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Transcriptional Analysis of the *Pseudomonas aeruginosa* Exoenzyme S Structural Gene

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The transcriptional regulation of the *Pseudomonas aeruginosa exoS* gene was investigated. Expression of *exoS* in *P. aeruginosa* PA103 was dependent upon growth in a low-cation environment and the presence of a functional *exsA* gene. Promoter fusion analysis indicated that a 285-bp *Pst*I-*Nsi*I fragment, located 5′ of the *exoS* coding region, contained a functional promoter for *exoS*. Expression of the reporter gene was inducible in a low-cation growth environment and required a functional copy of *exsA*. Divergent promoters, coordinately regulated with *exoS* transcription, were identified within the *Pst*I-*Nsi*I fragment. A fusion derivative of ExsA, MALA3A2, was shown to bind directly to the *Pst*I-*Nsi*I probe. DNase I protection analysis demonstrated that MALA3A2 bound to the intergenic region between the postulated −35 boxes of each promoter region. Northern (RNA) blot analysis with probes internal to and upstream of *exoS* demonstrated that separate, coordinately regulated mRNAs were expressed in *P. aeruginosa*. These data suggested that a locus, coregulated with *exoS* transcription, was located upstream of *exoS*. DNA sequence analysis of the *exoS* upstream region revealed three open reading frames, ORF 1, ORF 2, and ORF 3. ORF 1 demonstrated significant homology to the SycE/YerA protein of *Yersinia* sp. SycE/YerA is postulated to function as a chaperone for the YopE cytotoxin. The loci encoding YopE and ExoS show similarities in genetic organization, protein composition, and regulation.

Pseudomonas aeruginosa is an opportunistic pathogen capable of infecting immunocompromised individuals, including patients afflicted with extensive burns or wounds, cystic fibrosis, leukemia, and neutropenia (3). The bacterium synthesizes and secretes a number of virulence determinants that are postulated to enhance survival within the host by providing nutrients, evading the host immune response, and allowing spread of the organism to other tissues (29). A determinant whose expression has been correlated with the ability of *P. aeruginosa* to disseminate from epithelial colonization sites to the bloodstream is exoenzyme S (2, 4, 26–28). Exoenzyme S is an ADP-ribosyltransferase (15) whose preferred eukaryotic target proteins include members of the H-Ras and K-Ras family of GTP-binding proteins and vimentin, an intermediate filament protein (4–6). For exoenzyme S to exhibit ADP-ribosyltransferase activity in vitro, a eukaryotic activator protein, termed FAS, is required (6). Recent studies indicate that FAS belongs to the 14-3-3 family of proteins, which play key roles in eukaryotic cell growth and differentiation (12). The relationship between exoenzyme S-mediated ADP-ribosyltransferase activity and cellular toxicity has not been defined.

The synthesis of exoenzyme S by *P. aeruginosa* is highly regulated. Exoenzyme S expression requires the growth of *P. aeruginosa* under environmental conditions that correspond to low concentrations of cations (37). In addition, a regulatory operon termed the exoenzyme S *trans*-regulatory locus is required for exoenzyme S synthesis (10). The *trans*-regulatory locus encodes three protein products, ExsC, ExsB, and ExsA (10, 11). ExsA is a member of the AraC family of DNA-binding proteins and possesses at least one carboxy-terminal helix-turn-helix motif. In addition, ExsA is highly homologous to the VirF and LcrF proteins of *Yersinia* species, which control the expression of a series of genes involved in pathogenesis (13, 36). Recent studies indicate that transcriptional initiation of the regulatory operon requires the inclusion of chelators in the growth medium and a functional *exsA* gene (43). Taken together, these data suggest that in response to an environment with low cation levels, transcription of regulatory genes is enhanced, which results in high levels of exoenzyme S synthesis (43).

Exoenzyme S has been purified from the supernatant of *P. aeruginosa* 388 as an aggregate consisting of two proteins of 53 and 49 kDa (16, 27). The 49-kDa form of exoenzyme S possesses enzymatic activity in vitro, while the 53-kDa form appears to be inactive (16, 27). The gene encoding ExoS was cloned by designing degenerate oligonucleotides based on amino acid sequences of amino-terminal and tryptic peptides of the gel-purified 49-kDa form of exoenzyme S (18). Degenerate oligonucleotides were used in the PCR to produce specific probes for hybridization to a cosmid bank of strain 388 chromosomal DNA. The cloned gene was predicted to encode a protein of 453 amino acids with a molecular mass of 48,302 Da. As sequences within ExoS aligned to active-site residues of several members of the ADP-ribosyltransferase family, it appeared that the gene corresponding to the enzymatically active 49-kDa form of exoenzyme S (*exoS*) had been cloned.

The regulation of *exoS* was studied to determine if ExsA directly interacted with the *exoS* promoter region to activate transcription. Transcriptional initiation of *exoS* was examined by using promoter fusion, Northern (RNA) blot, and transcriptional start site mapping analyses. In this study, we report that *exoS* requires ExsA for transcriptional initiation, that ExsA binds to the *exoS* promoter region, and that a second, coordinately regulated, divergent promoter transcribes into a region located upstream of the *exoS* promoter and open reading frame (ORF). This region was subjected to nucleotide sequence analysis and appears to encode a homolog to the *Yersinia* sp. SycE/YerA protein (9, 41).

