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Expression of Recombinant Exoenzyme S of *Pseudomonas aeruginosa*

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The structural gene for the 49-kDa form of exoenzyme S (*exoS*) isolated from *Pseudomonas aeruginosa* 388 was expressed in both *Escherichia coli* and *P. aeruginosa* PA103. Expression of *exoS* in *E. coli* under the transcriptional regulation of the T7 promoter yielded a soluble cytosolic protein with an apparent molecular mass of 49 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Expression of *exoS* in *P. aeruginosa* PA103 under the transcriptional regulation of the 0.9 kbp of *Pseudomonas* chromosomal DNA flanking the 5′ end of *exoS* yielded a nitrilotriacetic acid-inducible extracellular protein with an apparent molecular mass of 49 kDa. Recombinant ExoS (rExoS) reacted with the anti-49-kDa form of exoenzyme S immunoglobulin G, existed as an aggregate as determined by gel filtration chromatography, and ADP-ribosylated soybean trypsin inhibitor at a specific activity that was similar (within twofold) to that of native exoenzyme S. Allelic exchange of *exoS* with a tetracycline gene cartridge yielded a strain of *P. aeruginosa* 388 that did not express detectable amounts of either ExoS in an immunoblot analysis using the anti-49-kDa form of exoenzyme S immunoglobulin G or ADP-ribosyltransferase activity under standard enzyme assay conditions. Expression of catalytically active rExoS in *E. coli* demonstrated that *exoS* was necessary and sufficient for the factor-activating exoenzyme S-dependent ADP-ribosyltransferase activity of exoenzyme S. Expression of nitrilotriacetic acid-inducible rExoS in *P. aeruginosa* PA103 demonstrated that the 0.9 kbp of *Pseudomonas* chromosomal DNA flanking the 5′ end of *exoS* encoded a functional exoenzyme S promoter. Expression analysis and allelic exchange experiments suggest that the 49- and 53-kDa forms of exoenzyme S are encoded by separate genes.

Conditions such as cystic fibrosis, leukemia, neutropenia, and burn wounds predispose individuals to infections by *Pseudomonas aeruginosa* (1). Recently, *P. aeruginosa* has been reported to cause infections in children infected with human immunodeficiency virus (28). Numerous factors, including the production of exoenzyme S (26), have been shown to contribute to the virulence of *P. aeruginosa* (37). Exoenzyme S has been reported to contribute to the dissemination of *P. aeruginosa* from burn wounds and to tissue destruction in chronic lung infections (24–26).

Expression of exoenzyme S is not constitutive but, like expression of many bacterial virulence determinants (22), is modulated by environmental factors (12, 35). Although the molecular mechanisms responsible for the expression of exoenzyme S are not completely understood, genes that comprise a chromosomally encoded *trans*-regulatory locus have been shown to be required for exoenzyme S synthesis (9, 10).

Exoenzyme S is a member of the family of bacterial exoenzymes that catalyze the ADP-ribosylation of eucaryotic proteins. Exoenzyme S has been shown to ADP-ribosylate several proteins within the family of low-molecular-weight GTP-binding proteins (4, 6) and to require a eucaryotic protein, termed factor-activating exoenzyme S (FAS), for expression of enzymatic activity in vitro (5). Although the molecular nature of exoenzyme S is not completely understood, two *Pseudomonas* proteins possessing apparent molecular masses of 53 and 49 kDa have been observed to copurify with the enzymatic activity of exoenzyme S in *P. aeruginosa* 388 (17, 25). The 49-kDa protein, termed the 49-kDa form of exoenzyme S, possessed ADP-ribosyltransferase activity upon extraction from sodium dodecyl sulfate (SDS)-polyacrylamide gels (5, 18, 25), while the 53-kDa protein, termed the 53-kDa form of exoenzyme S, did not possess enzymatic activity upon extraction from polyacrylamide gels (25). It has been proposed that the 53- and 49-kDa forms of exoenzyme S exist in a precursor-proteolytic product relationship wherein the 53-kDa form of exoenzyme S undergoes a carboxy-terminal cleavage to yield the enzymatically active 49-kDa form of exoenzyme S (3). This model was based on the observation that these proteins share properties such as immunological cross-reactivity (17, 25), a common amino-terminal amino acid sequence (3), and common peptides following proteolytic and cyanogen bromide cleavage (14). However, the functional and genetic relationships between the 53- and 49-kDa forms of exoenzyme S are not currently understood.

The structural gene encoding the 49-kDa form of exoenzyme S (*exoS*) has been cloned from *P. aeruginosa* 388 (18). In this study, the biochemical and molecular properties of recombinant ExoS (rExoS) were determined when *exoS* was expressed in *Escherichia coli* and *P. aeruginosa* PA103. We report that *exoS* was necessary and sufficient for expression of exoenzyme S ADP-ribosyltransferase activity in *E. coli* and that the 2.3-kbp *Bam*HI *Pseudomonas* DNA fragment containing *exoS* and 0.9 kbp of the chromosomal DNA flanking the 5′ end of *exoS* possesses a functional nitrilotriacetic acid (NTA)-inducible promoter for exoenzyme S.

MATERIALS AND METHODS

Materials. pUC19, restriction enzymes, and DNA-modifying enzymes were purchased from New England Biolabs, except for the Klenow fragment of DNA polymerase I, which was purchased form Bethesda Research Laboratories. The *Taq* track DNA sequencing kit was purchased from Promega. Bovine serum albumin (BSA) standard and the bicinchoninic acid protein determination kit were purchased from Pierce. *E. coli* BL21(DE3) has been described previously (33).

Purification of native exoenzyme S. Native exoenzyme S was purified from the spent culture medium of *P. aeruginosa* 388 as described previously (17).

