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Cloning and Sequence Analysis of a *trans*-Regulatory Locus Required for Exoenzyme S Synthesis in *Pseudomonas aeruginosa*

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Exoenzyme S is an ADP-ribosyltransferase enzyme distinct from exotoxin A that is synthesized and secreted by *Pseudomonas aeruginosa*. Yields of exoenzyme S are variable and depend on strain and growth conditions. Since certain medium additives are required for exoenzyme S production, its regulation may be influenced by environmental stimuli. In this study, we have cloned a region that complements the exoenzyme S-deficient phenotype of strain 388 *exsI*::Tn*1*, a chromosomal Tn*1* insertional mutation. A large clone (28 kb) was shown to restore both synthesis and secretory functions to the mutant strain. Subcloning and Tn*501* mutagenesis experiments localized the region required for exoenzyme S synthesis to a 3.2-kb fragment. Nucleotide sequence analysis demonstrated several open reading frames. Comparison of the N-terminal amino acid sequence of purified exoenzyme S with predicted amino acid sequences of all open reading frames indicated that the structural gene was not encoded within the sequenced region. Homology studies suggested that the region encoded three regulatory genes, *exsC*, *exsB*, and *exsA*. ExsA was homologous to the AraC family of transcriptional activator proteins, with extensive homology being found with one member of this family, VirF of *Yersinia enterocolitica*. VirF and ExsA both contain carboxy-terminal domains with the helix-turn-helix motif of DNA-binding proteins. The ExsA gene product appeared to be required for induction of exoenzyme S synthesis above a low basal level. Expression of ExsA was demonstrated by cloning the region under the control of the T7 promoter. Gene replacement experiments suggested that the expression of ExsC affects the final yield of exoenzyme S.

Pseudomonas aeruginosa is an opportunistic pathogen capable of producing a wide array of extracellular proteins important to pathogenesis (20, 34). One extracellular product, exoenzyme S, appears to be a major virulence determinant involved in the dissemination of *P. aeruginosa* from the initial site of colonization in the skin to the bloodstream of burned and infected animals (17, 19). The ability to disseminate correlates to an increased incidence of fatal sepsis in these animals. Passive administration of antiserum to exoenzyme S protects animals from sepsis but has no significant effect on the numbers of organisms recovered from the site of colonization (17). An exoenzyme S-deficient phenotype also correlates to a reduction in the amount and severity of tissue damage associated with *P. aeruginosa* chronic lung infections (35, 36).

Exoenzyme S is an ADP-ribosyltransferase enzyme that is secreted from *P. aeruginosa* cells in at least two forms (18). The highest-molecular-mass form is 53 kDa and is enzymatically inactive. A lower-molecular-mass form of 49 kDa is enzymatically active and functions by transferring ADP from NAD to a variety of eukaryotic target proteins (13). Preferred target proteins are modified at arginine residues and include monomeric vimentin (3) and small GTP-binding proteins, including $p21^{c\text{-}H\text{-}ras}$ (4). The significance of the eukaryotic targets identified in vitro, and the pathology associated with infection by exoenzyme S-producing strains has not been defined. The molecular analysis of exoenzyme S has begun with the cloning of the structural gene from *P. aeruginosa* DG1 (24). The protein product synthesized from cloned DNA in expression plasmids is enzymatically inactive and migrates at a higher molecular mass (68 kDa) than do the products excreted by *P. aeruginosa* (24). Sequence comparison between exoenzyme S and other members of the ADP-ribosyltransferase family of proteins and further analysis of the gene will lead to a better understanding of the structure and function of this enzyme.

Exoenzyme S production in *P. aeruginosa* is not constitutive and can be altered by growing the bacteria under different conditions. Yields are enhanced by the addition of a chelator such as nitrilotriacetic acid or EDTA, glycerol as a carbon source, and monosodium glutamate to dialyzed Trypticase soy broth (27). Inclusion of subinhibitory concentrations of the antibiotics ciprofloxacin, tobramycin, and ceftazidime in growth medium results in reduced exoenzyme S yields (10). The addition of iron to exoenzyme S medium has no effect on yield of this protein, suggesting that the regulation of exoenzyme S production is not related to other iron-repressed *Pseudomonas* products such as exotoxin A, elastase, or the proteins involved in iron uptake (12a). The increase in exoenzyme S yields in the presence of a chelator appears to be partly but not completely related to the inhibition of protease production and reduced breakdown of the protein (27). These observations suggest that environmental stimuli may also play a role in modulating net exoenzyme S production. In this report, we describe the cloning, sequence analysis, and expression of part of the regulatory pathway involved in exoenzyme S synthesis in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* strains were cultivated in LB broth or on LB agar at 37°C with antibiotics or selective salts in the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference(s)
Strains		
P. aeruginosa		
388	Hyperproducer of exoenzyme S	1, 13
388 *exsI*::Tn*1*	Tn*1* chromosomal insertion, ExoS$^-$	18
PAO1	Prototypical strain of *P. aeruginosa*	12
PAOS1	Tn*501* chromosomal insertion with reduced exoenzyme S expression	This study
PAOS21	Tn*501* chromosomal insertion with reduced exoenzyme S expression	This study
E. coli		
HB101	*supE44 hsdS20* $r_B^-m_B^-$ *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	2
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	37
Plasmids		
pLAFR	Broad-host-range cosmid cloning vector, Tc^r *mob*$^+$	9
pDF100	pLAFR containing a 28-kb insert of 388 chromosomal DNA	This study
pDF102	Subclone of pDF100 containing an 8.2-kb *Eco*RI fragment in pLAFR	This study
RSF1010::Tn*501*/λ*c*I857	IncQ Sm^r Hg^r/λ phage for Tn*501* mutagenesis of cloned DNA	21
pDF102::Tn*501*S1	Tn*501* insertion in pDF102 used for gene replacement	This study
pDF102::Tn*501*S21	Tn*501* insertion in pDF102 used for gene replacement	This study
pDF114	Subclone of pDF102::Tn*501*S1 containing a 3.2-kb *Eco*RI fragment in pLAFR	This study
pGp1-2, pT7-5	Cloned T7 RNA polymerase gene and expression vector	26
pT7-5A1A2	ExsA under the control of the T7 promoter	This study