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype	Reference or source
P. aeruginosa strains		
388	Wild type	2
PAK	Wild type	S. Lory
PAK*exsA*::Ω	Mutation in the chromosomal copy of *exsA*	11
PA103	Wild type, 49-kDa ExoS$^-$	11
PA103*exsA*::Ω	Mutation in the chromosomal copy of *exsA*	11
E. coli strains		
TB1	F$^-$ *ara* Δ(*lac-proAB*) *rpsL* f80d*lacZ*Δ*M15 hsdR17*(r_K^- m_K^+)	1
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	21
Plasmids		
pQF26	CAT reporter vector	8
pS	*Pst*I-*Nsi*I-CAT fusion	This study
pS′	*Nsi*I-*Pst*I-CAT fusion	This study
pUC*exoS*	pUC19 containing the exoenzyme S gene	18
pUCP18	*E. coli-P. aeruginosa* shuttle vector	34
pUCP*exoS*	pUCP18 containing the exoenzyme S gene	17

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used are summarized in Table 1. *Escherichia coli* cultures were cultivated in Luria-Bertani broth or agar at 37°C with ampicillin (100 μg/ml) as needed. *P. aeruginosa* was grown in Luria-Bertani broth at 37°C or on Vogel-Bonner minimal agar (39) at 37°C with carbenicillin (400 μg/ml) as necessary. For induction of exoenzyme S production and chloramphenicol acetyltransferase (CAT) assays, *P. aeruginosa* strains were grown at 32°C in a deferrated dialysate of Trypticase soy broth containing 10 mM nitrilotriacetic acid (NTA), 1% glycerol, and 100 mM monosodium glutamate as previously described (10, 11, 37).

Construction of complementation and promoter fusion clones. For use in complementation studies, the *Bam*HI 2.3-kb restriction fragment from pUC*exoS* was cloned into an *E. coli-P. aeruginosa* shuttle vector, pUCP18 (34). The *exoS* ORF was oriented opposite the *lac* promoter sequences such that *exoS* was expressed from native promoter sequences. The construct was transformed into *P. aeruginosa* PA103 and PA103*exsA*::Ω by the $MgCl_2$ method (30). Plasmids were isolated from *P. aeruginosa* transformants and confirmed by restriction endonuclease mapping.

Promoter fusions were constructed by cloning the 285-bp *Pst*I-*Nsi*I restriction fragment from pUC*exoS* into the *Pst*I site of pUC18. After establishing the orientation of the insert by restriction mapping, both orientations were subcloned as *Xba*I-*Hin*dIII fragments into the promoterless CAT reporter vector, pQF26 (8). The reporter clone in which the putative promoter region was oriented to transcribe the predicted exoenzyme S ORF was termed pS. The clone containing the opposite orientation of the promoter fragment was designated pS′. The orientation of each promoter fragment was confirmed by restriction endonuclease and nucleotide sequence analyses after subcloning in M13 vectors. Following confirmation, promoter fusion clones were transformed into *P. aeruginosa* PAK and PAK*exsA*::Ω as described previously (30) and screened by restriction endonuclease mapping.

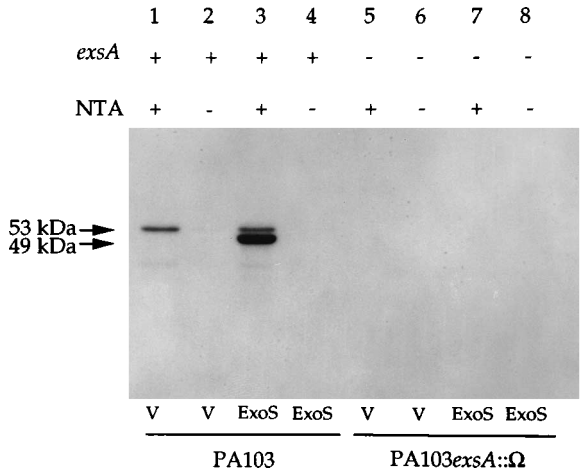

FIG. 1. Expression of rExoS in PA103 and PA103*exsA*::Ω. *P. aeruginosa* PA103 (lanes 1 to 4) and PA103*exsA*::Ω (lanes 5 to 8) transformed with pUCP18 or pUCP*exoS* were grown under inducing (+NTA) and noninducing (−NTA) conditions for exoenzyme S synthesis. Cellular extracts were subjected to Western blot analysis. Lanes 1, 2, 5, and 6 show strains containing the vector pUCP18 (V), while the strains in lanes 3, 4, 7, and 8 contain pUCP*exoS* (ExoS). Positions of migration of the 49- and 53-kDa forms of exoenzyme S are shown at the left.

Analysis of exoenzyme S antigen. *P. aeruginosa* PA103 and PA103*exsA*::Ω containing pUCP18 or pUCP*exoS* were grown under inducing (inclusion of 10 mM NTA in the medium) or noninducing (absence of NTA during growth) conditions for exoenzyme S production (10, 11, 37). Bacterial cells were harvested, washed, and lysed by suspension in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer as previously described (11). Three microliters of lysate material was loaded per lane, and proteins in each sample were separated by electrophoresis on an SDS–11% polyacrylamide gel (19) under reducing conditions. After transfer to nitrocellulose (38), samples were probed with antiserum that recognized both the 53- and 49-kDa forms of exoenzyme S (10, 11, 27). Bound immunoglobulin G was detected with ^{125}I-protein A and autoradiography.

CAT assays. Promoter fusion strains were grown under inducing and noninducing conditions for exoenzyme S synthesis and assayed for CAT as previously described (25, 43). Briefly, 1.6×10^9 cells were harvested, washed in 100 mM Tris-Cl (pH 7.8), and lysed by sonication. Cell lysates were assayed in the presence of 1 mM chloramphenicol, 10 μl of [*butyryl*-1-^{14}C]butyryl coenzyme A (4.0 mCi/mmol; New England Nuclear Research Products, Wilmington, Del.), 100 mM Tris-HCl (pH 7.8), and 5 ml of Econofluor II (New England Nuclear). Scintillation counts were monitored, and units of CAT were determined from a standard curve generated by using purified CAT (Boehringer Mannheim Corporation, Indianapolis, Ind.). Units of CAT were normalized by protein content and calculated as units per microgram of total protein.