Expression of rExoS in *E. coli*. (i) Construction of *E. coli* expression vectors. Expression of rExoS in *E. coli* was tested by using constructs that contained *exoS*

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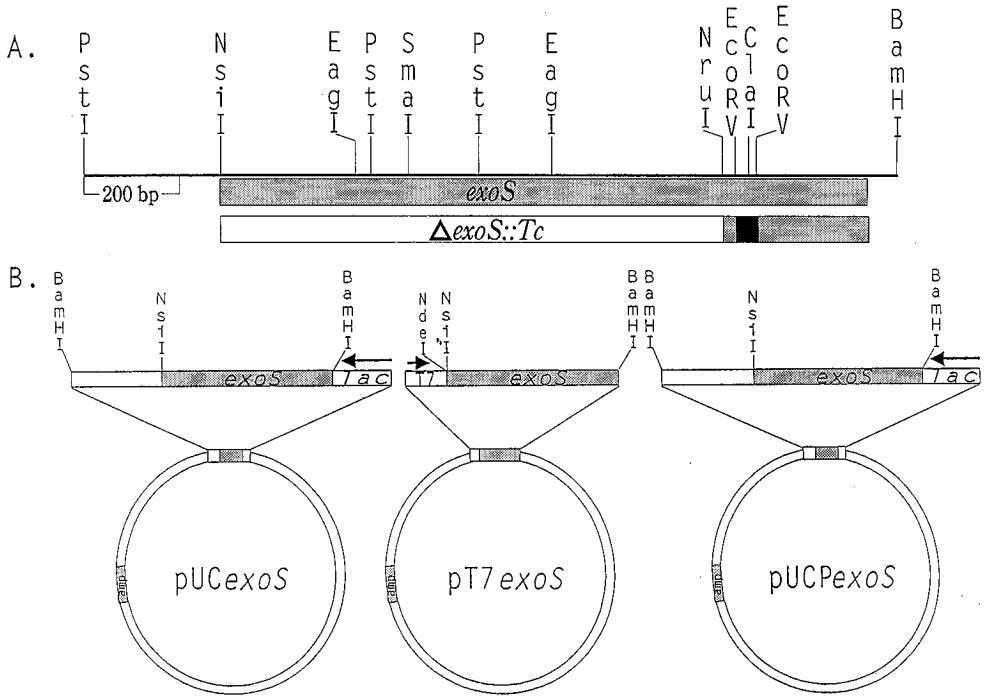

FIG. 1. Schematic of *exoS* and expression vectors containing *exoS*. (A) A 2.3-kbp *Bam*HI *Pseudomonas* DNA fragment containing *exoS* and 0.9 kbp of chromosomal DNA flanking the 5′ end of *exoS* (18). The *Nsi*I-*Bam*HI DNA fragment containing *exoS* (shaded box) and the restriction endonuclease sites lying within *exoS* are also illustrated. Δ*exoS*::*Tc* represents a schematic of the allelic replacement of *exoS*. The white and black areas represent the regions of *exoS* that were deleted. The black region also represents the region where the tetracycline gene cartridge was inserted. (B) The pUC*exoS*, pT7*exoS*, and pUCP*exoS* expression vectors. Methods used to engineer these vectors are described in Materials and Methods. The *Nsi*I restriction endonuclease site contains the predicted initiation methionine of ExoS. Arrows indicate the direction of transcription from vector promoters which are located adjacent to the polycloning restriction endonuclease recognition sites.

under the transcriptional control of its proposed promoter region (pUC*exoS*) and *exoS* under the transcriptional control of the T7 promoter (pT7*exoS*).

(a) pUC*exoS*. A 2.3-kbp *Bam*HI *Pseudomonas* DNA fragment containing *exoS* and 0.9 kbp of chromosomal DNA flanking the 5′ end of *exoS* (18) was subcloned into the *Bam*HI site of pUC19 to create pUC*exoS* (Fig. 1). Restriction endonuclease analysis of pUC*exoS* showed that *exoS* was aligned in the opposite orientation relative to the *lac* promoter.

(b) pT7*exoS*. DNA encoding the amino terminus of ExoS was amplified by using overlap PCR mutagenesis (13) to introduce a unique *Nde*I restriction endonuclease recognition site immediately upstream of the predicted initiator methionine of ExoS and a unique *Sal*I restriction endonuclease recognition site downstream of the predicted initiator methionine of ExoS. The amplified fragment was subcloned into the *Nde*I and *Sal*I restriction endonuclease sites in pT7-7 (34) to create pT7*exoS*-nterm. An *Nsi*I-*Bam*HI fragment which contained the entire *exoS* open reading frame was subcloned from pUC19*exoS* into the corresponding restriction sites in pT7*exoS*-nterm to create pT7*exoS* (Fig. 1). DNA containing *exoS* was subcloned from pT7*exoS* into M13mp18 and subjected to dideoxy DNA sequence analysis (29) to confirm that secondary mutations had not been introduced into *exoS* during subcloning and DNA amplification procedures.

(ii) Production of rExoS in *E. coli*. (a) pUC*exoS*. *E. coli* TG1(pUC*exoS*) or *E. coli* TG1(pUC19) was grown overnight at 37°C in L broth containing 0.1 mg of ampicillin per ml with aeration. Overnight cultures were diluted 1/50 in L broth containing ampicillin, cultured for 2 h at 37°C with aeration, and then incubated in either the presence or the absence of 0.5 mM (final concentration) isopropylthiogalactopyranoside (IPTG) for an additional 2 h. Cells were centrifuged at 8,000 × *g* for 10 min and lysed by the addition of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and boiling for 5 min, and the resulting cell lysates were subjected to SDS-PAGE (19) in the presence of β-mercaptoethanol. Gels were stained for total protein with Coomassie blue or subjected to Western blot (immunoblot) analysis (2) using anti-49-kDa protein immunoglobulin G (IgG) as described previously (17).

To test the effect of NTA on the expression of rExoS in *E. coli* TG1(pUC*exoS*), cells were cultured with aeration overnight at 32°C in either the presence or the absence of 10 mM NTA as described previously (17) with the exception that 100 μg of ampicillin per ml (final concentration) was included in the Trypticase soy broth dialysate (TSBD) medium to select for pUC*exoS*. The overnight culture was centrifuged at 10,000 × *g* for 10 min at 4°C. Cell lysates were prepared by boiling cell pellets in SDS-PAGE sample buffer containing β-mercaptoethanol for 5 min while the soluble culture medium was subjected to ammonium sulfate precipitation (60%, final concentration; 1.5 ml of saturated ammonium sulfate added per ml of soluble culture medium) for 1 h at 4°C. The ammonium sulfate-precipitable material was collected by centrifugation at 20,000 × *g* for 30 min and suspended in SDS-PAGE sample buffer containing β-mercaptoethanol at 1/20 of the original volume of culture medium.

(b) pT7*exoS*. *E. coli* BL21(DE3) (pT7*exoS*) or *E. coli* BL21(DE3) (pT7-7) was grown overnight at 30°C in L broth containing 0.1 mg of ampicillin per ml with aeration. Overnight cultures were diluted 1/100 in L broth containing ampicillin, cultured for 3 h at 30°C with aeration, and then incubated in either the presence or the absence of 0.5 mM (final concentration) IPTG for an additional 2 h. Cell lysates were analyzed for total protein and Western blot by SDS-PAGE as described above.

