JOURNAL OF BACTERIOLOGY, Mar. 1996, p. 1412–1419 Vol. 178, No. 5
0021-9193/96/$04.00+0

Genetic Relationship between the 53- and 49-Kilodalton Forms of Exoenzyme S from *Pseudomonas aeruginosa*

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Received 28 August 1995/Accepted 27 December 1995

Exoenzyme S is an ADP-ribosylating extracellular protein of *Pseudomonas aeruginosa* that is produced as two immunologically related forms, a 49-kDa enzymatically active form and a 53-kDa inactive form. The postulated relationship between the two proteins involves a carboxy-terminal proteolytic cleavage of the 53-kDa precursor to produce an enzymatically active 49-kDa protein. To determine the genetic relationship between the two forms of exoenzyme S, *exoS* (encoding the 49-kDa form) was used as a probe in Southern blot analyses of *P. aeruginosa* chromosomal digests. Cross-hybridizing bands were detected in chromosomal digests of a strain of *P. aeruginosa* in which *exoS* had been deleted by allelic exchange. A chromosomal bank was prepared from the *exoS* deletion strain, 388Δ*exoS*::Tc, and screened with a probe internal to *exoS*. Thirteen clones that cross-hybridized with the *exoS* probe were identified. One representative clone contained the open reading frame *exoT*; this open reading frame encoded a protein of 457 amino acids which showed 75% amino acid identity to ExoS. The *exoT* open reading frame, cloned into a T7 expression system, produced a 53-kDa protein in *Escherichia coli*, termed Exo53, which reacted to antisera against exoenzyme S. A histidine-tagged derivative of recombinant Exo53 possessed approximately 0.2% of the ADP-ribosyltransferase activity of recombinant ExoS. Inactivation of *exoT* in an allelic-replacement strain resulted in an Exo53-deficient phenotype without modifying the expression of ExoS. These studies prove that the 53- and 49-kDa forms of exoenzyme S are encoded by separate genes. In addition, this is the first report of the factor-activating-exoenzyme-S-dependent ADP-ribosyltransferase activity of the 53-kDa form of exoenzyme S.

Exoenzyme S from *Pseudomonas aeruginosa* was first described as an ADP-ribosyltransferase activity that was distinct from exotoxin A (15). Several laboratories, utilizing a variety of purification protocols, found that exoenzyme S activity was associated with a large aggregate consisting predominantly of two proteins with molecular masses of 49 and 53 kDa (3, 18, 26, 33). Analysis of gel-purified proteins suggested that the 49-kDa protein was enzymatically active whereas the 53-kDa protein possessed little to no ADP-ribosyltransferase activity (3, 26). The two forms of exoenzyme S were immunologically related (26). Purified 49- and 53-kDa proteins had similar amino-terminal sequences and proteolytic patterns when exposed to cyanogen bromide, trypsin, or chymotrypsin (14). Other experiments demonstrated coordinate expression and regulation of the 49- and 53-kDa forms of exoenzyme S (10, 26). These data were consistent with a model in which the 53-kDa protein was proteolytically processed at the carboxy terminus to yield an active molecule (3). The model predicted that a single gene encoded exoenzyme S.

To genetically characterize the structural gene for exoenzyme S, Kulich et al. purified the 49-kDa form of exoenzyme S and used amino-terminal and tryptic-peptide amino acid sequences to design a PCR strategy to amplify DNA probes encoding the amino terminus and an internal portion of the molecule (20). With both probes, *exoS* was cloned and sequenced from *P. aeruginosa* 388. The translated product was predicted to encode a protein of 453 amino acids with a predicted molecular mass of 48,302 Da. In studies using both *Escherichia coli* and *P. aeruginosa* hosts (19), recombinant ExoS (rExoS) was expressed as a protein which comigrated with the 49-kDa form of native exoenzyme S in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Allelic-replacement studies with *P. aeruginosa* demonstrated that deletion of *exoS* eliminated expression of the 49-kDa protein but did not effect the synthesis of the 53-kDa protein (19). These data indicated that the 53- and 49-kDa polypeptides were encoded by separate genes.

In this report, we show that the 49- and 53-kDa forms of exoenzyme S are encoded by separate genes. The gene encoding the 53-kDa form of exoenzyme S (*exoT*) was cloned, sequenced, and expressed. rExo53 possessed 0.2% of the ADP-ribosyltransferase activity of rExoS. Enzyme activity was dependent on the eukaryotic protein, factor activating exoenzyme S or FAS (4). Inactivation of *exoT* was achieved by insertional mutagenesis. These studies should aid in defining the role of exoenzyme S in *P. aeruginosa* pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α and BL21 were cultured in Luria-Bertani (LB) broth or agar with ampicillin (100 μg/ml) at 37°C unless otherwise noted. *E. coli* TG1 was used as a host strain for M13 bacteriophage propagation, using standard protocols (23). For exoenzyme S production, *Pseudomonas* strains were cultivated in a deferrated dialysate of trypticase soy broth containing 10 mM nitrilotriacetic acid as previously described (9).