Gel retardation assay with the putative exoenzyme S promoter. A 285-bp *Pst*I-*Nsi*I fragment, containing the first 5 bp of the exoenzyme S ORF and 280 bp of 5′ sequences, was isolated from pUC*exoS* (18) as a restriction fragment. The restriction fragment was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) and end labeled with [γ-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear), using T4 polynucleotide kinase (21) (Promega Biochemicals,

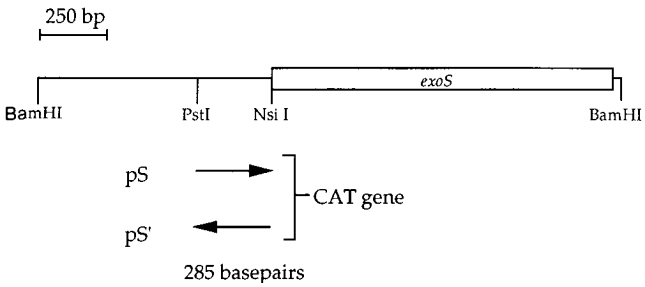

FIG. 2. Construction of *exoS* promoter fusion clones. The map of the 2.3-kb *Bam*HI fragment containing the exoenzyme S ORF (open box) is shown. The *Pst*I-*Nsi*I region was cloned into the promoterless CAT reporter vector, pQF26, and the resulting clones were designated pS and pS′. Arrows represent the orientation of the proposed promoter region relative to the *cat* gene.

TABLE 2. CAT activities of promoter clones

Promoter clone	CAT activity (U/μg of total protein)							
	P. aeruginosa PAK				*P. aeruginosa* PAK*exsA*::Ω, A_{540} = 5.0		*E. coli* TB1, A_{540} = 5.0	
	A_{540} = 0.9		A_{540} = 5.0					
	−NTA[a]	+NTA[b]	−NTA	+NTA	−NTA	+NTA	−NTA	+NTA
pS	ND[c]	0.49 ± 0.17	ND	0.51 ± 0.19	ND	ND	0.08 ± 0.02	0.08 ± 0.01
pS′	ND	1.08 ± 0.23	ND	0.60 ± 0.19	ND	ND	ND	ND

[a] −NTA, transformants grown in medium without NTA.
[b] +NTA, transformants grown in medium containing 10 mM NTA.
[c] ND, not detectable (less than 0.06 U/μg of total protein).

Madison, Wis.). Nonspecific DNA fragments consisting of two internal *Pst*I fragments of *exoS* (315 and 225 bp) and a 218-bp fragment of the exoenzyme S *trans*-regulatory locus corresponding to pB (43) were labeled and used in gel retardation assays.

Recombinant ExsA was purified as a fusion derivative (MALA3A2) by using the pMAL-c2 expression vector (New England Biolabs, Beverly, Mass.). The construction, purification, and DNA-binding activity of this fusion protein will be described elsewhere (14). Gel retardation assay reaction mixtures (13) contained labeled DNA (0.01 pmol), 500 ng of poly(dI-dC) (Sigma Chemical Company, St. Louis, Mo.), 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 5% glycerol, and the MALA3A2 fusion protein (0.25 to 25 pmol) in a final volume of 20 μl. Assay mixtures were incubated for 15 min at 37°C, and then DNA loading dye (21) was added. Samples were electrophoresed on a 5% low-ionic-strength TAE (6.7 mM Tris-Cl [pH 7.5], 3.3 mM sodium acetate, 1 mM EDTA) polyacrylamide gel (1:30 bisacrylamide-acrylamide), dried, and exposed to autoradiography film.

Purified MalE served as a nonspecific control to determine if the MalE fusion partner contributed to the observed DNA-binding activity of MALA3A2. In gel retardation experiments using both specific and nonspecific labeled DNA probes, purified MalE failed to bind to DNA fragments (data not shown).

DNase I protection analysis. MALA3A2 and labeled sense and antisense strands were used in DNase I protection assays. To obtain labeled sense and antisense strands for the pS promoter region (*Pst*I-*Nsi*I fragment), a set of pUC subclones containing the *Pst*I-*Nsi*I fragment in both orientations relative to the *Bam*HI restriction site was used. Each orientation of the fragment was excised by using *Eco*RI and *Hin*dIII, end labeled, and treated with *Bam*HI to cleave off one labeled end.

DNase I protection assays were performed as described by Lambert de Rouvroit et al. (20). The binding reaction mixture consisted of 500 ng of poly(dI-dC), labeled probe (15,000 to 30,000 dpm), and MALA3A2 (2.5 to 50 pmol) in 50 μl of modified FP buffer (10 mM Tris-HCl [pH 7.9], 100 mM KCl, 6.25 mM $MgCl_2$, 0.05 mM EDTA, 8.5% glycerol). Binding reaction mixtures were incubated at room temperature for 30 min. After incubation, 50 μl of a 5 mM $CaCl_2$–10 mM $MgCl_2$ solution and then 0.01 μg of DNase I (Worthington Biochemical Corporation, Freehold, N.J.) were added. Reaction mixtures were incubated for 1 min at room temperature, and reactions were stopped with a 100-μl aliquot of 200 mM NaCl–20 mM EDTA–1% SDS. Following a phenol-chloroform extraction, DNA was precipitated in the presence of ethanol and 10 μg of carrier tRNA. Precipitated DNA was collected by centrifugation at 13,000 × *g* for 15 min at 5°C, and the pellet was suspended in a formamide dye mix (Promega) and heated to 85°C for 5 min prior to electrophoresis on 8 M urea–polyacrylamide (6.6%) sequencing gels. Chemical cleavage reactions (22) were analyzed on the same gels to determine the nucleotide sequence of regions protected by MALA3A2. The gels were fixed, dried, and subjected to autoradiography.