(c) Purification of rExoS from *E. coli*. Overnight cultures of *E. coli* BL21(DE3) (pT7*exoS*) or *E. coli* BL21(DE3) (pT7-7) were diluted 1/50 in 400 ml of L broth containing ampicillin and cultured for 2 h at 30°C with aeration, and then 0.5 mM (final concentration) IPTG was added. After a 2-h induction, cells were centrifuged at 8,000 × *g* for 10 min at 4°C; washed with 15 ml of 25 mM Tris-HCl (pH 7.6); centrifuged at 8,000 × *g* for 10 min at 4°C; suspended in 15 ml of 10 mM Tris-HCl (pH 7.6) containing 0.1 M NaCl, 1 mM $MgCl_2$, and 10 μg of DNase I and RNase A per ml; and lysed by passage twice through a French pressure cell (piston diameter of 2.5 mM; Aminco). Lysed cells were subjected to ultracentrifugation (104,000 × *g* for 30 min at 4°C), and 10 ml of the soluble fraction was subjected to gel filtration chromatography using a 475-ml Sephacryl S400-HR column equilibrated in 25 mM Tris-HCl (pH 7.6). The column was run at 4°C at a flow rate of 0.5 ml/min, and 6-ml fractions were collected.

Expression of rExoS in *P. aeruginosa* PA103. (i) Construction of pUCP*exoS*. The 2.3-kbp *Bam*HI *Pseudomonas* DNA fragment containing *exoS* and 0.9 kbp of chromosomal DNA flanking the 5′ end of *exoS* (18) was subcloned into the *Bam*HI site of pUCP18 (30) to create pUCP*exoS* (Fig. 1). Restriction endonuclease analysis of pUCP*exoS* showed that *exoS* was aligned in the opposite orientation relative to the *lac* promoter.

(ii) Expression and purification of rExoS in *P. aeruginosa* PA103. The procedures used were those described previously (21). *P. aeruginosa* PA103 (pUCP *exoS*) or *P. aeruginosa* PA103(pUCP18) was cultured as described previously (17) with the exception that 400 μg of carbenicillin per ml (final concentration) was included in the Vogel-Bonner minimal medium plates and TSBD medium to select for the pUCP18 plasmids. Cell lysates and 60% ammonium sulfate precipitates of the spent culture media were prepared as described above with the

exception that the 60% ammonium sulfate-insoluble material was suspended in 25 mM Tris-HCl (pH 7.6). The solubilized ammonium sulfate-precipitable material was subjected to gel filtration chromatography using Sephacryl S400-HR resin equilibrated in 25 mM Tris-HCl (pH 7.6). Specific chromatographic conditions are described in the figure legends. Column fractions were subjected to SDS-PAGE, and gels were analyzed for total protein and Western blotting as described above. Column fractions containing rExoS were pooled and subjected to DEAE-Sephacel chromatography (10 ml of resin per 150 ml of culture medium equilibrated in 25 mM Tris-HCl [pH 7.6]). Bound rExoS was eluted from the DEAE column in 25 mM Tris-HCl (pH 7.6) containing 125 mM NaCl to yield purified rExoS.

Measurement of exoenzyme S ADP-ribosyltransferase activity. Exoenzyme S ADP-ribosyltransferase activity was measured as the incorporation of radiolabel from [*adenylate phosphate*-^{32}P]NAD into soybean trypsin inhibitor (SBTI) essentially as previously described (17). Briefly, reaction mixtures 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), 40 nM recombinant FAS (11), and enzyme. Preparations of exoenzyme S were diluted in 25 mM Tris-HCl (pH 7.6) containing 0.1 mg of chicken egg albumin per ml to normalize protein concentrations in the reaction mixtures. Reactions mixtures were incubated at 25°C and at timed intervals spotted onto trichloroacetic acid-saturated Whatman 3MM paper. Radiolabel was quantitated by scintillation counting.

In experiments that measured the effect of preimmune IgG and anti-49-kDa protein IgG on exoenzyme S ADP-ribosyltransferase activity, *E. coli* cell lysates which contained rExoS were incubated with an equal volume of IgG (3 μM, final concentration) or phosphate-buffered saline for 10 min at 25°C prior to measurement of exoenzyme S ADP-ribosyltransferase activity.

Construction of *P. aeruginosa* 388ΔexoS. The 2.3-kb *Bam*HI fragment encoding *exoS* was subcloned into the allelic replacement vector pNOT19 to produce pNOT19*exoS* (31). pNOT19*exoS* was digested with *Nsi*I and *Nru*I and treated with mung bean nuclease. The vector portion of digested pNOT19*exoS* was purified from agarose gels and ligated to produce pNOT19*exoS*ΔNsiI-NruI, which possessed a deletion in the DNA encoding the amino-terminal 350 amino acids of ExoS. Transformants containing pNOT19*exoS*ΔNsiI-NruI were identified by restriction endonuclease digestion. pNOT19*exoS*ΔNsiI-NruI was digested with *Eco*RV to delete an additional 42 bp within the *exoS* open reading frame, termed pNOT19*exoS* double deletion, to provide a unique blunt end site for the insertion of a tetracycline gene cartridge. The tetracycline gene cartridge, from mini-Tn*5* Tc (7), was isolated as a 2.1-kb *Eco*RI fragment, subjected to treatment with the Klenow fragment of DNA polymerase I, and ligated into pNOT19*exoS* double deletion at the unique *Eco*RV site, to produce pNOT19Δ*exoS*::Tc. Transformants containing pNOT19Δ*exoS*::Tc were selected by tetracycline resistance and confirmed by restriction endonuclease digestion. Allelic replacement was performed following insertion of the MOB cartridge from pMOB3 (31) into the unique *Not*I site of pNOT19Δ*exoS*::Tc. Conjugation conditions and selection of *P. aeruginosa* 388Δ*exoS*::Tc allele were performed as described previously (31) and confirmed by Southern blot analysis using internal *exoS* and Tc probes (18). *P. aeruginosa* 388Δ*exoS*::Tc was termed *P. aeruginosa* 388Δ*exoS*.