following concentrations (in micrograms per milliliter) as required: ampicillin, 100; kanamycin, 50; tetracycline, 25; and $HgCl_2$, 10. *P. aeruginosa* strains were grown on minimal medium (29) plates with tetracycline (100 μg/ml) or $HgCl_2$ (15 μg/ml) added for selection of plasmids. For maximal production of exoenzyme S, *P. aeruginosa* 388 or derivatives of 388 were grown in a defined medium at 32°C as previously described (18). PAO1 strains were grown in a deferrated dialysate of Trypticase soy broth supplemented with 10 mM nitrilotriacetic acid (Sigma Chemical Co.), 1% glycerol, and 100 mM monosodium glutamate at 32°C (18). For complementation, clones constructed in pLAFR were transferred to *P. aeruginosa* by conjugation using pRK2013 as described elsewhere (8).

Immunological detection of exoenzyme S. Colony blot analysis on lysed cells was performed as described by Helfman et al. (11). Cultures for exoenzyme S analysis were harvested at optical density at 540 nm of 4.0. Supernatant material used as antigen in Western immunoblots was concentrated 20-fold by a 40% ammonium sulfate precipitation. Equal volumes (5 μl) were loaded per lane for each sample. Cell-associated exoenzyme S was examined by harvesting 2×10^9 cells in microfuge tubes, washing the cells twice in 1 ml of cold 50 mM Tris-HCl, pH 7.2, and resuspending the cell pellet in 300 μl of sample buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (14); 5 μl of lysate material was loaded per lane. Transfer of proteins separated by SDS-PAGE to nitrocellulose was accomplished by the method of Towbin et al. (28). For detection of exoenzyme S in colony and Western blots, specific rabbit antiserum to the 49- and 53-kDa forms of the protein was used as previously described (18). Staphylococcal protein A labeled with ^{125}I was used to detect binding of the primary antibody in Western blots. Affinity-purified, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim Biochemicals) and 4-chloro-1-naphthol (Sigma) were used to detect antibody binding in colony blots (18).

Cloning procedures and mutagenesis with Tn*501*. Subcloning of DNA, plasmid purification, restriction mapping, and Southern blot procedures were performed as described by Maniatis et al. (16). Transposon mutagenesis was accomplished by using methods developed by Ohman et al. (21) and White et al. (33). Briefly, HB101(pDF102) was transformed with RSF1010::Tn*501*. Transformants were selected for both tetracycline and mercury resistance. Because the vector, pLAFR, contains the lambda *cos* site, in vitro packaging of large DNA fragments is possible. A lambda *c*I857 lysate (21) was prepared from transformants containing both plasmids and used to transduce recombinant plasmids into HB101. Transductants containing pDF102 with a Tn*501* insertion were selected on medium containing both tetracycline and mercury salts. The approximate location of the insertion was mapped by using restriction endonuclease cleavage analysis. Plasmids were conjugated to 388 *exsI*::Tn*1* to determine the effect on exoenzyme S production by colony blot analysis.

Gene replacement. Results obtained during initial screening of the cosmid bank in 388 *exsI*::Tn*1* suggested that pLAFR plasmids containing cloned DNA regions which complemented the mutation for exoenzyme S production were unstable. If the vector, pLAFR, or the insert DNA was contributing to the loss of the plasmid, we reasoned that growth in the absence of antibiotic selection may hasten this process and perhaps lead to chromosomal insertion of cloned DNA. Our strategy was to select for the insertion of Tn*501*-containing DNA segments and the resolution of plasmid DNA by differential sensitivity to tetracycline (pLAFR) and mercury (Tn*501*). Tn*501* insertions that expressed (pDF102::Tn*501*S1) and did not express (pDF102::Tn*501*S21) exoenzyme S antigen were conjugated to *P. aeruginosa* PAO1. These strains were grown in LB medium and sequentially transferred six times without antibiotic selection. The cultures were plated, and single colonies were picked onto minimal medium with tetracycline or mercury salts. Colonies that exhibited a tetracycline-sensitive, mercury-resistant phenotype were selected, and chromosomal DNA was isolated for Southern blot analysis. A 1.6-kb *Bgl*II fragment from pLAFR (*cos* site) and an 1,198-bp *Sal*I fragment located within the smallest complementing clone were used as probes to compare the patterns of *Sal*I-digested chromosomal and plasmid sequences. DNA fragments were

labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear Research Products), using the random-primed DNA labeling kit (United States Biochemical Corp.).