RNA isolation, Northern blot analysis, and transcriptional start site mapping. Total RNA was isolated as previously described, using an acid phenol procedure (40, 43). For Northern blot analysis, RNA (20 μg) was denatured by using glyoxal-dimethyl sulfoxide and separated by electrophoresis using 1.2% agarose gels (21). After vacuum transfer to Magnagraph nylon membranes (Micron Separations Inc., Westboro, Mass.), the RNA was attached to the nylon support by UV cross-linking (Spectronics Corporation, Westbury, N.Y.). DNA probes were isolated from agarose gels as restriction fragments by using a Geneclean II kit (Bio 101, La Jolla, Calif.) and labeled with [^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear) by using a random primer extension reaction (Bethesda Research Laboratories, Gaithersburg, Md.). Start sites were mapped as previously described (43). Primers (Operon Technologies, Inc., Alameda, Calif.) used to map start sites are shown in Fig. 5.

DNA sequence analysis. The DNA sequence of a *Bam*HI-*Pst*I fragment, which lies upstream of sequence reported by Kulich et al. (18), was determined by using standard M13 cloning techniques (33) and a TaqTrack sequencing kit from Promega. Oligonucleotides used for sequencing were synthesized at the Shared Protein-Nucleic Acid Core Facility at the Medical College of Wisconsin.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper is available from GenBank and the EMBL Data Bank under accession number L27629.

RESULTS

Expression of the 49-kDa form of exoenzyme S in *P. aeruginosa* PA103 and PA103*exsA*::Ω. Exoenzyme S activity copurifies as two antigenically related peptides of 53 and 49 kDa. Previous work has attributed the ADP-ribosylating activity of exoenzyme S to the 49-kDa form (16, 27). The gene encoding the 49-kDa form of exoenzyme S (*exoS*) has been cloned and sequenced and is located on a 2.3-kb *Bam*HI restriction fragment of *P. aeruginosa* 388 chromosomal DNA (18). This clone contains the entire coding sequence for *exoS* as well as 922 bases 5′ of the initiation codon. Studies conducted by Kulich et al. have shown that the *Bam*HI 2.3-kb region will express the 49-kDa form of exoenzyme S when provided in *trans* in *P. aeruginosa* PA103 (17). Expression of rExoS required that transformants be grown in the presence of a chelator. The same construct transformed in *E. coli* was unable to synthesize exoenzyme S either in the absence or in the presence of a chelator. These data suggested that expression of rExoS in *P. aeruginosa* required a positive regulatory factor that may not be present in *E. coli.*

Earlier studies identified a protein, ExsA, encoded by the *P. aeruginosa trans*-regulatory locus, that possessed homology to the AraC family of transcriptional activators (10). Insertional

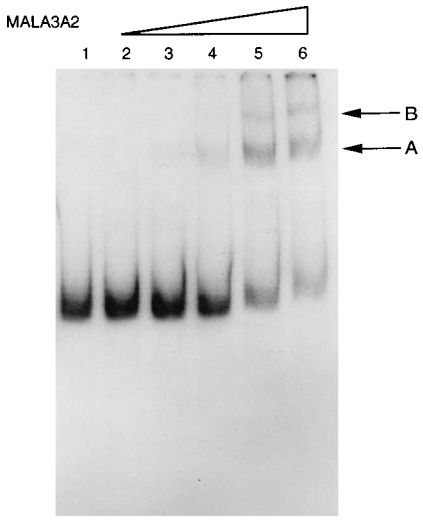

FIG. 3. Gel shift analysis of the binding of MALA3A2 to the *Pst*I-*Nsi*I *exoS* promoter. Ten femtomoles of end-labeled *Pst*I-*Nsi*I restriction fragment (285 bp) was incubated with increasing concentrations of MALA3A2 fusion protein and then subjected to electrophoresis on a low-ionic-strength TAE gel. Lane 1, labeled DNA probe in the absence of the ExsA fusion protein, MALA3A2; 2 to 6, labeled probe incubated with 0.25, 1.25, 5.0, 12.5, and 25 pmol, respectively, of the purified MALA2A3 fusion protein.

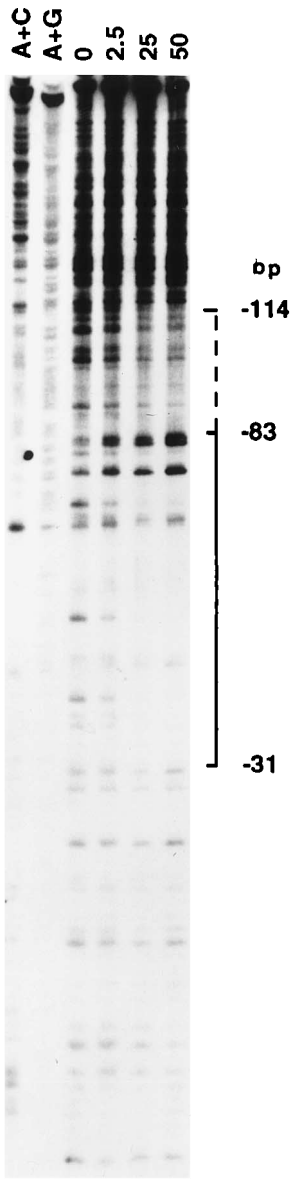

FIG. 4. DNase I protection of pS. The labeled antisense strand of the *Pst*I-*Nsi*I fragment was subjected to DNase I digestion in the presence of increasing concentrations (0 to 50 pmol) of the ExsA fusion derivative, MALA3A2. The lanes labeled A+C and A+G represent chemical cleavage ladders, and numbered lanes represent increasing concentrations of added MALA3A2. The solid bracket to the right indicates an area of strong protection in the presence of MALA3A2. A dotted bracket indicates an area of weaker but detectable protection.