Protein determinations. Total protein was determined with the bicinchoninic acid protein determination kit, using a BSA standard for normalization and assuming equal molar dye binding. The 53- and 49-kDa forms of exoenzyme S from *P. aeruginosa* and rExoS were quantitated with a densitometer (AMBIS, San Diego, Calif.) from Coomassie blue-stained SDS-polyacrylamide gels, using a BSA standard for normalization and assuming equal molar staining. Anti-49-kDa protein IgG immunoreactive protein was determined by densitometric analysis of autoradiograms of Western blots. Western blots were probed with anti-49-kDa protein IgG followed by ^{125}I-protein A.

RESULTS

Expression of rExoS in *E. coli*. Earlier studies (18) showed that a 2.3-kbp *Bam*HI *Pseudomonas* DNA fragment contained a 1,362-bp open reading frame (*exoS*) which was predicted to encode the 49-kDa form of exoenzyme S (Fig. 1). The 2.3-kbp *Bam*HI *Pseudomonas* DNA fragment containing *exoS* and 0.9 kbp of chromosomal DNA flanking the 5′ end of *exoS* was subcloned into pUC19 in the opposite direction relative to the *lac* promoter (Fig. 1, pUC*exoS*) and transformed into *E. coli* TG1. When incubated in either the presence or the absence of IPTG, *E. coli* TG1(pUC*exoS*) did not produce detectable levels of rExoS, as determined by analysis of cell lysates for the presence of a novel 49-kDa Coomassie blue-stainable protein or a 49-kDa protein that reacted with anti-49-kDa protein IgG (data not shown). When cultured in TSBD medium containing NTA, conditions which have been shown to induce the production of exoenzyme S in *P. aeruginosa* (35), *E. coli* TG1 (pUC*exoS*) did not produce detectable levels of rExoS, as determined by analysis of cell lysates and the spent culture medium for the presence of a novel 49-kDa Coomassie blue-stainable protein or a 49-kDa protein that reacted with anti-49-kDa protein IgG (data not shown).

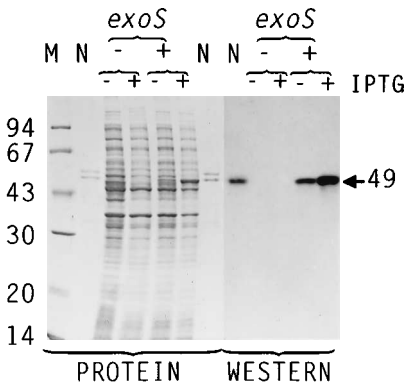

FIG. 2. Expression of rExoS in *E. coli*. Equivalent volumes of cell lysates from *E. coli*(pT7-7) (*exoS* −) or *E. coli*(pT7*exoS*) (*exoS*+) which had been incubated in either the presence (+) or the absence (−) of 0.5 mM IPTG were subjected to SDS-PAGE (10% gel) in the presence of β-mercaptoethanol. Gels were stained for total protein with Coomassie blue (PROTEIN) or transferred to nitrocellulose and probed with anti-49-kDa protein IgG (WESTERN). An autoradiogram of a 24-h exposure with intensifying screens of the Western blot is pictured. Lane N contains native exoenzyme S purified from *P. aeruginosa* 388. The migration of the 49-kDa form of exoenzyme S (0.9 pmol) is indicated by the arrow. Lane M contains Coomassie blue-stained protein standards with molecular masses of 94, 67, 43, 30, 20, and 14 kDa.

Since *exoS* was not expressed in *E. coli* when *exoS* was under the transcriptional control of its own promoter, we engineered a vector (pT7*exoS*) which allowed for the expression of *exoS* under the transcriptional control of the T7 promoter (Fig. 1). pT7*exoS* was transformed into *E. coli* BL21(DE3), a strain of *E. coli* which harbored an IPTG-inducible T7 RNA polymerase (33). Cell lysates of *E. coli* BL21(DE3) (pT7*exoS*) expressed an IPTG-inducible, anti-49-kDa protein IgG immunoreactive protein which possessed an apparent molecular mass of 49 kDa (Fig. 2). The soluble fraction of cell lysates of *E. coli* BL21 (DE3) (pT7*exoS*) expressed exoenzyme S activity with a specific activity of 11 pmol of ADP-ribosylated SBTI per min per pmol of anti-49-kDa protein IgG immunoreactive protein (range, 6 to 15; $n = 2$). Addition of anti-49-kDa protein IgG to the soluble fraction of cell lysates expressing exoenzyme S activity inhibited the ADP-ribosyltransferase activity by >90%. Addition of an equivalent amount of preimmune IgG to the soluble fraction of cell lysates expressing exoenzyme S activity did not inhibit ADP-ribosyltransferase activity (data not shown). This 49-kDa protein was termed rExoS.

Cell lysates from control cells, *E. coli* BL21(DE3) (pT7-7), did not express detectable levels of rExoS when assayed for total protein or probed by Western blotting with the anti-49-kDa protein IgG (Fig. 2). In addition, the soluble fraction of cell lysates from control cells did not express detectable exoenzyme S ADP-ribosyltransferase activity (data not shown).

Gel-filtration of rExoS from *E. coli*. Gel filtration chromatography of the soluble fraction of cell lysates of *E. coli* BL21(DE3) (pT7*exoS*) showed that the majority of rExoS eluted near the void volume while a small amount of rExoS, <5% of total rExoS in the column fractions, eluted as predicted for a nonaggregated monomeric form of rExoS (data not shown). Elution of rExoS in the void volume (the molecular mass cutoff of the resin is 8,000 kDa for globular proteins) indicated the rExoS existed as a high-molecular-mass aggregate as has been observed for native exoenzyme S produced in

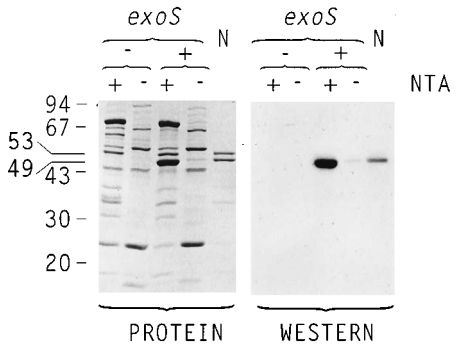

FIG. 3. Expression of rExoS in *P. aeruginosa* PA103. *P. aeruginosa*(pUCP18) (*exoS* −) or *P. aeruginosa* (pUCPexoS) (*exoS* +) was cultured overnight in either the presence (+) or absence (−) of 10 mM NTA. Equivalent volumes of ammonium sulfate concentrates of the spent culture media were subjected to SDS-PAGE (10% gel) in the presence of β-mercaptoethanol. Gels were stained for total protein (PROTEIN) or transferred to nitrocellulose and probed with anti-49-kDa protein IgG (WESTERN). An autoradiogram of a 72-h exposure with intensifying screens is pictured. Lane N contains native exoenzyme S purified from *P. aeruginosa* 388. To the left are the positions of migration of the 53- and 49-kDa forms of exoenzyme S and protein standards with molecular masses of 94, 67, 43, 30, and 20 kDa.