Southern blot analysis. Chromosomes from *P. aeruginosa* 388, 388Δ*exoS*::Tc, and 388*exoT*::Tc were isolated (10), digested with restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, Md.), and subjected to Southern blot analysis (23). Restriction fragments containing DNA internal to the coding region for *exoS* (*Nsi*I-*Nru*I), *exoT* (*Sma*I 900), and a tetracycline interposon (TnTc) were isolated from agarose gels, radiolabeled with [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear Research Products, Wilmington, Del.) by utilizing a random-primer kit (Bethesda Research Laboratories), and used as

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)	Reference or source
P. aeruginosa strains		
388	Wild type	2
388Δ*exoS*::Tc	*exoS* mutant	19
388*exoT*::Tc	Tetracycline-inactivated *exoT*	This study
E. coli strains		
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	23
BL21(DE3)	*hsd5 gal*(λcIts857 *ind1 Sam*7 *nin5 lacUV5*-T7 gene 1)	31
DH5α	*supE44* Δ*lacU169* (f80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	12
SM10	*thi thr leu tonA lacY supE recA*::Rp4-2Tc::Mu Km	30
Plasmids		
pUC18	Wild type	25
pUC18N79	Bank clone containing 6.5-kb *Bgl*II fragment encoding Exo53	This study
pUC18*Pst*I4.5	pUC18 containing 4.5-kb *Pst*I fragment	This study
pT7-7	Wild type	32
pT7*exoS*	pT7-7 containing *exoS* (*Nsi*I-*Bam*HI)	19
pT7*exoT*	pT7-7 containing *exoT* (*Nsi*I-*Bam*HI)	This study
pET16b	Wild type	Novagen
pET*exoS*	pET16b containing *exoS* (*Nsi*I-*Bam*HI)	19
pET*exoT*	pET16b containing *exoT* (*Nsi*I-*Bam*HI)	This study
Mini-Tn*5* Tc	Tetracycline interposon	5
pNOT19	Wild type	29
pNOT*exoT*	pNOT19 containing 4.5-kb *Pst*I fragment	This study
pNOT*exoT*::Tc	pNOT*exoT* containing a tetracycline marker within the *Nsi*I site	This study
pMOB3	Wild type	29
pMOB*exoT*::Tc	pNOT*exoT*::Tc containing the MOB cassette	This study

probes. Blots were washed under high-stringency conditions (23) and subjected to autoradiography.

Construction and screening of the *P. aeruginosa* 388Δ*exoS*::Tc chromosomal bank. Chromosomal DNA from *P. aeruginosa* 388Δ*exoS*::Tc was digested to completion with *Bgl*II, phenol-chloroform extracted, and precipitated. Digested DNA was ligated into pUC18 (digested with *Bam*HI), transformed into competent *E. coli* DH5α (12), and plated on ampicillin plates (100 μg/ml) coated with 50 μl of a 2% solution of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Transformants containing inserts (2,200) were picked onto LB agar plates, transferred to nitrocellulose filters (Schleicher and Schuell, Keene, N.H.), and screened by colony blot hybridization (23) with the *exoS Nsi*I-*Nru*I restriction fragment labeled with [α-^{32}P]dCTP as a probe. Blots were washed under high-stringency conditions and subjected to autoradiography.

DNA sequence analysis. Southern blot analysis indicated that the coding region for Exo53 was localized on a 4.5-kb *Pst*I restriction fragment. Subclones of the region were made in M13 vectors (25), and DNA sequencing reactions were performed (28) by using a TaqTrack kit (Promega Biochemicals, Madison, Wis.). Oligonucleotide primers were designed to fill gaps in the sequence.

Expression of *exoT* in *E. coli*. The pT7*exoT* and pET*exoT* expression clones were constructed as follows. pUC18*Pst*I4.5 was partially digested with *Sal*I. A fragment of 2.7 kb, corresponding to the *exoT* open reading frame plus an additional 700 bases of 5′ sequence, was excised from an agarose gel and purified. The *Sal*I 2.7-kb fragment was ligated into the *Xho*I restriction site of pET16b (Novagen, Madison, Wis.) and screened for orientation. This resulted in the positioning of a *Bam*HI site on the 3′ end of *exoT* and allowed for the removal of the entire *exoT* gene as an *Nsi*I-*Bam*HI restriction fragment. The *Nsi*I-*Bam*HI fragment of pT7*exoS* (encoding the 49-kDa form of exoenzyme S) (19) was then replaced with the *exoT Nsi*I-*Bam*HI fragment, resulting in pT7*exoT*. In the same manner, the *Nsi*I-*Bam*HI fragment of pET*exoS* was replaced with the *exoT Nsi*I-*Bam*HI fragment, resulting in pET*exoT* (19). The clones were then transformed into *E. coli* BL21 for expression studies.

For expression of pT7 and pET clones, cells were grown, induced with 0.3 mM isopropylthio-β-galactoside (IPTG), lysed with SDS, and subjected to SDS-PAGE as previously described (16, 19, 21). For Western blots (immunoblots), proteins were transferred to nitrocellulose (34) and probed with two different polyclonal rabbit antisera. One anti-exoenzyme S immunoglobulin G (IgG) antiserum reacted with both ExoS and Exo53 (9, 26), and the second IgG antiserum reacted predominantly with ExoS (18). Bound IgG was detected by using ^{125}I-labeled protein A and autoradiography (18).

