAT-ABC is a negative control construct in which the ExsA-CAT fusion is in the opposite orientation relative to the T7 promoter. CAT antigen was detected with an anti-CAT immunoglobulin G followed by incubation with ^{125}I-labeled protein A. The predicted positions of the ExsA-CAT and ExsB-CAT fusion derivatives are indicated by a labeled arrow.

mRNA demonstrated that full-length CAT transcript was detectable from all of the CAT fusion constructs but not from the vector control strain (data not shown). Thus, CAT was transcribed in pUCP18CA-CAT but not translated in the absence of the *exsB* region. These data suggest that *exsB* is required for translation of ExsA and that in the absence of ExsA translation, the *exsA* mRNA may be unstable due to degradation or processing.

Determination of the nature of the *exsB* element. If the element within the *exsB* region were an RNA structure, we reasoned that the regulatory effect on ExsA translation in *P. aeruginosa* could be reproduced in another host with different transcriptional signals. CAT fusion constructs were generated in T7 vectors for expression in *E. coli*. Expression of CAT antigen was analyzed by Western blot analysis using an anti-CAT antibody (Fig. 7). A full-length construct with CAT fused to ExsA, in the opposite orientation relative to the T7 promoter, did not produce CAT (Fig. 7, lane CAT-ABC). The same construct in the correct orientation relative to the T7 promoter produced a CAT fusion protein (Fig. 7, lane CBA-CAT). Deletion of the *exsB* region (Fig. 7, lane CA-CAT) inhibited ExsA-CAT expression. In the absence of both *exsC* and *exsB* (Fig. 7, lane A1A2-CAT), ExsA-CAT expression was restored. An in-frame fusion with *exsB* did not produce CAT protein in *E. coli* (Fig. 7, lane CB-CAT). Thus, in the absence of *exsB* in *E. coli*, ExsA was not translated. When both *exsC* and *exsB* were deleted, however, ExsA could be translated as a CAT fusion. These results suggest that ExsA expression is affected by an interaction between ExsC and *exsB* and mirror the effect of an *exsC* and *exsB* deletion in the complementation analyses. In the absence of *exsC* and *exsB*, ExsA was translated, as demonstrated by the abundance of *exoS* mRNA (Fig. 5, lanes 7 and 8). Exoenzyme S protein and activity levels were found to be low in this situation, suggesting that ExsC may play a role in ExoS stability or translation.

DISCUSSION

The production of exoenzyme S by *P. aeruginosa* is regulated at several levels. Exoenzyme S synthesis and secretion are induced by growth in a low-cationic environment, which is maintained in vitro by the addition of chelators to the medium (12). Promoter fusion and transcriptional start site analyses have demonstrated that growth in low-cationic medium induces transcription of several operons required for exoenzyme S production (46). Coordinate activation of the exoenzyme S regulon is mediated by the transcriptional activator, ExsA, which binds to a specific sequence in each promoter identified to date (21, 47). ExsA is required to initiate its own transcription as part of a regulatory operon (the *trans*-regulatory locus) that also contains *exsC* and *exsB* (21, 46). Data presented in this study suggest that posttranscriptional checkpoints play a significant role in exoenzyme S production. ExsC-*exsC* and an RNA element located in a region originally defined as *exsB* affect the expression of ExsA, which in turn regulates the rest of the pathway at the level of transcription. Preliminary evidence that implicates ExsC as a mediator of ExoS translation or stability is presented. The tight regulation of exoenzyme S production at both transcriptional and posttranscriptional checkpoints may ensure that expression occurs only under the appropriate conditions, which in respect to pathogenesis may be defined as the environment of damaged host tissue. It will be important to determine the in vivo induction signal to understand the roles that exoenzyme S and other coordinately regulated products play in *P. aeruginosa* infections.

The goal of these studies was to clarify the function of ExsC and ExsB in exoenzyme S production. Based on codon usage and the presence of a functional ribosome binding site at an appropriate distance from the start codon, *exsB* was predicted to encode a protein with a molecular mass of 15,026 Da (12). Allaoui et al. reported that the sequence of a *Yersinia enterocolitica* lipoprotein, VirG, was homologous to the predicted ExsB sequence (26.2% identity) (1). VirG appears to play a role in the secretion of a subset of the Yops virulence determinants (1). Although these data would implicate a similar role for ExsB in *P. aeruginosa*, a start site mutation failed to affect exoenzyme S synthesis or secretion. In addition, ExsB expression was not detectable in *P. aeruginosa* or *E. coli* hosts as a CAT fusion protein or from *E. coli* T7 expression constructs. We concluded that *exsB* did not encode a protein and hypothesized that the *exsB* RNA may mediate some sort of regulatory effect. RNase protection analysis indicated that antisense RNA was not a factor. By analyzing complementation constructs in a host in which *exsC*, *exsB*, and *exsA* were inactivated by a polar mutation, the absence of the *exsB* region appeared to enhance the processing of *exsA* mRNA. The processing of mRNA appeared more severe with a probe located toward the end of the message than with a probe that overlapped the translational start signals. In the absence of *exsB* and when *cat* was fused in frame with *exsA*, processing of the *cat* message was not observed in *P. aeruginosa*, indicating that *exsA* mRNA was less stable than *cat* mRNA. Although the *cat* message appeared stable, CAT expression was not observed in *P. aeruginosa*, suggesting that ExsA translation may not occur in the absence of *exsB*. If ExsA is not translated, the mRNA may be susceptible to processing or degradation. Alternatively, processing or degradation of the *exsA* part of the message may prevent translation. The dynamic processes involved in transcription and translation, as well as mRNA and ExoS degradation or processing, favor expression under inducing conditions for ExoS production and apparently serve to efficiently shut off expression in the absence of induction. This model predicts that other coordinately regulated members of the exoenzyme S regulon may inhibit protein or mRNA processing or enhance translational efficiency during induction. One likely candidate for these functions may be ExsC.