P. aeruginosa 388 (17, 35). Gel filtration chromatography separated rExoS from the majority of the *E. coli* cellular proteins and provided a preparation enriched for rExoS (data not shown). Attempts to further purify rExoS were unsuccessful, as we were unable to define conditions which allowed rExoS to bind to either DEAE-Sephacel or Bio-Rex 70 resin.

E. coli purified rExoS possessed full-length rExoS and several lower-molecular-weight proteins that reacted with anti-49-kDa protein IgG. These immunoreactive proteins, which became more predominant during the purification protocol (compare immunoreactive proteins in Fig. 2 [a cell lysate] and Fig. 5 [*E. coli* purified rExoS]), appeared to be degradation products of full-length rExoS, which indicated that rExoS was susceptible to *E. coli* proteases.

In control experiments, identically prepared soluble fractions of cell lysates from *E. coli* BL21(DE3) (pT7-7) showed an elution profile for *E. coli* cellular proteins similar to that of *E. coli* BL21(DE3) (pT7*exoS*) but did not possess detectable levels of exoenzyme S ADP-ribosyltransferase activity or a protein which reacted with anti-49-kDa protein IgG (data not shown).

Expression of rExoS in *P. aeruginosa* PA103. The 2.3-kbp *Bam*HI *Pseudomonas* DNA fragment containing *exoS* and 0.9 kbp of chromosomal DNA flanking 5′ end of *exoS* was subcloned into pUCP18 such that *exoS* was aligned in the opposite orientation relative to the *lac* promoter (Fig. 1, pUCP*exoS*). pUCP*exoS* was transformed into *P. aeruginosa* PA103. *P. aeruginosa* PA103 was chosen as the host for the expression of rExoS since it has been shown to possess only low levels of exoenzyme S ADP-ribosyltransferase activity and does not express detectable amounts of the 49-kDa form of exoenzyme S, as determined by Western blot analysis (10). Previous studies showed that NTA induced the expression of exoenzyme S in *P. aeruginosa* 388 (35). The spent culture medium of *P. aeruginosa* PA103(pUCP*exoS*) contained an NTA-inducible 49-kDa protein which reacted against anti-49-kDa protein IgG (Fig. 3). This 49-kDa protein, which was not detectable in Western blot analysis of either cell lysates (8a) or spent culture medium (Fig. 3) of *P. aeruginosa* PA103 (pUCP18), was termed rExoS.

In linear velocity reactions, ammonium sulfate concentrates of the spent culture medium of *P. aeruginosa* PA103 (pUCP*exoS*) that had been cultured in the absence of NTA catalyzed the ADP-ribosylation of SBTI with a specific activity of 7.8 ± 1.9 pmol of SBTI per min per μg of total protein. Ammonium sulfate concentrates of the spent culture medium of strain PA103(pUCP*exoS*) that had been cultured in the presence of NTA catalyzed the ADP-ribosylation of SBTI with a specific activity of 210 ± 40 pmol of SBTI per min per μg of total protein. This 30-fold induction of rExoS ADP-ribosyltransferase activity by NTA was similar to NTA induction of exoenzyme S in *P. aeruginosa* 388 (35).

Ammonium sulfate concentrates of the spent culture medium obtained from control cells, *P. aeruginosa* PA103 (pUCP18), which had been cultured in the absence of NTA did not possess exoenzyme S ADP-ribosyltransferase activity above the activity of control reactions which contained diluent (25 mM Tris-HCl [pH 7.6]) in place of enzyme. Ammonium sulfate concentrates of the spent culture medium of strain PA103(pUCP18) that had been cultured in the presence of NTA catalyzed the ADP ribosylation of SBTI with a specific activity of 0.03 ± 0.01 pmol of SBTI per min per μg of total protein.

Consistent with earlier reports, NTA induced the expression of the 53-kDa form of exoenzyme S in *P. aeruginosa* PA103 (10). Induction of the 53-kDa form of exoenzyme S was independent of pUCP*exoS* (Fig. 3). These data indicated that production of both the 53- and 49-kDa forms of exoenzyme S were induced by NTA but were regulated independently and that the 49-kDa form of exoenzyme S was not produced as a 53-kDa precursor.

Gel filtration of rExoS from *P. aeruginosa*. Ammonium sulfate concentrates of the spent culture medium of *P. aeruginosa* PA103(pUCP*exoS*) or *P. aeruginosa* PA103(pUCP18) cultured in either the absence or the presence of NTA were subjected to Sephacryl S400-HR gel filtration chromatography and analyzed for the expression of rExoS and the 53-kDa form of exoenzyme S (Fig. 4). In *P. aeruginosa* PA103(pUCP*exoS*), NTA induced the extracellular expression of both rExoS and the 53-kDa form of exoenzyme S. Both proteins eluted in the void volume of the S400-HR column (Fig. 4B). In *P. aeruginosa* PA103(pUCP18), extracellular rExoS was not detected, but extracellular expression of the NTA-inducible 53-kDa form of exoenzyme S was detected (Fig. 4A). The 53-kDa form of exoenzyme S eluted as an aggregate in the absence of rExoS. In the absence of NTA, neither the 53-kDa form of exoenzyme S nor rExoS was expressed (Fig. 4C and D).

Purification of rExoS in *P. aeruginosa*. The peak fractions of rExoS obtained following S400-HR chromatography from NTA-induced spent culture medium of *P. aeruginosa* PA103 (pUCP*exoS*) were pooled and subjected to DEAE-Sephacel chromatography. Bound rExoS was batch eluted from the column with 125 mM NaCl (Fig. 5). In contrast to *E. coli*-expressed rExoS, *Pseudomonas*-expressed rExoS did not show detectable degradation during purification from the spent culture media.