Enrichment of rHisExo53. After overnight growth, strains containing plasmids encoding rHisExoS and rHisExo53 were diluted 50-fold into 800 ml of LB broth with ampicillin (100 μg/ml) and incubated at 30°C. After a 2-h incubation, 0.3 mM IPTG was added, cultivation was continued for an additional 2 h, and cellular extracts were prepared as described previously (16). Cellular extracts were filtered (0.45-μm-pore-size cellulose nitrate filter) and loaded onto a 2-ml nickel column (Novagen). The column was washed with 20 ml of binding buffer, followed by binding buffer containing 0.1 M imidazole. Bound proteins were

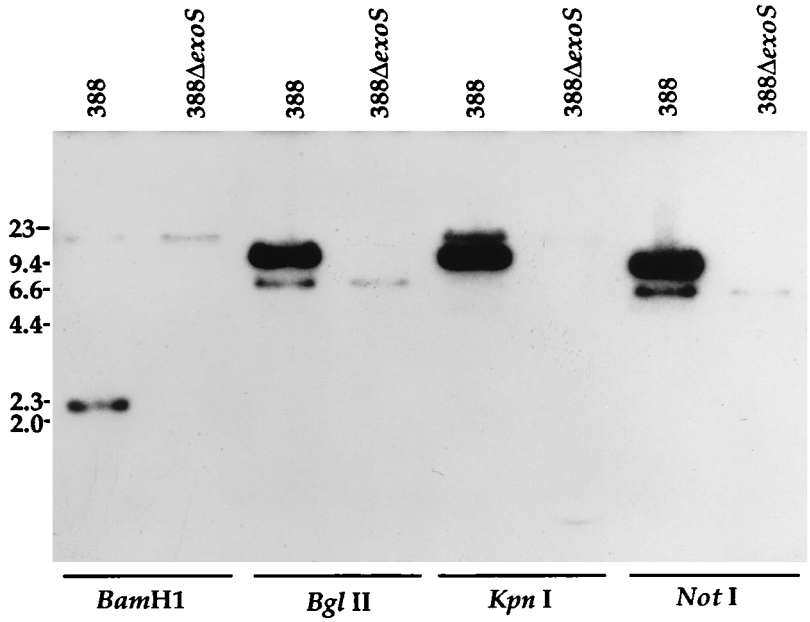

FIG. 1. Southern blot comparison of *P. aeruginosa* 388 and 388Δ*exoS*. Southern blot of restriction endonuclease digests of 388 and 388Δ*exoS*::Tc chromosomes. The probe used was an internal fragment of *exoS* which was deleted by allelic exchange in 388Δ*exoS*::Tc. Numbers on the left are molecular size markers, in kilobases.

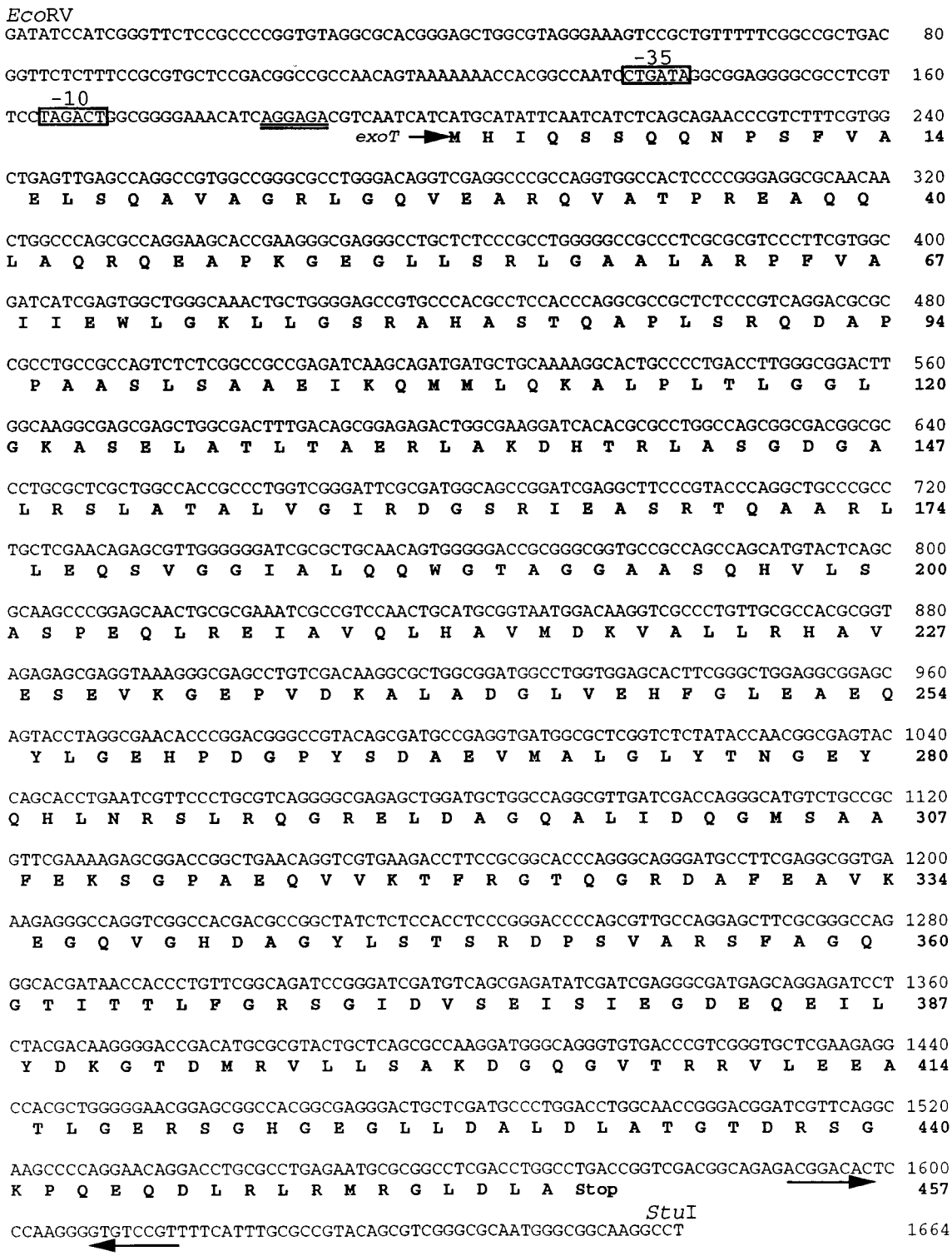

FIG. 2. Nucleotide sequence of *exoT*. The nucleotide sequence of an *Eco*RV-*Stu*I fragment containing *exoT* is shown. Listed below the nucleotide sequence in boldface is the deduced amino acid sequence of Exo53. The proposed −35 and −10 promoter consensus sequences are enclosed by boxes. A consensus Shine-Dalgarno sequence is double underlined. An inverted repeat sequence proposed to act as a transcriptional terminator is shown with convergent arrows.