mutations within the *exsA* gene of several strains of *P. aeruginosa* resulted in a severe reduction in exoenzyme S production (11). Additionally, ExsA has been implicated in the initiation of transcription from promoters of genes required for exoenzyme S synthesis and possibly secretion (43). To test whether *exsA* was involved in the transcriptional regulation of *exoS*, the pUCP*exoS* clone and a vector control (pUCP18) were transformed into either strain PA103 (wild type for *exsA*) or PA103*exsA*::Ω, which contained an interposon mutation within the chromosomal copy of the *exsA* gene. Host strain PA103 was chosen because it was deficient in the expression of the 49-kDa form of exoenzyme S by Western blot (immunoblot) analysis and enzyme activity studies (11). Transformants were grown under inducing and noninducing conditions for exoenzyme S synthesis, and whole-cell lysates were subjected to Western blot analysis (Fig. 1). The 53-kDa form of exoenzyme S was expressed when PA103(pUCP18) was grown in the presence of NTA but was not detected when cells were grown in the absence of NTA (lane 2). When PA103 was provided the 49-kDa *exoS* gene in *trans*, both forms of exoenzyme S antigen were detected from transformants grown in the presence (lane 3) but not in the absence (lane 4) of NTA. In the *exsA* mutant strain (PA103*exsA*::Ω), neither the 53- nor the 49-kDa form of exoenzyme S was detected regardless of the plasmid used for transformation or the growth conditions used. Supernatant material from the same experiment demonstrated an identical pattern of exoenzyme S expression (data not shown). These results suggested that *exsA* or a gene regulated by *exsA* was involved in the expression of the cloned *exoS* gene as well as the chromosomally encoded 53-kDa gene (11).

Promoter fusion analysis. The nucleotide sequence immediately 5′ of the ATG start codon for *exoS* (285-bp *Pst*I-*Nsi*I fragment) exhibited A+T-rich regions similar to those of other *exsA*-regulated promoters (43) and several −10/−35 RNA polymerase binding consensus boxes. To identify a functional *exoS* promoter and to determine if transcriptional initiation of *exoS* required a functional *exsA* gene, a promoter fusion clone was constructed by subcloning the 285-bp *Pst*I-*Nsi*I fragment into the promoterless CAT vector, pQF26 (Fig. 2). Both orientations of the insert relative to the CAT reporter gene were isolated. The clone in which the putative promoter was oriented to transcribe the predicted ORF for exoenzyme S was designated pS, while the clone with the opposite orientation was termed pS′. Promoter clones were transformed into both *E. coli* and *P. aeruginosa* hosts, and CAT activity was measured under inducing and noninducing conditions for exoenzyme S synthesis.

In *E. coli*, CAT activity was undetectable in the pS′ clone regardless of the growth conditions. The pS clone expressed CAT activities that were only slightly above control levels under both inducing and noninducing conditions (Table 2). These data are consistent with the inability to detect ExoS production in *E. coli* when the cloned DNA containing the native promoter was tested in expression studies (17).

Promoter clones (pS and pS′) were transformed into *P. aeruginosa* PAK and PAK*exsA*::Ω. When *P. aeruginosa* transformants were assayed under noninducing conditions for exoenzyme S, CAT activity was not detected regardless of the strain (PAK or PAK*exsA*::Ω), the orientation of the promoter insert (pS or pS′), or the A_{540} at which the cells were harvested (Table 2). In wild-type PAK, under inducing conditions for exoenzyme S production, the pS promoter demonstrated CAT activity at both early (A_{540} = 0.9) and late (A_{540} = 5.0) stages of growth (Table 2). The DNA fragment cloned in the opposite orientation (pS′) also exhibited CAT activity when cells were grown under inducing conditions for exoenzyme S production. pS′ exhibited higher levels of CAT activity when cells were harvested at an early stage of growth (A_{540} = 0.9) (Table 2). These data suggested that a promoter for the *exoS* gene was located within the *Pst*I-*Nsi*I region and that a second, divergently transcribed promoter may also be located within the same DNA fragment.

To determine the role of *exsA* in the transcriptional regulation of the *exoS* gene, promoter fusion clones (pS and pS′) were transformed into PAK*exsA*::Ω and assayed for CAT activity. CAT activity of promoter fusion clones was not detected in PAK*exsA*::Ω regardless of the growth conditions (Table 2).