DNA sequence analysis. Subclones of the *trans*-regulatory region were prepared in M13mp18, M13mp19, and pUC18. DNA sequence was determined for both strands of each clone, using the chain termination techniques developed by Sanger et al. (23) and [^{35}S]dATP (1,000 Ci/mmol; New England Nuclear). The entire region (3,055 bp) was sequenced once with the Sequenase system and once with Sequenase and 7-deaza-dGTP reagents as recommended by the manufacturer (United States Biochemical). The insertion point of Tn*501* in pDF102::Tn*501*S21 was derived by using primers and double-stranded DNA sequencing techniques. Tn*501* from the S1 insertion donated the *Eco*RI site used for subcloning. Oligonucleotides used as primers for sequence analysis and the polymerase chain reaction were produced at the Core Nucleic Acid Laboratory of the University of Rochester and the Protein/Nucleic Acid Shared Facility of the Medical College of Wisconsin.

Expression of ExsA. The polymerase chain reaction was used to amplify two DNA fragments corresponding to ExsA. One fragment (A1A2, 966 bp) included the resident GAGG ribosome binding site and was cloned downstream of the *lac* promoter in the pUC18 *Sma*I site to form pUC18A1A2. This fragment was removed from pUC18 by using the multiple cloning sites *Eco*RI and *Hin*dIII and inserted into the same sites in pT7-5 to form pT7-5A1A2 for expression of ExsA. A second fragment (A3A2, 933 bp) that did not include the ribosome binding site of ExsA was amplified and was designed to have an *Eco*RI site in the 5′ region. This fragment was cloned in pKK223-3 (Pharmacia) by using the *Eco*RI and *Sma*I restriction sites to form pKK223-3A3A2. Reagents and enzymes used in amplification reactions were purchased from New England Nuclear.

Clones using the *lac* and *tac* promoters (pUC18A1A2 and pKK223-3A3A2, respectively) were transformed into *E. coli* JM109. Transformants were grown to early logarithmic phase in L broth and split into induced and uninduced aliquots. Then 1 to 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the induced cultures, and cell samples from both aliquots were removed periodically. Cells were washed and resuspended in an amount of SDS-PAGE sample buffer such that all samples contained the same number of cells. Larger-scale cultures (10 ml) were grown to early logarithmic phase, induced for 4 h, harvested, washed, and lysed in an Aminco French pressure cell (3/8-in. [ca. 1-cm]-diameter piston). Unbroken cells were removed by centrifugation at 14,000 × *g* for 15 min at 4°C. The lysate was separated into membrane (pellet) and cytoplasmic (supernatant) fractions by a 1-h centrifugation at 100,000 × *g* in a Beckman SW55 swinging-bucket rotor (4°C). Protein patterns in each sample were analyzed with Coomassie- or silver-stained SDS-polyacrylamide gels. Growth and labeling conditions for expression of ExsA from pT7-5A1A2 was performed as described by Tabor and Richardson (26).

Exoenzyme S assays. Supernatant and cell lysate samples from *P. aeruginosa* strains were assayed for ADP-ribosyltransferase activity as described previously except that 2.5 μl (instead of 5 μl) of [U-^{14}C]NAD (518 mCi/mmol) was used per reaction (18). Each sample was titrated so that reported enzyme activity fell within the linear range of the assay. *P. aeruginosa* cultures were grown to stationary phase (optical density at 540 nm of 5.0 to 6.0); 2 × 10^{10} cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C and washed twice in 10 ml of a 50 mM Tris-HCl buffer, pH 7.2. The cell pellet was resuspended in 2 ml of cold 50 mM Tris-HCl–20 mM EDTA–10 mM benzamidine–1 μg of leupeptin per ml, pH 7.5, and lysed by passage through an Aminco French pressure cell twice. Unbroken cells were removed by centrifugation at 14,000 × *g* for 15 min at 4°C. Lysates were immediately assayed for exoenzyme S activity. Protein determinations were made on lysate fractions and concentrated supernatant material by the method of Lowry et al. (15). Absolute values for exoenzyme S activity varied from experiment to experiment since we were measuring material that had accumulated during an entire growth cycle.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number M64975.

RESULTS

Cloning of a genetic region involved in exoenzyme S synthesis. Previous mutagenesis experiments with Tn*1* had resulted in the isolation of a chromosomal insertion in *P. aeruginosa* 388 that eliminated exoenzyme S production (18). This strain, 388 *exs1*::Tn*1*, was negative for exoenzyme S in both cell lysate and supernatant material, as determined from Western blot analysis and ADP-ribosyltransferase activity. To clone the genes that restored exoenzyme S activity, a cosmid bank of 388 chromosomal DNA in the broad-host-range vector pLAFR was transferred en masse by conjugation to 388 *exs1*::Tn*1*. Individual *P. aeruginosa* transconjugants were picked onto a defined medium that allows maximal exoenzyme S production. Colonies were tested for exoenzyme S antigen expression by a colony blot technique using rabbit antisera to the 49-kDa form of the molecule. This antiserum was made specific by previous absorption with 388 *exs1*::Tn*1* whole cells. Several clones that expressed exoenzyme S antigen were isolated; however, we were unable to recover plasmid DNA from *P. aeruginosa*. These results suggested that the region required for restoration of exoenzyme S production might contain sequences that lead to chromosomal insertion resulting in a functional region and complementation.

Since we were unable to detect exoenzyme S after direct screening of a cosmid bank in *E. coli* HB101, a second protocol that consisted of transferring cosmid clones to strain 388 *exs1*::Tn*1* by batch mating was initiated. Each batch contained 96 individual colonies. *P. aeruginosa* transconjugants were tested en masse by the colony blot assay. The presence of a single transconjugant producing exoenzyme S indicated that the batch contained a cosmid clone expressing the genes required for exoenzyme S antigen production. Smaller batches were transferred into 388 *exs1*::Tn*1* to locate the clone responsible for complementation. This resulted in the isolation of a single clone, pDF100 (Fig. 1). When pDF100 was transferred by conjugation to 388 *exs1*::Tn*1*, both synthesis and secretion of exoenzyme S were restored (Fig. 2, lanes 3 and 8).