eluted with binding buffer containing 0.5 M imidazole. As a negative control, an extract (rHis) was also prepared from *E. coli* BL21 pET16b. Recombinant-protein concentrations were determined by SDS-PAGE and Coomassie blue staining, using bovine serum albumin as a standard. Dried gels were analyzed by using an Ambis 4000 optical imaging system (Ambis, San Diego, Calif.).

Enzymatic activity of rHisExo53. Partially purified rHis, rHisExoS, and rHis Exo53 were assayed for ADP-ribosyltransferase activity as previously described (16). Recombinant proteins (diluted in 10 mM Tris-Cl [pH 7.6] containing egg albumin [0.1 mg/ml]) were incubated in a solution containing 0.2 M sodium acetate (pH 6.0), 30 μM soybean trypsin inhibitor (target), and 30 μM [adeny-

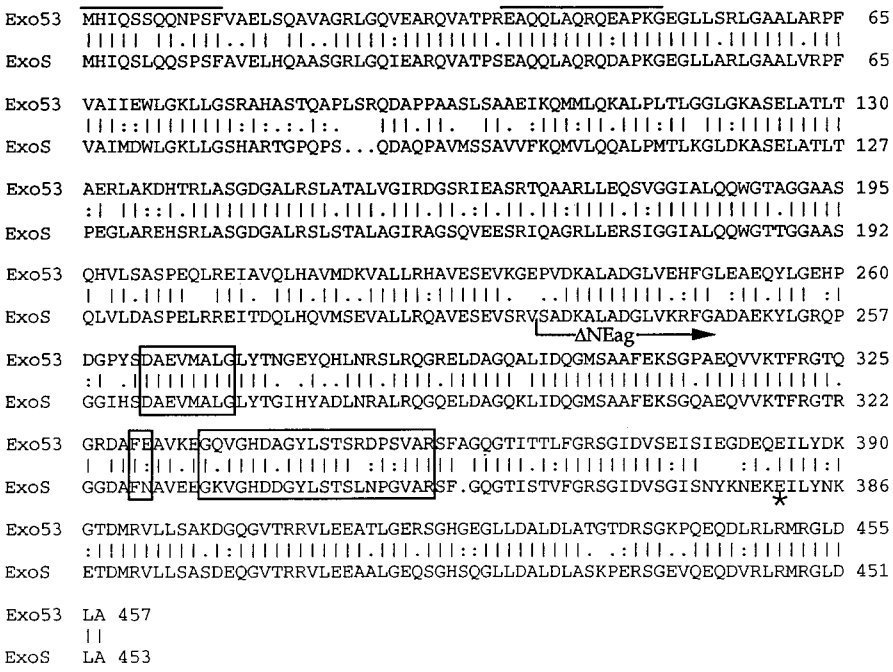

FIG. 3. BESTFIT alignment of the ExoS and Exo53 amino acid sequences. ExoS and Exo53 are 75% identical over the entire lengths of the proteins. The sequences of both amino-terminal and tryptic peptides obtained from partially purified Exo53 are indicated by a solid line. ExoS (453 amino acids) has a predicted molecular mass of 48,312 Da, while Exo53 (457 amino acids) has a predicted molecular mass of 48,532 Da. Amino acid residues demonstrating homology to the sequences of other members of the bacterial ADP-ribosyltransferase family are boxed. An asterisk marks the position of an active-site glutamic acid residue identified in ExoS. An aggregation domain has been mapped to the amino-terminal 100 residues of ExoS. The location of a catalytically active truncated form of ExoS (ΔNEag) is indicated with an arrow. Similarity between amino acid residues is indicated by the number of dots.

latephosphate-^{32}P] NAD (1 Ci/mmol; New England Nuclear Research Products) in the presence or absence of 34.5 nM FAS at 25°C. Reactions were terminated by the addition of SDS-polyacrylamide gel sample buffer and electrophoresed on SDS–13% polyacrylamide gels. Stained bands corresponding to soybean trypsin inhibitor were excised from the gels, and radioactivity was monitored by scintillation counting.

Construction of an *exoT* chromosomal mutation. An allelic-replacement technique was used to introduce a specific mutation into the *P. aeruginosa* 388 chromosome (10, 29). Briefly, the 4.5-kb *Pst*I restriction fragment containing *exoT* was cloned into pNOT19. A tetracycline cartridge (5) was inserted into the unique *Nsi*I site which overlaps the ATG start codon of *exoT*, resulting in the plasmid pNOT*exoT*::Tc. A mobilization cartridge (MOB) (29) containing the counterselectable marker *sacB* was added to pNOT*exoT*::Tc, resulting in pMOB*exoT*::Tc. pMOB*exoT*::Tc was transferred to *P. aeruginosa* 388 by conjugation, and tetracycline-resistant transconjugants were selected on Vogel Bonner minimal media (35) with tetracycline (100 μg/ml). Merodiploids were forced to resolve plasmid sequences by subculture on LB plates containing 5% sucrose and 100 μg of tetracycline per ml (29). The allelic-replacement strain was subjected to Western blot analysis, ADP-ribosyltransferase assays, and Southern blot analysis to confirm genotype and phenotype.