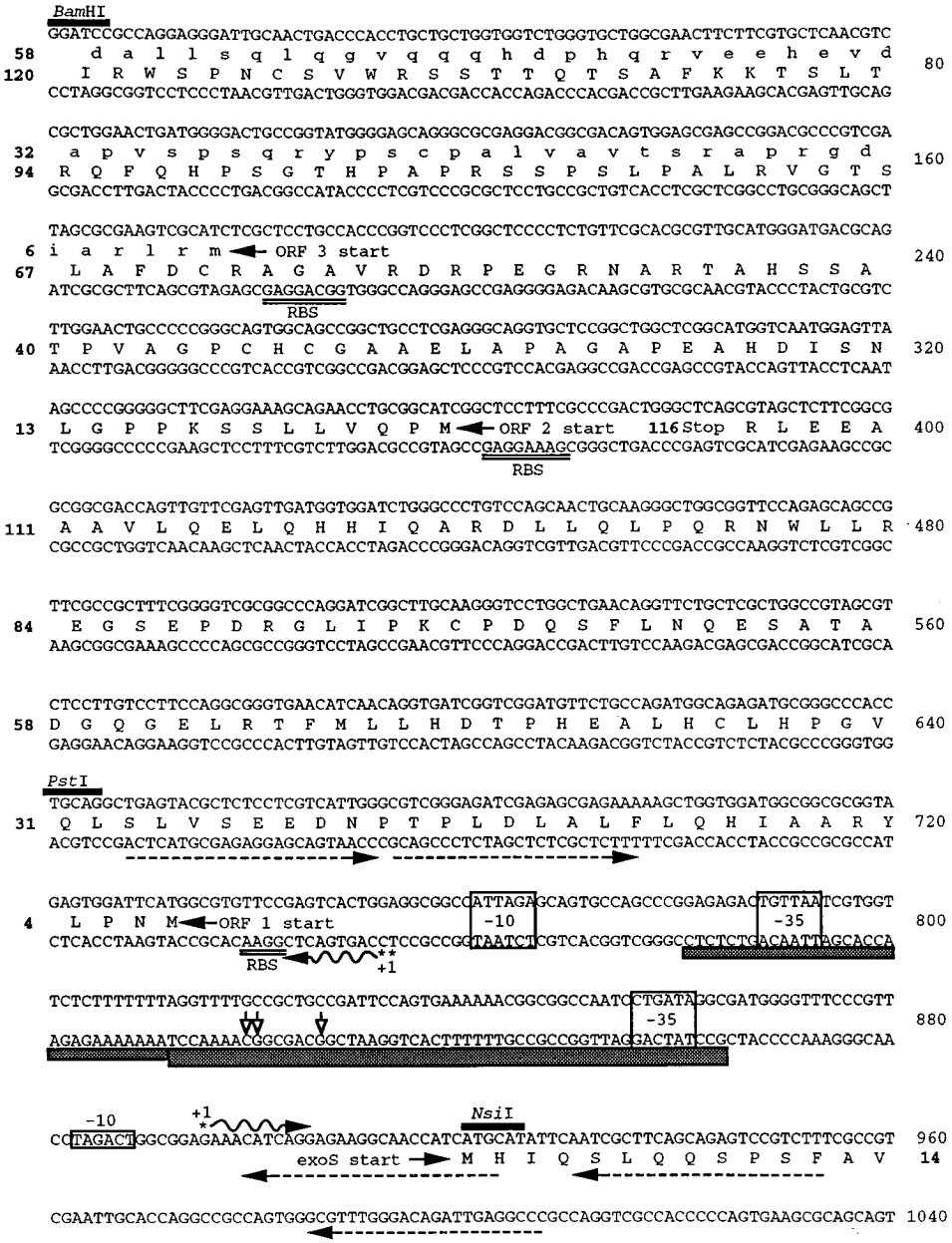

FIG. 5. Nucleotide sequence of the region upstream of the exoenzyme S structural gene. The positions of the *Bam*HI, *Pst*I, and *Nsi*I restriction sites are shown as solid black boxes. Boldface numbers refer to the amino acid residues for peptides encoded by the locus. Primers used to map transcriptional start sites are designated as dashed arrows. Transcriptional start sites are shown with asterisks, and the direction of transcription is indicated with a wavy arrow. The sequences protected from DNase I digestion by bound MALA3A2 are shown as hatched boxes. The thinner box indicates weaker protection, and the thicker box indicates stronger protection. Vertical arrows show the nucleotides that demonstrate increased susceptibility to DNase I cleavage in the presence of MALA3A2. Double-underlined sequences show the positions of proposed ribosomal binding sites. As portions of ORF 2 and ORF 3 overlap, the ORF 3 gene product is shown in lowercase single-letter designations for amino acids.

This result localized the *exsA*-related activation of pS and pS′ to the *Pst*I-*Nsi*I region.

Gel retardation using pS as a probe. To address whether ExsA directly interacted with the *Pst*I-*Nsi*I region, DNA band shift assays were performed. The ExsA ORF was fused in frame to the MalE protein to produce the fusion derivative MALA3A2. MALA3A2 was purified by amylose affinity and DEAE chromatography and used in DNA binding assays. In the absence of MALA3A2, the migration of the labeled *Pst*I-*Nsi*I fragment was not altered (Fig. 3, lane 1). As the concentration of MALA3A2 increased, the migration of the probe was retarded (lanes 2 to 6). Purified MalE failed to bind to the *Pst*I-*Nsi*I probe, suggesting that the fusion partner was not contributing to the observed DNA-binding activity of MALA3A2 (data not shown). In addition, the mobilities of nonspecific DNA fragments which included two probes located within the *exoS* ORF (*Pst*I 225- and 315-bp fragments) and a probe from the exoenzyme S *trans*-regulatory locus termed pB (218-bp fragment) were unaltered in the presence of different concentrations of purified MALA3A2 or MalE (data not