A map of pDF100 was constructed and showed that the plasmid contained approximately 28 kb of *Pseudomonas* DNA that could be divided into four *Eco*RI fragments of 11, 5, 8.2, and 3.8 kb. Each *Eco*RI fragment was cloned into pLAFR, transferred to 388 *exs1*::Tn*1* by conjugation, and tested in colony blots for expression of exoenzyme S antigen. One clone, pDF102, containing an 8.2-kb *Eco*RI insert, was found to complement 388 *exs1*::Tn*1*. However, this clone was deficient in the secretion of exoenzyme S antigen (Fig. 2, lane 4). Equal amounts of antigen resided in the

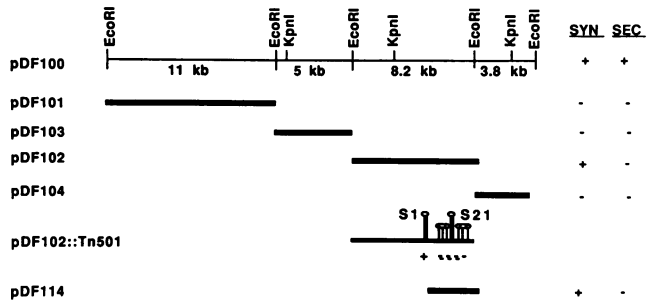


FIG. 1. Cloning strategy and complementation pattern for expression of exoenzyme S antigen in 388 *exsI*::Tn*1*. The top line represents a 28-kb cosmid clone in pLAFR that complements *P. aeruginosa* 388 *exsI*::Tn*1* for both synthesis (SYN+) and secretion (SEC+) functions. Subcloned DNA *Eco*RI fragments are depicted as thick lines that vary in size from 3.8 to 11 kb as shown. Tn*501* insertions (lollipop symbols) in pDF102 were used to localize the region required for synthesis. Exoenzyme S phenotype is shown below as + or − in terms of reactivity in colony blot analysis. The S1 and S21 insertions (bold Tn*501* symbols) were mapped by nucleotide sequence analysis and used in gene replacement experiments. An *Eco*RI site donated by the S1 Tn*501* insertion was used to construct the smallest clone (pDF114) complementing 388 *exsI*::Tn*1* for exoenzyme S synthesis.

cell-associated material when 388 *exsI*::Tn*1*(pDF102) was compared with 388 *exsI*::Tn*1*(pDF100) (Fig. 2, lane 8) or with 388 parental lysates (Fig. 2, lane 6).

To identify the smallest region required for exoenzyme S synthesis in 388 *exsI*::Tn*1*, pDF102 was subjected to mutagenesis with Tn*501*. Two predominant phenotypes were observed when pDF102::Tn*501* insertions were conjugated into 388 *exsI*::Tn*1* for analysis. One class of insertions consisted of transconjugants that expressed low amounts of exoenzyme S, and the second class produced no exoenzyme

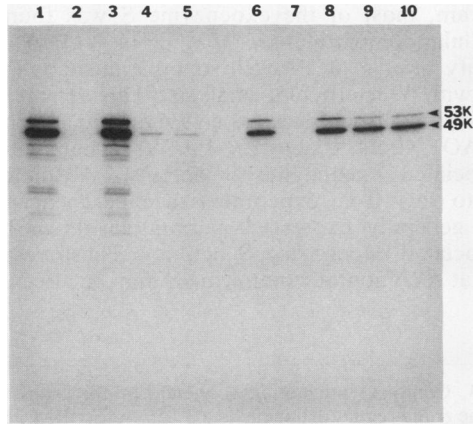


FIG. 2. Western blot analysis of clones that restore exoenzyme S production in 388 *exsI*::Tn*1*. Concentrated supernatants (lanes 1 to 5) and cell-associated material (lanes 6 to 10) from strains 388(pLAFR) (lanes 1 and 6), 388 *exsI*::Tn*1*(pLAFR) (lanes 2 and 7), 388 *exsI*::Tn*1*(pDF100) (lanes 3 and 8), 388 *exsI*::Tn*1*(pDF102) (lanes 4 and 9), 388 *exsI*::Tn*1*(pDF114) (lanes 5 and 10) were grown for exoenzyme S production in defined medium and harvested at stationary phase. Specific rabbit antisera to the 49- and 53-kDa forms of exoenzyme S was the primary antibody used to detect exoenzyme S antigen. Smaller-molecular-mass bands are breakdown products of exoenzyme S.

S in colony blot analysis. Restriction endonuclease mapping and Southern blot analysis localized several transposon insertions that eliminated exoenzyme S production to one end of pDF102. Only two transposon insertions were mapped that produced low amounts of exoenzyme S. One insertion mapped to plasmid sequences (not shown), and the other insertion, S1, mapped to a site approximately 3.2 kb upstream of one of the *Eco*RI sites (Fig. 1). Since Tn*501* contains *Eco*RI sites on each end, a 3.2-kb *Eco*RI fragment was isolated from pDF102::Tn*501*S1 and cloned into pLAFR to form pDF114. Conjugation of pDF114 into 388 *exsI*::Tn*1* resulted in the same phenotype that was exhibited by pDF102. Exoenzyme S was produced but remained cell associated (Fig. 2, lanes 5 and 10).