Nucleotide sequence accession number. The accession number for the nucleotide sequence reported for *exoT* is L46800.

RESULTS

Southern blotting analysis. To determine the genetic relationship between the 49- and 53-kDa forms of exoenzyme S, a Southern blot comparison of *P. aeruginosa* 388 and 388Δ*exoS*::Tc chromosomes was performed with the *Nsi*I-*Nru*I *exoS* fragment as a probe. With wild-type 388, both high- and low-intensity signals were detected (Fig. 1). In lanes containing chromosomal fragments from 388Δ*exoS*::Tc, only the low-intensity band was detected (Fig. 1). The high-intensity band corresponded to *exoS* from a previously performed (2.3-kb *Bam*HI fragment or a 9.0-kb *Kpn*I fragment) restriction fragment analysis (20). The lower-intensity signal appeared to be a sequence related to *exoS*, possibly encoding the 53-kDa form of exoenzyme S.

Construction and screening of the *P. aeruginosa* 388Δ*exoS*::Tc bank. A plasmid bank of the *P. aeruginosa* 388Δ*exoS*::Tc chromosome was probed with the *exoS Nsi*I-*Nru*I restriction fragment under high-stringency conditions. After autoradiography, 13 positive clones were identified. Plasmid DNA was isolated from each clone, digested with *Sal*I and *Pst*I, and subjected to Southern blot analysis. When probed with the *Nsi*I-*Nru*I fragment, a 2.0-kb *Sal*I fragment and a 4.5-kb *Pst*I restriction fragment hybridized with plasmid DNA isolated from all 13 clones (data not shown). One representative clone, pUC18N79, was selected for subsequent characterization. The 4.5-kb *Pst*I fragment of pUC18N79 was isolated from an agarose gel and cloned into pUC18 (pUC18*Pst*I4.5).

DNA sequence analysis. Southern blot analysis of pUC18N79 identified several restriction fragments (*Sal*I 2000, *Eco*RV 1300, *Sma*I 900, and *Sal*I 750) which hybridized with the internal *Nsi*I-*Nru*I *exoS* probe (data not shown). These hybridizing fragments were cloned into M13 vectors and subjected to DNA sequence analysis. Additional subclones were produced in M13 vectors, and gaps in the sequence analysis were resolved with synthetic oligonucleotide primers. The nucleotide sequence of the 1,664-bp *Eco*RV-*Stu*I fragment is reported here (Fig. 2).

Alignment of the *Eco*RV-*Stu*I nucleotide sequence with *exoS* identified a region of homology (80%) which started at the −62 position of *exoS* (base 147 of *Eco*RV-*Stu*I) and continued throughout the entire coding sequence, terminating 33 nucleotides (base 1607 of *Eco*RV-*Stu*I) downstream of the *exoS* stop codon. Open reading frame and codon usage analyses (37) of the *Eco*RV-*Stu*I region identified one sequence encoding a protein of 457 amino acids with a predicted molecular mass of 48,532 Da. BESTFIT alignment (6) to ExoS revealed a high degree of identity (75%) and an overall similarity of 82% when conservative amino acid substitutions were

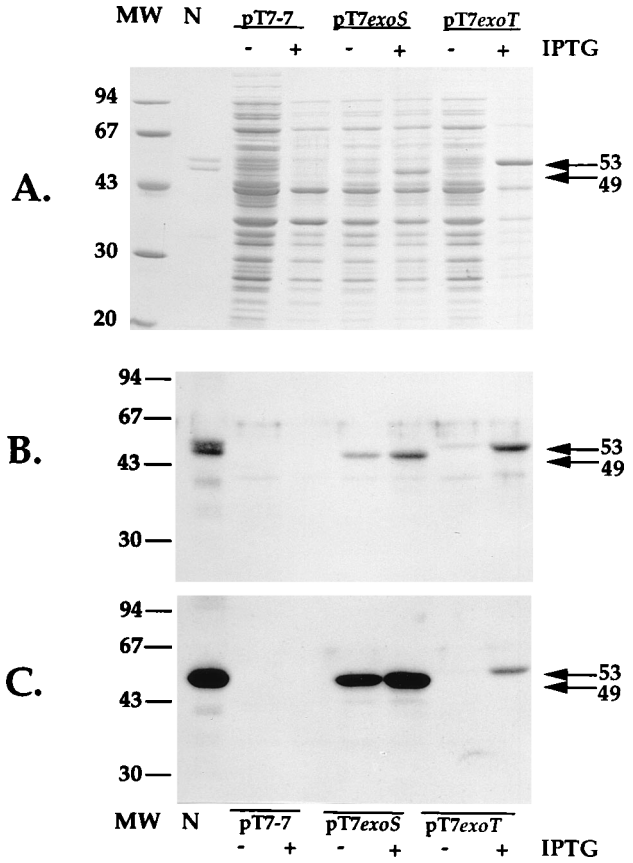

FIG. 4. Expression of rExo53 in *E. coli* BL21. (A) Coomassie-stained gel. Lane N shows the 49- and 53-kDa forms of exoenzyme S as purified from *P. aeruginosa* 388. pT7-7 is a vector control lacking an inserted sequence. pT7*exoS* contains the structural gene for the 49-kDa form of exoenzyme S. pT7*exoT* contains the structural gene for the 53-kDa form of exoenzyme S. (B) Western blot with anti-exoenzyme S IgG which reacts with both the 49- and 53-kDa forms of exoenzyme S. (C) Western blot with anti-exoenzyme S IgG which predominantly reacts with the 49-kDa form of exoenzyme S. Arrows indicate the positions of the 49- and 53-kDa forms of exoenzyme S. Numbers at the left indicate molecular weight (MW), in thousands.

considered (Fig. 3). On the basis of the observed homology to ExoS, this protein was termed Exo53 and the corresponding gene was termed *exoT*.