ADP-ribosyltransferase activity of purified recombinant ExoS. In a linear velocity reaction, rExoS purified from either *E. coli* or *P. aeruginosa* (Fig. 5) ADP-ribosylated SBTI at a specific activity that was within twofold the activity of native exoenzyme S (5.4 ± 1.0, 5.8 ± 0.7, and 9.8 ± 2.6 pmol of ADP-ribosylated SBTI per min per pmol of 49-kDa protein for *E. coli* rExoS, *P. aeruginosa* rExoS, and native exoenzyme S, respectively). As reported for native exoenzyme S and the purified 49-kDa form of exoenzyme S isolated from native exoenzyme S (5), the in vitro ADP-ribosyltransferase activity of rExoS was dependent upon the presence of the FAS protein; no detectable ADP-ribosyltransferase activity above that de-

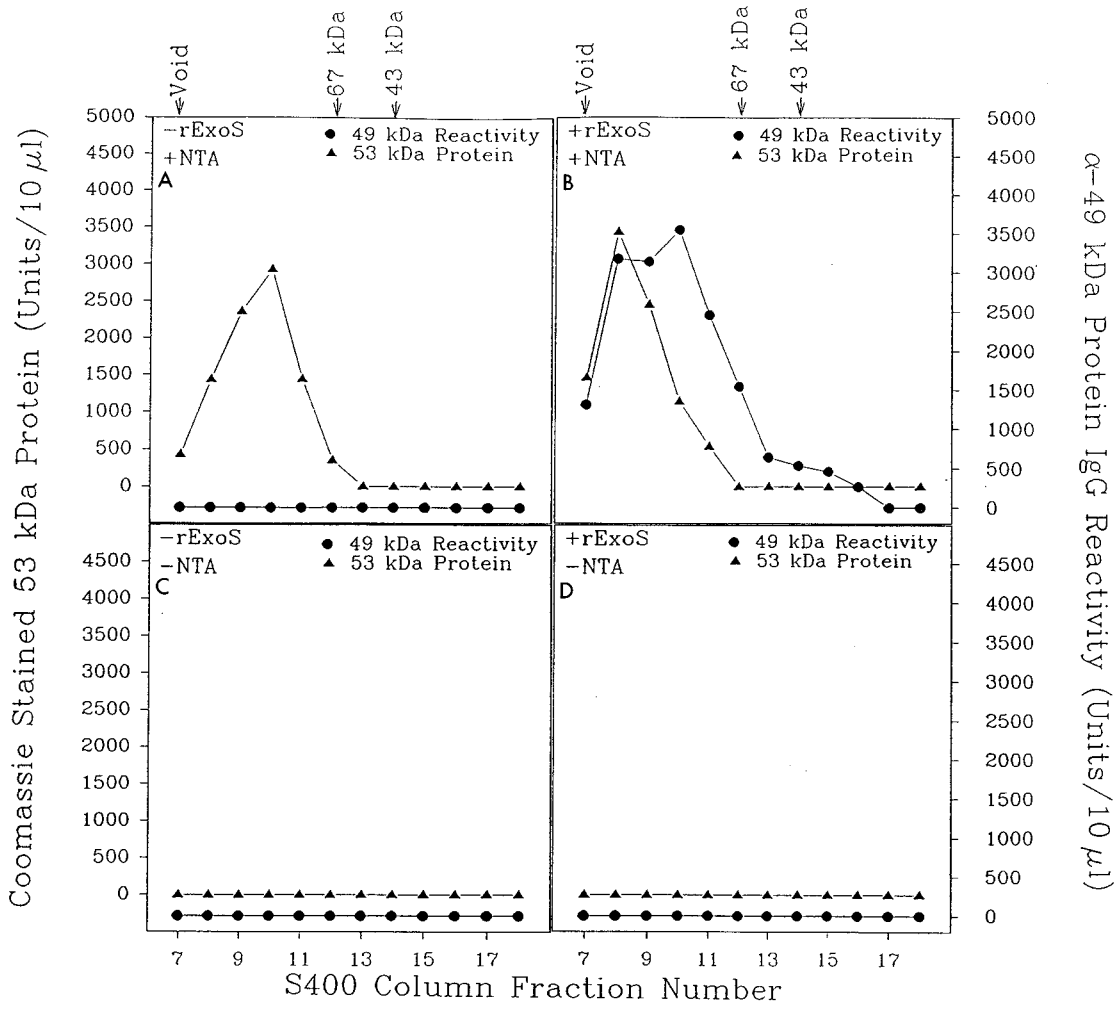

FIG. 4. Gel filtration of rExoS from *P. aeruginosa* PA103. Five hundred microliters of ammonium sulfate concentrates of the spent culture media from *P. aeruginosa*(pUCP18) (−rExoS) or *P. aeruginosa*(pUCP*exoS*) (+rExoS) cultured in the presence (+NTA) or absence (−NTA) of 10 mM NTA were subjected to gel filtration chromatography using Sephacryl S400-HR resin (21 ml of resin equilibrated in 25 mM Tris-HCl [pH 7.6]). Column fractions (1.2 ml) were analyzed for rExoS by measuring immunoreactivity against anti-49-kDa (α-49-kDa) protein IgG in Western blots (●) and for the 53-kDa form of exoenzyme S by densitometry of Coomassie blue-stained gels (▲). Datum points for levels of anti-49-kDa protein IgG immunoreactivity or 53-kDa form of exoenzyme S below the limits of detection were assigned values of zero. The fraction where blue dextran first appeared (Void) and the fraction containing the peak of BSA (67 kDa) and chicken egg albumin (43 kDa) as determined by densitometric analysis of SDS-polyacrylamide gels are indicated by arrows.

tected in a reaction containing diluent (25 mM Tris-HCl [pH 7.6]) in place of enzyme was found for native exoenzyme S, *E. coli* rExoS, or *P. aeruginosa* rExoS in the absence of FAS. There is a difference in the intensity of the amount of immunoreactive materials in rExoS expressed in *E. coli* with respect to native exoenzyme S and rExoS expressed in *P. aeruginosa*. This difference in immunoreactivity may be due to the facts that the protein band representing rExoS expressed in *E. coli* was diffuse and showed considerable degradation and that these degradation products may have a reactivity to the antibody different from that of full-length material.