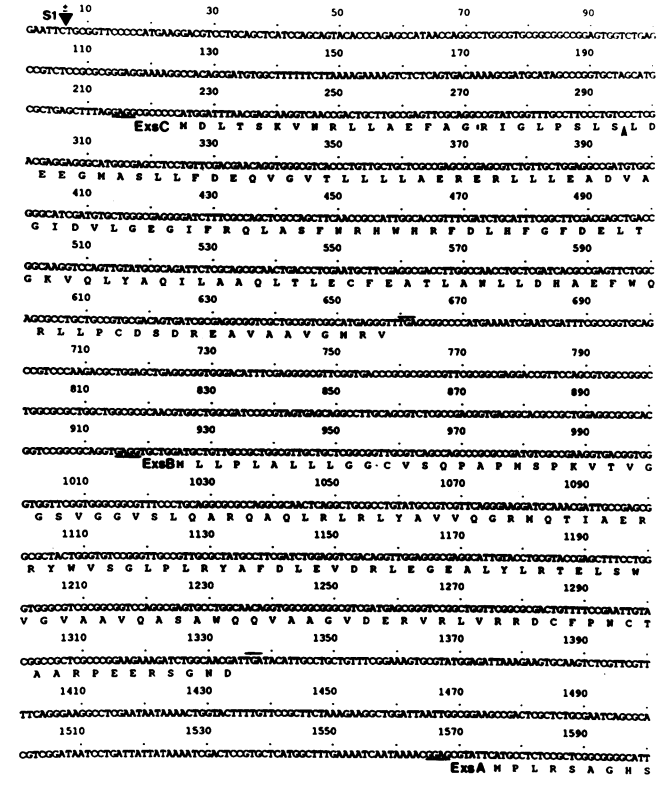
Nucleotide sequence analysis. The 3.2-kb region encoding synthesis but not secretion functions was cloned in pUC18, for which a more detailed and accurate restriction endonuclease map was constructed. Overlapping subclones were prepared and sequenced as described in Materials and Methods. Nucleotide sequences were analyzed with the Pustell programs (International Biotechnologies, Inc.) and the University of Wisconsin Genetics Computer Group software programs FRAMES, CODONPREFERENCE, and FASTA (7). Protein-encoding regions for homology studies were chosen on the basis of the presence of ribosome binding sites 7 to 14 bp upstream of the start codon and codon usage similar to that determined for other *Pseudomonas* genes (32). Homology searches using nucleotide and protein sequences failed to reveal a match between the cloned region and an amino-terminal amino acid sequence obtained for the 49-kDa form of exoenzyme S (12a). In addition, it was noted that none of the open reading frames were large enough to encode the structural gene (1.5 to 1.6 kb). We concluded that the cloned region represented regulatory sequences, functioning in *trans*, that were necessary for exoenzyme S synthesis in the mutant strain 388 *exsI*::Tn*1*. The sequence for the *trans*-regulatory locus is shown in Fig. 3.

Analysis of the *trans*-regulatory locus suggested that at least three proteins were encoded by this region. The first open reading frame, ExsC, was predicted to be a preprotein of 145 amino acids with a molecular weight of 16,228. The amino terminus of ExsC has the properties of a prokaryotic signal sequence (30). Potential cleavage sites were found between serine 24 and leucine 25 or between alanine 31 and serine 32. Although the second site has a higher probability of cleavage according to the standard weight-matrix approach of von Heijne (31), three polar, charged amino acids (Asp-26, Glu-27, and Glu-28) occur prior to this site. These amino acids are not associated with the hydrophobic core, indicating that the first cleavage site may be more likely (31). Two stretches of hydrophobic amino acids were observed between residues 40 and 56 and 96 and 116 which were of sufficient length to span the membrane. The carboxy terminus of ExsC shared slight homology with the carboxy terminus of the *E. coli* inner membrane protein, FecD (25). FecD functions in citrate-mediated iron transport across the cytoplasmic membrane via a binding-protein-dependent mechanism (25).

The second open reading frame, ExsB, was predicted to contain 137 amino acids with a molecular weight of 15,026. The amino terminus is hydrophobic and rich in leucine residues but does not appear to contain other properties of signal peptides (30). The carboxy terminus of ExsB shares significant homology (29.2% identity in a 65-amino-acid overlap) with the amino terminus of VirB, a protein from

Yersinia enterocolitica that is involved in the expression of Yop proteins (5). Loss of the Yop proteins correlates with the loss of pathogenicity in this organism (5, 6).

ExsA was the largest open reading frame, containing 298 amino acids. The predicted molecular weight of ExsA was 33,909. The protein contained neither a signal sequence nor stretches of hydrophobic amino acids associated with membrane interaction. Homology studies indicated that ExsA is part of the AraC family of positive regulatory elements (5). Strong homology (56% identity in a 266-amino-acid overlap) was found between ExsA and the transcriptional activator of the *yop* genes in *Y. enterocolitica*, VirF (Fig. 4). VirF and ExsA were almost identical in the carboxy-terminus region, which contained a Cro-like DNA-binding domain consisting of a helix-turn-helix motif (5, 22).



Gene replacement. Specific Tn*501* insertions in cloned DNA were transferred from plasmid sequences to the chromosome of the prototypical *P. aeruginosa* strain, PAO1, through homologous recombination. Plasmids pDF102::Tn*501*S1 (ExoS$^+$) and pDF102::Tn*501*S21 (ExoS$^-$) were conjugated into PAO1, and transconjugants were selected on the basis of resistance to mercury (Tn*501*) and tetracycline (pLAFR). Cells in which homologous recombination occurred were selected as described in Materials and Methods. Southern blot analysis demonstrated that chromosomal and plasmid preparations had the same hybridization patterns with specific probes (Fig. 5). Hybridization with a probe for the vector, pLAFR, was not detectable in chromosomal preparations, indicating that the entire construct had not integrated into the chromosome.