Comparison of the nucleotide sequences of *exoS* and *exoT* identified unique and common regions. The promoter of *exoS* and the putative promoter of *exoT* possess identical RNA polymerase binding elements and an ExsA consensus binding site positioned the same distance from the −35 region (13, 38). These data suggest that *exoS* and *exoT* are coordinately regulated by ExsA, which is consistent with genetic data (10, 26). A sequence with characteristics of a rho-independent transcriptional termination signal was located downstream of *exoT* (1, 27). No similar element was identified in the *exoS* gene. These data and the fact that the *exoS* mRNA appears to be larger than the predicted open reading frame (38) suggest that additional sequences downstream of *exoS* may be transcribed.

Expression of *exoT* in *E. coli*. Native exoenzyme S from *P. aeruginosa* 388 is produced as an aggregate of two proteins with molecular masses of 49 and 53 kDa as determined by SDS-PAGE. From the predicted amino acid sequences, ExoS and Exo53 differed in molecular mass by 230 Da. To determine the relative mobility of Exo53 on SDS-polyacrylamide gels, the coding sequence was cloned into pT7-7 for expression analysis. As controls, vector alone (pT7-7) and vector containing the *exoS* gene (pT7*exoS*) were utilized. Cellular extracts were prepared and subjected to SDS-PAGE and Western blot analyses (Fig. 4). Native exoenzyme S (including both the 49- and 53-kDa forms) purified from culture supernatants of *P. aeruginosa* 388 (18) was used for comparison of molecular masses. In Coomassie-stained gels, the control pT7-7 lanes expressed no additional protein bands when induced with IPTG (Fig. 4A). Induction of both pT7*exoS* and pT7*exoT* with IPTG resulted in the appearance of an additional band. The induced band from pT7*exoS* migrated at the apparent molecular mass of native ExoS, while the apparent molecular mass of the induced product from pT7*exoT* was similar to that of native Exo53 (Fig. 4A, lane N). The blot in Fig. 4B was probed with anti-exoenzyme S IgG which reacted with both ExoS and Exo53, while the blot in Fig. 4C was probed with IgG which reacted primarily with ExoS. In pT7-7 vector control lanes, no reactivity with either IgG was detected. In the blot in Fig. 4B, both anti-exoenzyme S IgG preparations reacted with the induced bands of pT7*exoS* and pT7*exoT*. In the blot in Fig. 4C, the antigens recognized were identical to those recognized in the blot in Fig. 4B except that the signal intensity was stronger for ExoS. Products detected by Western blot analysis coincided with the induced protein products of the Coomassie-stained gel in Fig. 4A. The immunological cross-reactivity and the apparent molecular mass of 53 kDa were consistent with *exoT* being the *P. aeruginosa* gene responsible for production of the 53-kDa form of exoenzyme S.

ADP-ribosyltransferase activity of rHisExo53. To enzymatically characterize Exo53, a fusion derivative (rHisExo53) was partially purified by using the pET Ni^{2+} affinity column chromatography system (Novagen). Ten histidine residues encoded within a leader sequence provided by the vector (pET16b) were fused in frame to the amino terminus of Exo53. As controls, a fusion derivative of ExoS (rHisExoS [49 kDa]) and an extract (rHis) from *E. coli* BL21 carrying pET16b were also prepared. Ni^{2+} chromatography-purified fusion proteins were analyzed by SDS-PAGE and Western blotting. Antibody to exoenzyme S reacted with ExoS and Exo53 fusion derivatives and did not react with the negative control rHis extract (data not shown). Purified fusion proteins were assayed for ADP-ribosyltransferase activity by monitoring the incorporation of radiolabel from NAD to a target protein, soybean trypsin inhibitor. No ADP-ribosyltransferase activity was detected in the negative control, the rHis extract, under any of the conditions tested (data not shown). In the presence of FAS, both rHis ExoS and rHisExo53 catalyzed detectable levels of ADP-ribosyltransferase activity (Fig. 5A). The activity of rHisExo53 was 0.2% of that of rHisExoS. When rHisExo53 was assayed in the absence of FAS, no ADP-ribosyltransferase activity was detected (Fig. 5B). rHisExoS ADP-ribosyltransferase activity has previously been shown to be FAS dependent (16). The FAS dependence and linear kinetics of rHisExo53 ADP-ribosyltransferase activity demonstrate that Exo53 possesses ADP-ribosyltransferase activity.

***P. aeruginosa* mutant deficient in exoenzyme S synthesis.** To confirm that *exoS* and *exoT* were encoded by separate genes, a chromosomal mutation in *exoT* was introduced into *P. aeruginosa* 388. A tetracycline interposon (TnTc) was inserted into the *Nsi*I site of *exoT*, disrupting the ATG start codon, and the mutant allele was returned to the chromosome of *P. aeruginosa* 388 by allelic exchange. The *exoT*::Tc mutation was confirmed by Southern blotting of chromosomal digests (data not shown). As determined by SDS-PAGE and Western blot analyses, the