Sequences within the *exsB* region may be required for *exsA* mRNA stability, or they may be required for the message to assume a structure that is conducive to *exsA* translation. Since the *trans*-regulatory locus is transcribed as an operon (13, 46), the retention of the noncoding *exsB* region seems to be focused on coordinating the expression of ExsC and ExsA. In one model, ExsC and ExsA translation may be linked by RNA structure within *exsB*. This model would predict that ExsC plays a regulatory role and/or acts in concert with ExsA. In a second model, *exsB* may ensure that transcription starts at pC for maximal exoenzyme S production. Transcription starting 5′ of *exsA* may be poorly translated or processed in the absence of ExsC and *exsB*, which would effectively reduce the concentration of ExsA and keep exoenzyme S levels low. Vestiges of a promoter region containing sequences resembling an ExsA binding site are found in the region between *exsB* and *exsA*. These sequences no longer constitute an active promoter region as measured in promoter fusion (46), transcriptional start site (46), or ExsA-mediated DNA binding (21) assays. By requiring transcriptional initiation at pC for ExsA expression, the *exsB* region may again serve to coordinate the expression of ExsC and ExsA.

The function of ExsC remains somewhat elusive. The deletion of *exsC* in addition to *exsB* appears to reverse the negative effect on *exsA* transcript processing or ExsA translation seen with the deletion of only the *exsB* region. These results suggest

that an interaction between ExsC or *exsC* and *exsB* may modulate ExsA translation or transcript processing. Although ExsA translation and *exoS* transcription occurred in the absence of both *exsC* and *exsB*, translation of ExoS was reduced, as detected by both activity and antigen levels. Additionally, stop codons inserted within *exsC* consistently resulted in reduced levels of ExoS without affecting *exsA* or *exoS* mRNA. Thus, ExsC or *exsC* seems to affect the *exsB* element, thereby modulating ExsA expression as well as affecting the translation of ExoS. To accommodate these observations, one might predict that ExsC affects translation by binding to specific mRNAs; however, none of the tested mRNA probes cross-linked to purified ExsC (data not shown). These experiments indicate that ExsC may not directly bind to RNA to increase translational efficiency. Interpretation of these data, however, could be problematic since it may be that the purified ExsC fusion protein is not biologically active or that the experimental conditions for binding were suboptimal. Attempts at deleting only *exsC* either in complementation constructs or by allelic replacement in the *P. aeruginosa* genome have been unsuccessful (data not shown). Complementation constructs without *exsC* are stable in *E. coli* but, upon transfer to either wild-type or mutant strains of *P. aeruginosa*, rearrange regardless of the copy number of the vector. These data suggest that the combination of the *exsB* element and ExsA may be deleterious in the absence of ExsC, supporting the apparent strong selection pressure to maintain the operon organization as previously discussed.

The closest relatives of ExsA are the transcriptional regulators VirF and LcrF from yersiniae (9, 12, 27). Hoe et al. have shown that LcrF of *Yersinia pestis* is regulated at the level of translation (18, 19). At a low temperature (26°C), the Shine-Delgarno sequence is sequestered in a localized secondary structure. Growth at host temperatures (37°C) destabilizes the stem-loop structure to allow translation of LcrF, which in turn transcriptionally activates several operons associated with *Yersinia* virulence (19). ExsA expression appears to be more complex and involves *cis*-acting sequences that encompass a region larger than that postulated for LcrF. In addition, *exsC* and ExsC serve to modulate exoenzyme S production in *cis* and in *trans*. A homolog to ExsC has yet to be found in the yersiniae, supporting our findings of a novel regulatory loop in *P. aeruginosa*.

In conclusion, exoenzyme S synthesis is tightly regulated at multiple levels. Our data suggest that the regulatory operon controlling exoenzyme S expression has two coding regions, *exsC* and *exsA*, that are interrupted by a noncoding segment formerly designated *exsB*. The noncoding *exsB* region appears to have been retained to either stabilize the *exsA* part of the message or mediate ExsA translation, perhaps through the formation of an RNA structure. These functions appear to be required in the presence of ExsC or *exsC*. ExsC or *exsC* seems to serve in both *cis* and *trans* capacities. In *cis*, a processed *exsA* mRNA is observed in the presence of ExsC or *exsC* but the absence of *exsB*. Removal of both *exsC* and *exsB* from the operon relieves the processing effect on *exsA* mRNA (the *cis* effect) and allows translation of ExsA (as measured by the production of *exoS* transcript). However, the expression of ExoS is reduced, indicating that ExsC may be involved in ExoS translation or stability (the *trans* effect). The reconstitution of the *cis*-regulatory effects in *E. coli* under strong transcriptional controls indicates that accessory *P. aeruginosa* products are not involved and suggests that RNA structure may be responsible for the *cis*-acting controls on *trans*-regulatory locus expression. ExsC cannot be eliminated from having direct effects on transcript processing or translation of the *trans*-regulatory locus products, but preliminary experiments suggest that RNA binding may not be involved in the function of ExsC. One model to account for these observations is that ExsC works in *trans* as a protein product involved in ExoS expression and that the *trans*-regulatory operon message forms RNA structures (23, 32, 37) with elements of *exsC*, *exsB*, and perhaps *exsA*. Key questions that remain concern the induction of *trans*-regulatory locus transcription and the roles of other members of the exoenzyme S regulon in controlling ExoS translation or degradation. The interaction between the regulatory proteins will provide important insights for understanding each stage of bacterial signal transduction and the expression of coordinately regulated virulence determinants.

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