Exoenzyme S activity and antigen expression were measured in parental and gene replacement strains. Coomassie-stained gels of concentrated supernatants from PAOS1, PAOS21, and PAO1 did not appear significantly different in terms of protein content or banding patterns (Fig. 6A). Western blot analysis demonstrated that exoenzyme S antigen was not detectable in supernatant samples of strain PAOS21 (Fig. 6B, lane 2) and was reduced in supernatant samples of strain PAOS1 (Fig. 6B, lane 1). Unlike our experiments with strain 388 derivatives (Fig. 2), Western blot analysis was not sensitive enough to detect exoenzyme S antigen in cell lysates of PAO1. To ensure maximum sensitivity, we quantitated exoenzyme S in both supernatant and cell-associated material by ADP-ribosyltransferase assays (Table 2). We found that in the parental strain, PAO1, only a small proportion of exoenzyme S activity was cell associated. Therefore, at this stage of growth in the wild-type strain, most of the exoenzyme S was found in the extracellular compartment.

Activity assays of PAOS1 supernatants confirmed our results with Western blot analysis. This strain produced approximately 10-fold less exoenzyme S than did the wild-type PAO1 strain (Table 2). PAOS1 accumulated some cell-associated exoenzyme S activity. Absolute values tended to vary from experiment to experiment; however, PAOS1 generally expressed parental levels or below of cell-associated exoenzyme S activity. These results indicated that secretion of exoenzyme S may be affected by the

FIG. 3. Complete nucleotide sequence of the *trans*-regulatory locus. The *trans*-regulatory locus is a 3,055-bp *Eco*RI fragment and is predicted to encode three proteins, ExsC, ExsB, and ExsA. The single-letter code for the amino acid sequence is shown below the nucleotide sequence. Ribosome binding sites are underlined, and translational termination codons are overlined. The predicted signal peptide cleavage site for ExsC is shown by an arrow. Tn*501* insertions (S1 and S21) are shown by triangles. Exoenzyme S enzyme activity of supernatant material from gene replacement strains PAOS1 (±) and PAOS21 (−) is indicated above the exact location of each Tn*501* insertion. Amino acids that are homologous to a helix-turn-helix motif of DNA-binding proteins are bracketed in ExsA.

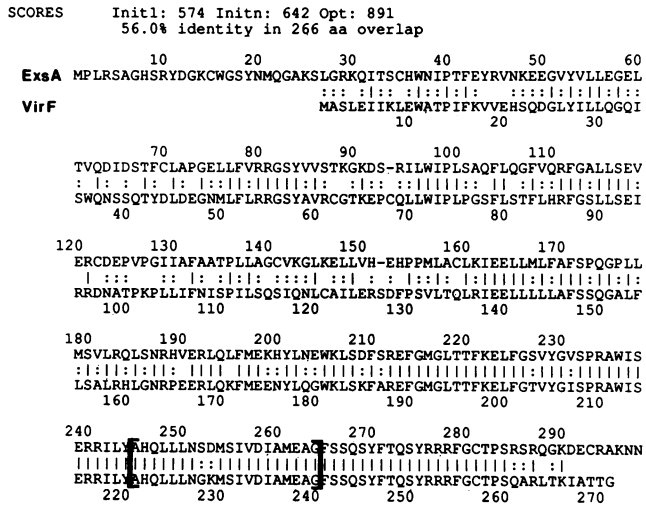


FIG. 4. Homology between ExsA of *P. aeruginosa* (top line of each row) and VirF of *Y. enterocolitica* (bottom line). FASTA analysis was performed between ExsA and VirF, using the University of Wisconsin Genetics Computer Group software (7). Identical amino acids are denoted by a line, and conservative changes are marked with dots. The region corresponding to a helix-turn-helix motif of DNA-binding proteins is bracketed. aa, amino acids.

S1 insertion. This effect could be due to interruption of another gene upstream of *exsC* or could be due to a disruption in the expression of ExsC by the transposon.

Although exoenzyme S was not detectable in the supernatant fraction of PAOS21 by using Western blot techniques, a small fraction of activity could be detected in cell lysates of this strain (Table 2). Standardization of the assay with respect to protein content tends to enhance activity levels in supernatants since the protein level in this compartment is quite small. Supernatant activity values have been divided by a constant factor that reflects the average difference in protein yields between supernatant and lysate fractions. This

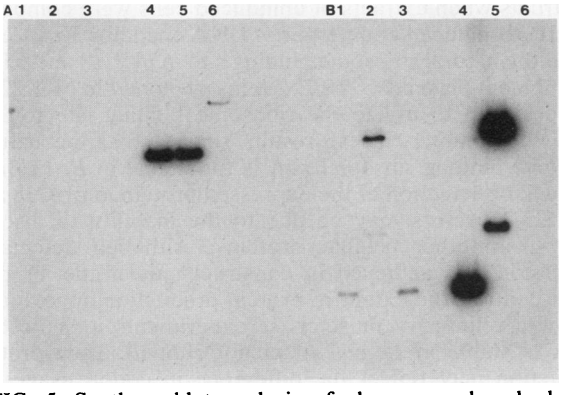


FIG. 5. Southern blot analysis of chromosomal and plasmid DNAs containing Tn*501* insertions. In panel A, a ^{32}P-labeled *Bgl*II fragment from the vector plasmid pLAFR was hybridized to the following *Sal*I-treated DNA preparations: PAOS1 chromosome (lane 1), PAOS21 chromosome (lane 2), PAO1 chromosome (lane 3), pDF102::Tn*501*S1 (lane 4), pDF102::Tn*501*S21 (lane 5), and pLAFR (lane 6). In panel B, a ^{32}P-labeled *Sal*I (1,198-bp) fragment located within the *trans*-regulatory locus was hybridized to the same set of DNA preparations.