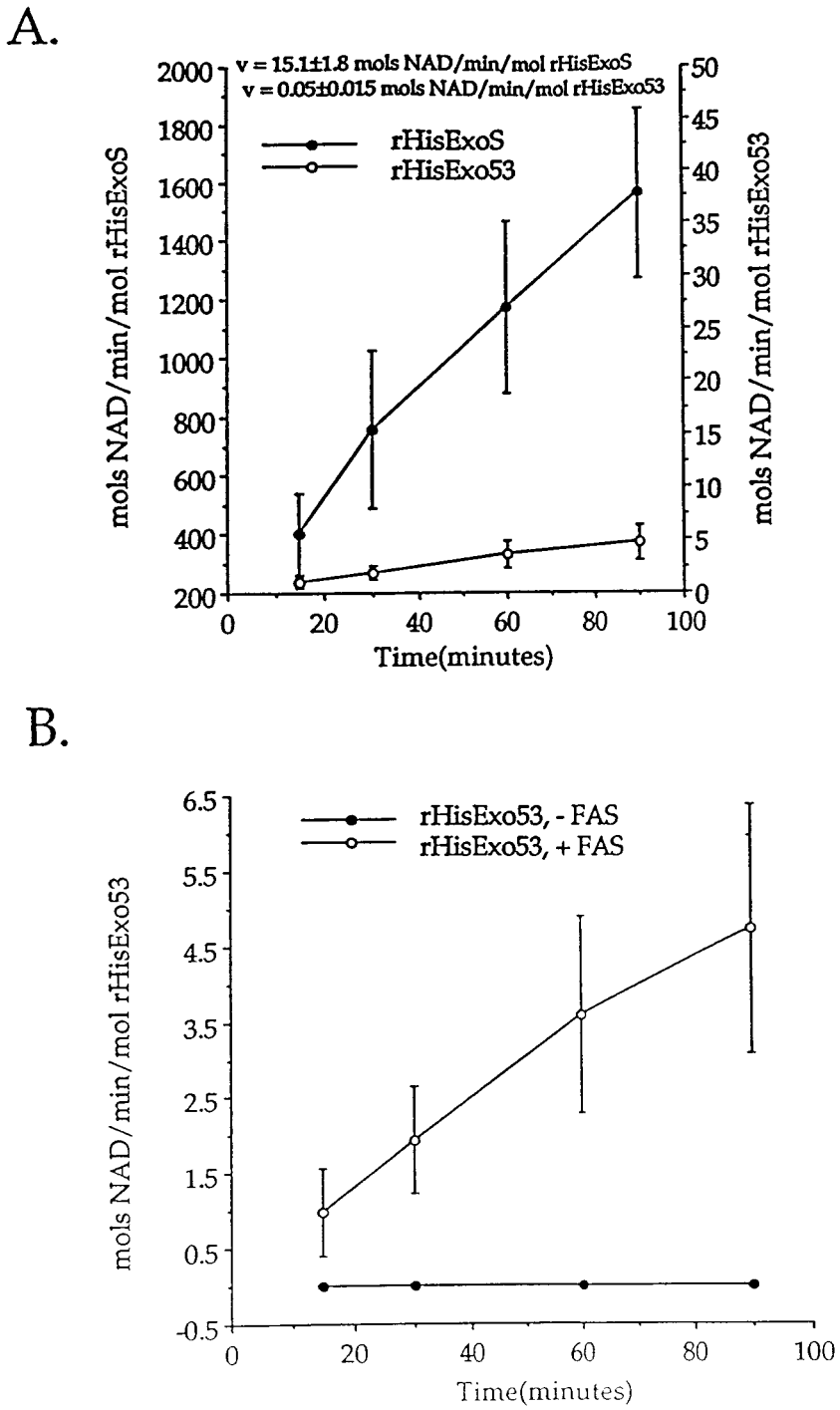

FIG. 5. ADP-ribosyltransferase activity of rHisExo53. (A) ADP-ribosyltransferase activities of rHisExoS and rHisExo53. Note that different scales are used for the *y* axes. (B) FAS dependence of rHisExo53. Negative controls include a reaction mixture containing FAS without enzyme (rHisExoS or rHisExo53). Counts present in the target protein of the negative control were subtracted from values reported in each curve.

mutant strain (388*exoT*::Tc) was deficient in the synthesis of Exo53 but continued to produce ExoS (Fig. 6).

DISCUSSION

In animal models of *P. aeruginosa* infection, exoenzyme S expression correlates with tissue destruction and an enhanced ability of *P. aeruginosa* to spread to the bloodstream (2, 17, 26). These conclusions were based on virulence analyses of a *P. aeruginosa* mutant that was deficient in the expression of both the 49- and 53-kDa forms of exoenzyme S. Subsequent genetic analysis mapped the mutation to a regulatory locus (9). Although exoenzyme S production was implicated in virulence, the contribution of other coordinately regulated factors could not be formally eliminated. Data presented in this report combined with earlier analyses demonstrate that separate genes

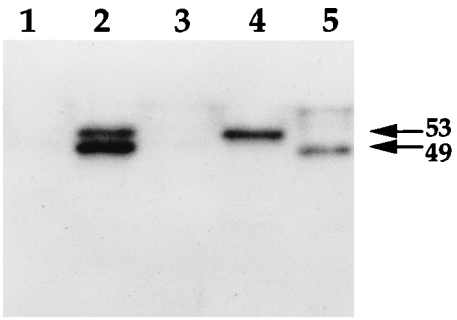

FIG. 6. Western blot analysis of concentrated culture supernatants. Lane 1 is wild-type *P. aeruginosa* 388 grown in the absence of nitrilotriacetic acid (noninducing conditions for exoenzyme S production). Lane 2 is 388 grown in the presence of nitrilotriacetic acid (inducing conditions). Lane 3 contains 388*exs1*::Tn*1*, a transposon-induced mutant deficient in the production of both ExoS and Exo53. Lanes 4 and 5 contain 388Δ*exoS*::Tc and 388*exoT*::Tc, respectively. The 53- and 49-kDa forms of exoenzyme S are indicated by arrows.

encode each form of exoenzyme S and that the 53-kDa form of exoenzyme S possesses ADP-ribosyltransferase activity. This new information will require a reassessment of the role of each gene product in *P. aeruginosa* infections.