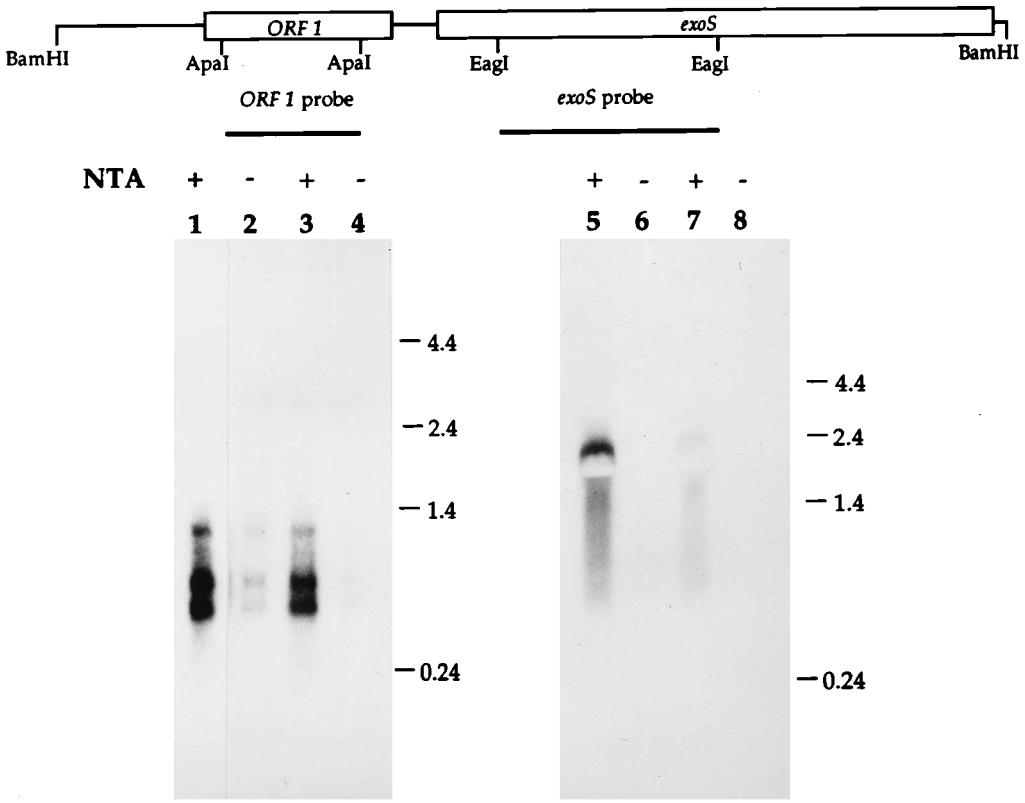

FIG. 6. Northern blot analysis using probes specific for ORF 1 and *exoS*. RNA was isolated from *P. aeruginosa* PAK (lanes 1, 2, 5, and 6) and 388 (lanes 3, 4, 7, and 8) grown under inducing (lanes 1, 3, 5, and 7) and noninducing (lanes 2, 4, 6, and 8) conditions for exoenzyme S synthesis. DNA regions used as specific gene probes are shown as solid dark lines, with the corresponding Northern blot shown below each probe. Positions of molecular weight standards (in kilobases) are shown at the right of each blot. Exposure times for lanes 2 to 4 were fourfold longer than for lane 1 in the blot using ORF 1 as a probe.

shown). These experiments indicated that MALA3A2 was binding specifically to pS.

At high levels of protein relative to the DNA probe, two bands were detected in autoradiograms. The multiple band pattern seen in Fig. 3 may be due to different forms (multimers) of MALA3A2 binding to a single site or may be due to the presence of more than one binding site on this fragment.

DNase I protection of pS. To map the region of DNA protected by MALA3A2, DNase I protection assays were performed with labeled sense and antisense strands of pS. Relatively few DNase I cleavage sites were contained within pS, which limited the resolution of the MALA3A2-protected region (Fig. 4), especially when the labeled sense strand was analyzed (data not shown). Strong protection was observed between bp −31 and −83, and weaker protection appeared to extend the region to bp −114 relative to the *exoS* transcriptional start site when the labeled antisense strand was examined (Fig. 4 and 5). Enhanced DNase I susceptibility was noted at bp −76, −75, and −69 (Fig. 5). These data indicated that MALA3A2 bound upstream of the RNA polymerase binding sites for both ORF1 and *exoS*. Hypersensitivity sites were located between two A+T-rich regions, indicating that DNA conformation may change upon MALA3A2 binding. A+T-rich regions have been noted for other promoter regions which require a functional *exsA* gene for transcriptional initiation (43). DNase I protection studies of all promoter regions that bind ExsA should more clearly define a consensus binding sequence.

DNA sequence analysis. Promoter fusion experiments suggested that a second, divergently transcribed promoter was located within the *Pst*I-*Nsi*I fragment. This second promoter exhibited NTA inducibility as well as a requirement for *exsA*, a common feature noted for promoter regions of genes which are postulated to be involved in exoenzyme S synthesis and secretion (43). Sequence analysis of this region (Fig. 5) revealed three ORFs which possessed possible Shine-Dalgarno sequences upstream of the ATG start codons (35). The first ORF (ORF 1) had typical codon usage patterns for *P. aeruginosa* (42). Translation of ORF 1 predicted a 13.1-kDa protein with a pI of 4.97. A FASTA search (7) with the ORF 1 gene product revealed homology to the *Yersinia* sp. SycE/YerA protein (9, 41). The proteins shared 44% identity over a 115-amino-acid overlap and conserved amino acid substitutions at 38% of the remaining residues (see Fig. 8). SycE/YerA, a 14.6-kDa protein with a pI of 4.55, has been implicated as a YopE-specific chaperone (41). Translation of ORF 2 and ORF 3 predicted truncated proteins of 12.5 and 6.4 kDa, respectively. Analysis using the FASTA program suggested that the ORF 2 and ORF 3 gene products were unique. As ORF 1 demonstrated significant homology to the SycE/YerA proteins of *Yersinia* sp., further analysis focused on this region.

Northern blot analyses of *exoS* and ORF 1. Specific probes (Fig. 6) for *exoS* and ORF 1 were isolated for use in Northern blot analysis. An *Apa*I probe specific for the ORF 1 gene hybridized to RNA species of 0.7, 0.9, and 1.2 kb (Fig. 6). All three bands were detected in two *P. aeruginosa* strains tested (388 and PAK). On long exposures of Northern blots using the ORF 1 probe, RNA was detected under noninducing growth