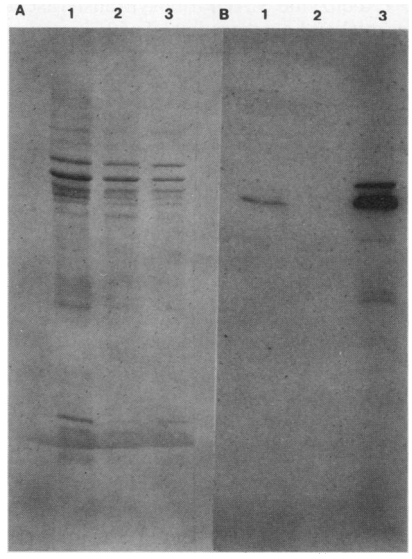


FIG. 6. Western blot analysis of exoenzyme S production in PAO1 and gene replacement strains. (A) Coomassie-stained gel of concentrated supernatant material from stationary-phase cultures of PAOS1 (lane 1), PAOS21 (lane 2), and PAO1 (lane 3). (B) Autoradiogram of a Western blot of a duplicate gel, using rabbit antibody specific for exoenzyme S and ^{125}I-labeled staphylococcal A protein to detect extracellular antigen. Supernatant material in lane 1 is from strain PAOS1, that in lane 2 is from strain PAOS21, and that in lane 3 is from parental strain PAO1.

adjustment makes activity levels comparable between these cellular compartments. Activity values of 0.01 U (threefold-higher counts per minute than background) or above are considered to indicate exoenzyme S activity. PAOS21 lysates consistently demonstrated activity at the 0.01-U level. These results indicate that exoenzyme S can be expressed at a low basal level without a functional ExsA region.

Expression of ExsA. The sequence homology studies and the pattern of exoenzyme S synthesis in strains and plasmids in which ExsA was interrupted suggested that this protein may act as a transcriptional activator. As a first step in the study of the function of ExsA, we used the polymerase chain reaction to specifically amplify the ExsA region with or without its predicted ribosome binding site. Cloned DNA from pDF114 served as a template in these reactions. Gel-purified amplified fragments were cloned under the *lac* promoter in pUC18, the *tac* promoter in pKK223-3, and the T7 promoter in pT7-5. Cell fractions were prepared from sets of induced and uninduced strains. The ExsA product was detected only in the pT7-5 clone, in which host transcription could be halted by the addition of rifampin and the cloned product could be detected by pulse-labeling of newly synthesized proteins. As shown in Fig. 7, a product with a calculated molecular weight of 33,890 was detected (lane 2) that did not appear in the vector control (lane 1). The close correlation between the predicted molecular weight and labeled protein molecular weight indicated that ExsA was expressed under these conditions.

DISCUSSION

A large DNA region has been cloned that encodes the functions necessary for complementation of the exoenzyme S-deficient phenotype of *P. aeruginosa* 388 *exs1*::Tn*1*. When

TABLE 2. Exoenzyme S ADP-ribosyltransferase activities of parental and chromosomal Tn*501* insertions

Strain	Exoenzyme S activity (U[a])			
	Supernatant	Fold reduction[b]	Lysate	Fold reduction
PAO1	1.01 ± 0.14		0.12 ± 0.03	
PAOS1	0.10 ± 0.06	9.9	0.06 ± 0.04	2
PAOS21	ND[c]		0.01 ± 0.002	12

[a] Arbitrarily defined as 500 cpm of [^{14}C]ADP-ribosyltransferase transferred from NAD to trichloroacetic acid-precipitable material in an ADP-ribosyltransferase assay normalized to protein content.

[b] Ratio of units of parental exoenzyme S activity divided by units of activity determined for PAOS1 and PAOS21 cultures.

[c] ND, not detectable.

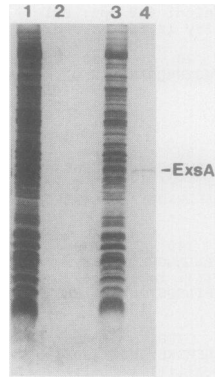


FIG. 7. Expression of ExsA in pT7-5A1A2. Lanes: 1 and 2, ^{35}S-labeled proteins from strain K38(pGp1-2, pT7-5) before (lane 1) and after (lane 2) treatment with rifampin; 3 and 4, labeled proteins from strain K38(pGp1-2, pT7-5A1A2) before (lane 3) and after (lane 4) rifampin treatment. The band corresponding to the ExsA protein is marked with an arrowhead.

pDF100 (28-kb insert) was conjugated into 388 *exs1*::Tn*1*, exoenzyme S was synthesized and secreted at levels equal to that of the parental strain, 388. Testing of smaller segments of the cosmid clone by subcloning and transposon mutagenesis localized the region required for synthesis but not secretion of exoenzyme S in 388 *exs1*::Tn*1*. These results suggest that the synthesis and secretion functions reside in separate loci. In contrast, the insertion of Tn*1* in the chromosome of strain 388 resulted in a mutation that was able to inactivate both synthesis and secretory functions. Tn*1* may be able to influence the expression of genes upstream or downstream of the point of insertion in addition to interrupting a coding region. We are in the process of mapping the location of the Tn*1* insertion to determine how this insertion inactivates the ability of strain 388 to synthesize and secrete exoenzyme S.