Alignment of the primary amino acid sequences showed that ExoS and Exo53 were 75% identical, with similar amounts of identity throughout the sequences. This is similar to the amino acid homology observed between cholera toxin and the heat-labile enterotoxin of *E. coli* (24). Although FASTA alignments between ExoS and other ADP-ribosylating exotoxins showed little primary amino acid homology, local homology between ExoS and regions of the active sites of cholera toxin and heat-labile enterotoxin was observed (20). These local regions of homology are also present within Exo53 (the alignments are indicated in the sequence comparison of ExoS and Exo53 in Fig. 3), with one exception in which the Phe-Asn sequence of ExoS is a Phe-Glu sequence in Exo53. The function of this sequence will be addressed in future studies.

Deletion mapping (16) showed that the carboxyl-terminal 222 amino acids constituted the FAS-dependent ADP-ribosyltransferase domain of ExoS. The high degree of homology in this region of ExoS and Exo53 challenges one to predict the mechanism for the lower level of ADP-ribosyltransferase activity of Exo53 relative to that of ExoS. Several mechanisms are envisioned for the lower amount of ADP-ribosyltransferase activity of Exo53: (i) relative to ExoS, Exo53 possesses a narrow range of target proteins that can be ADP-ribosylated, and its preferred target has not been identified; (ii) the lack of observed ADP-ribosyltransferase activity of Exo53 is not due to a low level of intrinsic ADP-ribosyltransferase activity but is due to stearic interference of the enzymatic domain by its amino-terminal region; or (iii) Exo53 lacks a specific amino acid residue(s) that prevents expression of ADP-ribosyltransferase activity at levels comparable to that of ExoS. With respect to the possibility that the low level of ADP-ribosyltransferase activity of Exo53 was due to the lack of a specific residue, E-381 of ExoS was recently shown to possess a catalytic function in the ADP-ribosyltransferase reaction (22). BESTFIT alignment predicts that E-385 of Exo53 is the homolog of E-381 of ExoS (Fig. 3). With respect to target specificity, as observed for SBTI, Exo53 is able to ADP-ribosylate H-ras as well as proteins within CHO cell extracts at a reduced rate relative to that of ExoS (1a). These results indicate that the reduced activity of Exo53 relative to that of ExoS is intrinsic to its catalytic domain. Studies are underway to resolve the mechanism for the low level of ADP-ribosyltransferase activity of Exo53 by biochemical and molecular genetic evaluation of its catalytic activity.

The structural genes for the 53- and 49-kDa forms of exoenzyme S may have arisen by a gene duplication event. To determine if the two genes were closely linked, Southern blot hybridization experiments with cosmid clones containing *exoS* were performed with a probe corresponding to the 3′ end of *exoS*. Cosmid clones contained an average of 20 kb of *P. aeruginosa* 388 DNA. *P. aeruginosa* 388 chromosomal DNA digested with the same enzyme as the cosmid clones was used as a control. Of the 11 clones examined, all contained a high-intensity band corresponding to *exoS* but none contained a cross-hybridizing band with a lower intensity (8). These results suggest that *exoS* and *exoT* are not contiguous on the *P. aeruginosa* chromosome.

The dissimilarity of sequences flanking the ExoS and Exo53 structural genes may have implications regarding the export of each protein. The *exoS* 5′ region contains divergent promoters that are coordinately regulated by ExsA (38). One promoter regulates *exoS* transcription, while the other promoter controls transcriptional initiation of an operon that contains several open reading frames (38). The predicted protein sequence of ORF 1 possesses homology to SycE (36) and YerA (7, 11) of *Yersinia enterocolitica* and *Y. pseudotuberculosis*, respectively (38). Functional analyses of SycE and YerA indicate that they serve as molecular chaperones for YopE, a cytotoxin of yersiniae. The binding of YerA or SycE to the amino terminus of YopE maintains the protein in a secretion-competent conformation. We have postulated that ORF 1 may facilitate the secretion of ExoS by a similar mechanism. From limited nucleotide sequence information for DNA flanking *exoT*, *orf1*-like sequences appear to be absent. These data indicate that the predicted ORF 1 of the ExoS locus may facilitate secretion of both ExoS and Exo53.

Although these studies have clarified the relationship between the 53- and 49-kDa forms of exoenzyme S, many new questions arise. The cloning and sequence analysis of both genes and construction of targeted mutations will allow definitive studies of the contribution of exoenzyme S to *P. aeruginosa* virulence and pathogenesis. Examination of the enzymatically active protein, ExoS, and the relatively enzymatically inactive form, Exo53, may aid in defining domains involved in NAD binding, binding by FAS, catalysis, and recognition of eukaryotic target proteins. Questions regarding the distribution of *exoS* and *exoT* among different *P. aeruginosa* isolates, their evolutionary relationship, and their association with acute or chronic infections can now be addressed.

ACKNOWLEDGMENTS

We thank Haian Fu for the generous gift of recombinant FAS. We also thank J. Goranson and P. Rooney for assistance in cloning *exoT*.

This work was supported by grants R29-AI31665 (to D.W.F.), KO4-AI01289 (to D.W.F.), RO1 AI31062 (to J.T.B.), and KO4-AI01087 (to J.T.B.) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Timothy Yahr was supported by Cystic Fibrosis Foundation grant G-797 (to D.W.F.).

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