ADP-ribosyltransferase activity of *P. aeruginosa* 388ΔExoS. *P. aeruginosa* 388 was subjected to allelic exchange to substitute *exoS* with a tetracycline gene cartridge which deleted the amino-terminal 350 amino acids and amino acids 361 to 374 of ExoS. This mutant was termed *P. aeruginosa* 388Δ*exoS*. Southern blot analysis of the chromosome of *P. aeruginosa* 388Δ*exoS* confirmed the deletion of the appropriate regions of *exoS* without perturbation to surrounding DNA (data not shown). SDS-PAGE analysis of spent culture media showed that *P. aeruginosa* 388Δ*exoS* did not express detectable amounts of ExoS by either Coomassie blue staining or immunoblot analysis using the anti-49/53-kDa form of exoenzyme S IgG (Fig. 6). Figure 6 also showed that the deletion of *exoS* did not influence the expression of the 53-kDa form of exoenzyme S. In the standard ADP-ribosylation assay of SBTI, spent culture media of *P. aeruginosa* 388Δ*exoS* did not express detectable amounts of ADP-ribosyltransferase activity and did not inhibit the ADP-ribosyltransferase activity of spent culture media of *P. aeruginosa* 388 (Fig. 7).

DISCUSSION

Exoenzyme S was originally defined by Iglewski and coworkers as an ADP-ribosylating activity produced by *P. aeruginosa* 388 which possessed biochemical properties that were different from exotoxin A, another ADP-ribosylating exoenzyme produced by *P. aeruginosa* (16). Two *Pseudomonas* proteins with

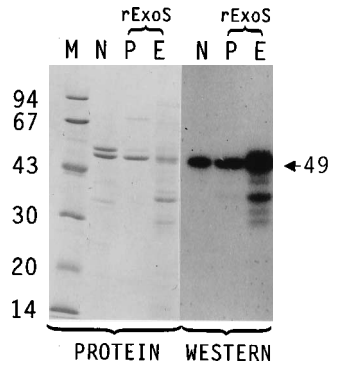

FIG. 5. Purified rExoS from *E. coli* and *P. aeruginosa* PA103. rExoS purified from either *E. coli* BL21(DE3)(pT7*exoS*) (lane E) or *P. aeruginosa* PA103 (pUCP*exoS*) (lane P) was subjected to SDS-PAGE (10% gel) in the presence of β-mercaptoethanol. Gels were stained for total protein with Coomassie blue (PROTEIN) or transferred to nitrocellulose and probed with anti-49-kDa protein IgG (WESTERN). An autoradiogram of a 24-h exposure with intensifying screens is pictured. Lane N shows the migration of native exoenzyme S purified from *P. aeruginosa* 388. The position where the 49-kDa form of exoenzyme S (2 pmol) migrated is indicated by the arrow. Lane M contains Coomassie blue-stained protein standards with molecular masses of 94, 67, 43, 30, 20, and 14 kDa.

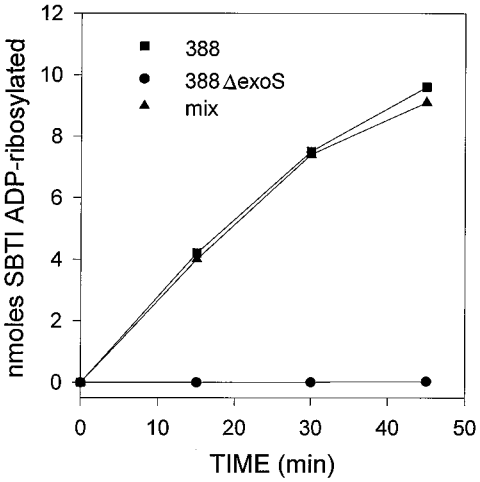

FIG. 7. ADP-ribosyltransferase activity of the spent culture media of *P. aeruginosa* 388 and *P. aeruginosa* 388Δ*exoS*. Equivalent volumes of ammonium sulfate concentrates of the spent culture media were analyzed for ADP-ribosyltransferase activity as described in Materials and Methods for the indicated times. Results are reported as nanomoles of SBTI ADP-ribosylated per microgram of total protein in the ammonium sulfate concentrate of *P. aeruginosa* 388 (■) or *P. aeruginosa* 388Δ*exoS* (●). ▲, level of ADP-ribosyltransferase activity in a mixture of ammonium sulfate concentrates of *P. aeruginosa* 388 and *P. aeruginosa* 388Δ*exoS*. Results are from a representative experiment of three independent experiments.

apparent molecular masses of 53 and 49 kDa were observed to copurify with the ADP-ribosyltransferase activity of exoenzyme S. The 49-kDa protein, termed the 49-kDa form of exoenzyme S (25), possessed ADP-ribosyltransferase activity upon extraction from polyacrylamide gels (5, 18, 25). Recently, the structural gene for the 49-kDa form of exoenzyme S (*exoS*) has been cloned from *P. aeruginosa* 388 (18).

rExoS was not detected in cell lysates of *E. coli* transformed with pUC19 containing the entire 2.3-kbp *Bam*HI *Pseudomonas* DNA fragment which contained *exoS* and 0.9 kbp of DNA flanking the 5′ end of *exoS*. Inclusion of NTA in the culture medium, an environmental signal that induces exoenzyme S production in *P. aeruginosa* (35), did not induce production of rExoS in *E. coli*. rExoS was expressed in *E. coli* under the regulation of the T7 RNA polymerase as a soluble cytosolic protein with an apparent molecular mass of 49 kDa, as determined by SDS-PAGE. Both the soluble fractions of cell lysates containing rExoS and purified rExoS possessed similar specific activities (within twofold) for the ADP-ribosylation of SBTI as native exoenzyme S purified from *P. aeruginosa* 388. The degradation of the rExoS expressed in *E. coli* limits an absolute determination of the specific activity of rExoS expressed in *E. coli*. These data showed that ExoS, the gene product of *exoS*, was responsible and sufficient for the expression of the enzymatic activity associated with exoenzyme S.

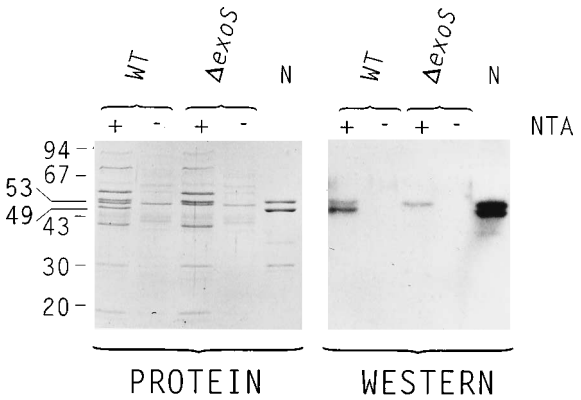

FIG. 6. Expression of ExoS in *P. aeruginosa* 388 and 388Δ*exoS*. *P. aeruginosa* 388 (wild type [WT]) and *P. aeruginosa* 388Δ*exoS* (Δ*exoS*) were cultured overnight in the presence of NTA. Equivalent volumes of ammonium sulfate concentrates of the spent culture media were subjected to SDS-PAGE (10% gel) in the presence of β-mercaptoethanol. Gels were stained for total protein (PROTEIN) or transferred to nitrocellulose and probed with anti-49/53-kDa protein IgG (WESTERN). An autoradiogram of a 48-h exposure with intensifying screens is pictured. Lane N contains native exoenzyme S purified from *P. aeruginosa* 388. To the left are the positions of migration of the 53- and 49-kDa forms of exoenzyme S and protein standards with molecular masses of 94, 67, 43, 30, and 20 kDa.