Nucleotide sequence analysis of the DNA complementing the lesion in 388 *exs1*::Tn*1* for synthesis of exoenzyme S indicated that a regulatory region had been cloned. Comparison studies using the N-terminal amino acid sequence of the purified 49-kDa enzymatically active form of exoenzyme S with all open reading frames in the cloned segment did not yield a match, indicating that the structural gene for exoenzyme S was not within the sequenced region. Homology studies further suggested that two open reading frames, ExsB and ExsA, were homologous to amino acid sequences of the VirB and VirF genes of *Y. enterocolitica*. Both *Yersinia* gene products regulate the synthesis of secreted virulence proteins called Yops. The role of VirB in *yop* expression is unknown, but mutants in *virB* affect the transcription of *yop51* (6). VirF plays a pivotal role in controlling Yop synthesis at the level of transcription through a mechanism involving a DNA-binding event (6). Both VirF and ExsA contain a carboxy-terminal domain exhibiting the helix-turn-helix motif of DNA-binding proteins. By analogy, we predict that ExsA may serve as a DNA-binding protein that enhances the transcription of the exoenzyme S structural gene and perhaps other virulence determinants. The requirement of ExsA for induction of exoenzyme S synthesis was further demonstrated in gene replacement experiments. Return of a Tn*501* insertion within the open reading frame of ExsA to the chromosome of strain PAO1 (PAOS21) resulted in a severe reduction in the amount of detectable exoenzyme S.

An unexpected result occurred when we returned a control Tn*501* insertion to the chromosome of strain PAO1 (PAOS1). This insertion mapped upstream of the ExsC, -B, and -A open reading frames and still produced exoenzyme S, as determined in qualitative immunocolony blots. Quantitative experiments demonstrated that strain PAOS1 tended to accumulate exoenzyme S in a cell-associated form. Only 9% of the parental level of exoenzyme S appeared in the supernatant. Secretion in general did not seem to be affected since the protein pattern of concentrated supernatant material was identical when PAO1 and PAOS1 lanes were examined in stained gels. Exoenzyme S is not secreted in 388 *exs1*::Tn*1* when a clone containing an intact *trans*-regulatory locus (*exsCBA*) as well as 5.0 kb of upstream DNA (pDF102) is provided in *trans*. Thus, the most likely explanation for this phenotype is that the Tn*501* insertion is affecting the expression of ExsC rather than interrupting a gene involved in secretion that lies upstream of *exsC*. We interpret these results as suggesting that *exsC* and upstream sequences are not sufficient to encode the secretion functions but that *exsC* may directly or indirectly facilitate exoenzyme S secretion.

Detection of a protein product was achieved when ExsA was cloned such that transcription was directed by the rifampin-resistant T7 polymerase. Products were undetectable in pUC18 (*lac* promoter) and pKK223-3 (*tac* promoter) constructs when extracts of uninduced cells were compared with IPTG-induced counterparts. DNA encoding ExsA contained the natural ribosome binding site in pUC18 and pT7-5 recombinant plasmids. The ExsA insert cloned in pKK223-3 was designed to utilize the ribosome binding site for the *lac*-UV5 promoter. These results suggest that the natural ribosome binding site for ExsA is functional in *E. coli* and that lack of detection of the expressed protein in pUC18 and pKK223-3 vectors may be due to the inability to discern ExsA from other cellular proteins. Although detectable expression was achieved in constructs under the T7 promoter control, the extent of protein production appeared to be low. Preliminary pulse-chase experiments indicate that ExsA is stable in *E. coli* (data not shown). Low protein production may reflect the difference in codon usage between *E. coli* and *P. aeruginosa* (32). Alternatively, two regions in the first one-third of ExsA show poor codon usage and an accumulation of rare codons. Pools of rare tRNAs may be required for translation, which may act to limit the amount of ExsA synthesized.

The homology between ExsB and ExsA and proteins involved in *yop* regulation in *Yersinia* spp. may be important clues for analyzing the regulation of exoenzyme S produc-

tion in *P. aeruginosa*. The Yop proteins are induced under conditions of growth at 37°C in the absence of calcium (5, 6). VirF has been shown to be the transcriptional activator of the *yop* regulon and appears to regulate its own transcription in a thermoinducible manner (5). The growth conditions required to elicit exoenzyme S production include the presence of a high concentration of a chelator (either nitrilotriacetic acid or EDTA). Whether inclusion of the chelator is required to remove some divalent cation (like Ca^{2+}) or whether this imposes a general stress on the cells is not clear. No data on the effect of growth temperature on the expression of exoenzyme S are currently available. Because of the requirement of ExsA, our current hypothesis is that it is a transcriptional activator protein that binds to a region upstream of the exoenzyme S structural gene to enhance transcription. The specific environmental stimuli that are required for ExsA to be active or for the gene to be transcribed have not been identified but may be related to stress or temperature induction. Whether the environmental stimulus affects the expression of the regulatory loci or the structural gene or both remains to be determined. Our efforts are currently focused on analyzing the expression of the regulatory loci promoter regions when cells are grown under different conditions. These experiments are designed to clarify the signal(s) required for induction of exoenzyme S synthesis in *P. aeruginosa*.

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