Like native exoenzyme S (17), rExoS expressed in *E. coli* was produced as a soluble aggregate as defined by gel filtration chromatography of the soluble fraction of cell lysates. This finding showed that the aggregation properties of exoenzyme S were inherent to rExoS and did not require the presence of other *Pseudomonas* proteins. Earlier studies showed that exoenzyme S was soluble following ultracentrifugation (17), which was consistent with exoenzyme S being associated with lipid. This lipid may contribute to the aggregated state of native or recombinant exoenzyme S. rExoS was also observed to be degraded during purification from *E. coli* (Fig. 5). Since little degradation was observed in crude cell lysates, it appeared that the degradation occurred during the purification steps. Future experiments will determine whether a rapid purification scheme can be developed to minimize rExoS degradation.

rExoS was expressed in *P. aeruginosa* PA103 as an NTA-inducible protein which was secreted into the culture medium in an aggregated form. These properties of rExoS were essentially identical to those of exoenzyme S from *P. aeruginosa* 388 (17, 35). As observed in *E. coli*, rExoS purified from *P. aeruginosa* PA103 possessed a similar specific activity (within twofold) for the ADP ribosylation of SBTI as native exoenzyme S purified from *P. aeruginosa* 388. These data indicated that the 2.3-kbp *Bam*HI *Pseudomonas* DNA fragment contained a functional NTA-inducible promoter for exoenzyme S in addi-

tion to the structural gene encoding for the 49-kDa form of exoenzyme S.

Expression of rExoS in *P. aeruginosa* PA103 appears to be advantageous compared with the *E. coli* expression system. *P. aeruginosa* PA103 secreted rExoS in the culture medium as a soluble, aggregated protein which could be purified directly from the spent culture medium. *P. aeruginosa* PA103, which has been shown to produce lower levels of extracellular proteases than other strains of *P. aeruginosa* (27), expressed rExoS without any detectable degradation upon purification from the culture media. Although the *P. aeruginosa* PA103 expression system provides a source of full-length rExoS for biochemical analysis, there are potential complicating factors within the *Pseudomonas* expression system. One potential complication in the *P. aeruginosa* PA103 expression system is the presence of exotoxin A in the spent culture medium, which could interfere with the analysis of exoenzyme S activity. Conditions in the exoenzyme S assays were adjusted to minimize the contribution of exotoxin A activity to the ADP-ribosyltransferase activity measured. Assays for exoenzyme S did not contain either the target for ADP-ribosylation by exotoxin A, eucaryotic elongation factor 2 (15), or conditions which have been shown to be required for the activation of exotoxin A in vitro (20, 36). A second complication in the *P. aeruginosa* PA103 expression system is the presence of low levels of endogenous exoenzyme S activity (10). Although the 49-kDa form of exoenzyme S was not detected in the spent culture media of *P. aeruginosa* PA103(pUCP18) by Western blot analysis, the spent culture media of *P. aeruginosa* PA103(pUCP18) cultured in the presence of NTA possessed exoenzyme S activity at a level of about 0.02% of the activity observed in *P. aeruginosa* PA103 (pUCP*exoS*) cultured under the same conditions. While this amount of endogenous exoenzyme S activity in *P. aeruginosa* PA103 does not prevent the use of this strain as an expression host, future experiments will address the engineering of a strain of PA103 that is devoid of exoenzyme S activity.

Exoenzyme S has been proposed to exist in two forms, a 53-kDa enzymatically inactive form and a 49-kDa enzymatically active form (25). On the basis of the observation that the 53- and 49-kDa forms of exoenzyme S share several properties, including immunological cross-reactivity (25) (Fig. 6), a common amino-terminal amino acid sequence (3), and common peptides following proteolytic and cyanogen bromide cleavage (14), a precursor-proteolytic product relationship has been proposed for the 53- and 49-kDa forms of exoenzyme S whereby the 53-kDa form of exoenzyme S undergoes a carboxyl-terminal cleavage to yield an enzymatically active 49-kDa form of exoenzyme S (3). While the observations that other bacterial ADP-ribosyltransferases undergo proteolytic activation lend some precedence to this hypothesis (8, 23, 36), the data presented in this study do not support this hypothesis. First, when expressed in either *E. coli* or *P. aeruginosa* PA103, rExoS comigrated with the 49-kDa form of native exoenzyme S purified from *P. aeruginosa* 388. If *exoS* encoded the precursor form of exoenzyme S, one would have predicted that rExoS would have migrated as a 53-kDa protein. Second, strain 388Δ*exoS* of *P. aeruginosa* that had undergone allelic exchange of *exoS* with a tetracycline gene cartridge showed the loss of the 49-kDa form of exoenzyme S without influencing expression of the 53-kDa form of exoenzyme S. This finding was consistent with the 53- and 49-kDa forms of exoenzyme S being encoded by different genes. Cloning the structural gene for the 53-kDa form of exoenzyme S will define the precise relationship between these two proteins.

The genetic relationship between *exoS* and the gene for exoenzyme S isolated from *P. aeruginosa* DG1 is unclear at this time (32). When provided in *trans* to *P. aeruginosa* PA103, the gene for exoenzyme S cloned from *P. aeruginosa* DG1 resulted in a 17-fold increase in exoenzyme S activity. However, when expressed in *E. coli*, the structural gene for exoenzyme S isolated from *P. aeruginosa* DG1 yielded an enzymatically inactive 68-kDa protein. Coupled with the observation that the reported cyanogen bromide fragment used to isolate the exoenzyme S gene from *P. aeruginosa* DG1 was not found within the predicted amino acid sequence of *exoS* (18), these data suggest that *exoS* differs from the gene for exoenzyme S cloned from *P. aeruginosa* DG1. Comparison of the nucleotide sequences will define the relationship of *exoS* and the gene for exoenzyme S isolated from *P. aeruginosa* DG1.

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