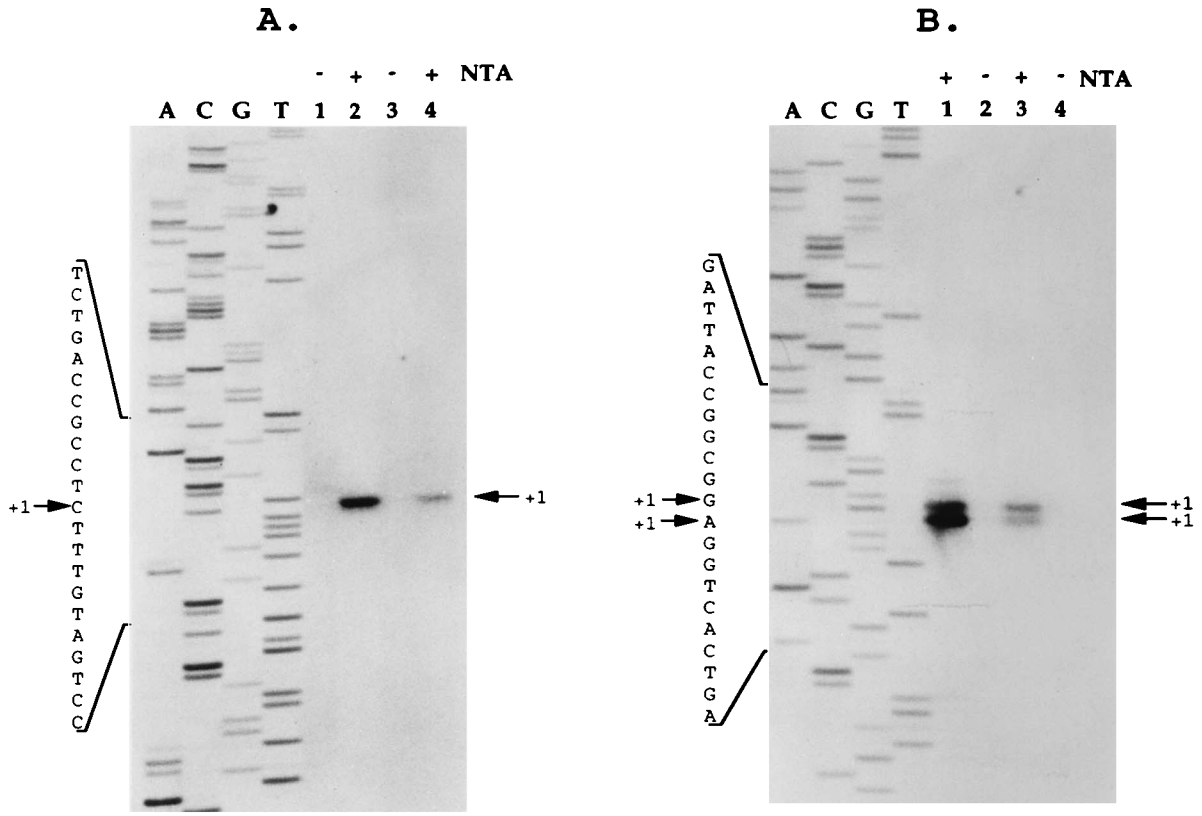

FIG. 7. Transcriptional start site mapping for *exoS* (A) and ORF 1 (B) by primer extension assay. Lanes 1 and 2 show primer extension products obtained by using RNA from *P. aeruginosa* PAK as a template for reverse transcriptase; lanes 3 and 4 shows primer extension products obtained by using *P. aeruginosa* 388 RNA as the template. RNA was harvested from cells grown under inducing (+NTA) and noninducing (−NTA) conditions for exoenzyme synthesis. The nucleotide sequence shown in lanes A, C, G, and T was generated by using the mapping primers in a dideoxy nucleotide sequence reaction.

conditions for exoenzyme S production (lanes 2 and 4); however, signals were stronger when RNA was harvested from cells grown under inducing conditions for exoenzyme S synthesis.

The internal *exoS* probe (*Eag*I fragment) hybridized to a single band when RNA was isolated from cells induced for exoenzyme S synthesis. The length of the *exoS* mRNA was estimated to be 2.1 kb; however, the size of the message proved difficult to determine because of the comigration of *exoS* mRNA with the 23S rRNA subunit. *exoS* mRNA was not detected even on longer exposures when RNA was isolated from cells grown in the absence of NTA.

Transcriptional start site mapping of *exoS* and ORF 1. Promoter fusion experiments suggested that transcription from both *exoS* and the divergently transcribed upstream locus (ORF 1) originated within the *Pst*I-*Nsi*I region. In addition, Northern blot analysis using probes located within (*Eag*I probe) and upstream (*Apa*I probe) of *exoS* showed that different transcripts were detected. To define the 5′ ends of the mRNA, primers were designed for each proposed promoter and used in primer extension assays. End-labeled primers were annealed to total RNA and extended with reverse transcriptase. For the pS promoter, three different primers (Fig. 5) were used, all of which mapped a nucleotide 25 bases upstream of the ATG start codon of *exoS* as the transcriptional start site (Fig. 7). This start site was mapped only when RNA was harvested from cells grown under inducing conditions for exoenzyme S synthesis. The start site for the pS′ promoter was mapped by using two different primers (Fig. 5 and 7). Under inducing conditions for exoenzyme S, the start site for ORF 1 mapped evenly to the thymine and cytosine bases located 22 and 23 bases upstream of the ATG start codon. For both transcriptional start sites (pS and pS′), the signal intensity was stronger for PAK RNA samples than for 388 RNA samples.

Analysis of the pS promoter revealed −10 (TAGACT) and −35 (CTGATA) regions with moderate homology (four of six matches for each region) to the *E. coli* consensus sequences for these elements (−10, TATAAT; −35, TTGACA) (23). The pS′ promoter region possessed −10 and −35 regions with strong homology (five of six matches for each) to the *E. coli* consensus sequences. The presence of −10 and −35 regions with homology to the *E. coli* consensus sequences suggested that the primer extension products represented the true 5′ ends of each mRNA rather than RNA processing sites. It should be noted that the spacing between the *P. aeruginosa* −10 and −35 regions was somewhat longer than the optimal spacing in *E. coli*, which is 17 bases. The pS −10 and −35 boxes were separated by 20 bases, and the pS′ −10 and −35 regions were separated by 22 bases. As in other promoters that demonstrate *exsA* dependence (43), A+T-rich regions were noted in the 62-bp intervening region between the *exoS* and ORF 1 −35 boxes. In addition, DNase I protection analysis demonstrated that MALA3A2 bound to this region.

DISCUSSION

The initiation of transcription from the *P. aeruginosa exoS* gene, encoding the 49-kDa enzymatically active form of exoenzyme S, depends on a direct interaction with the ExsA protein.

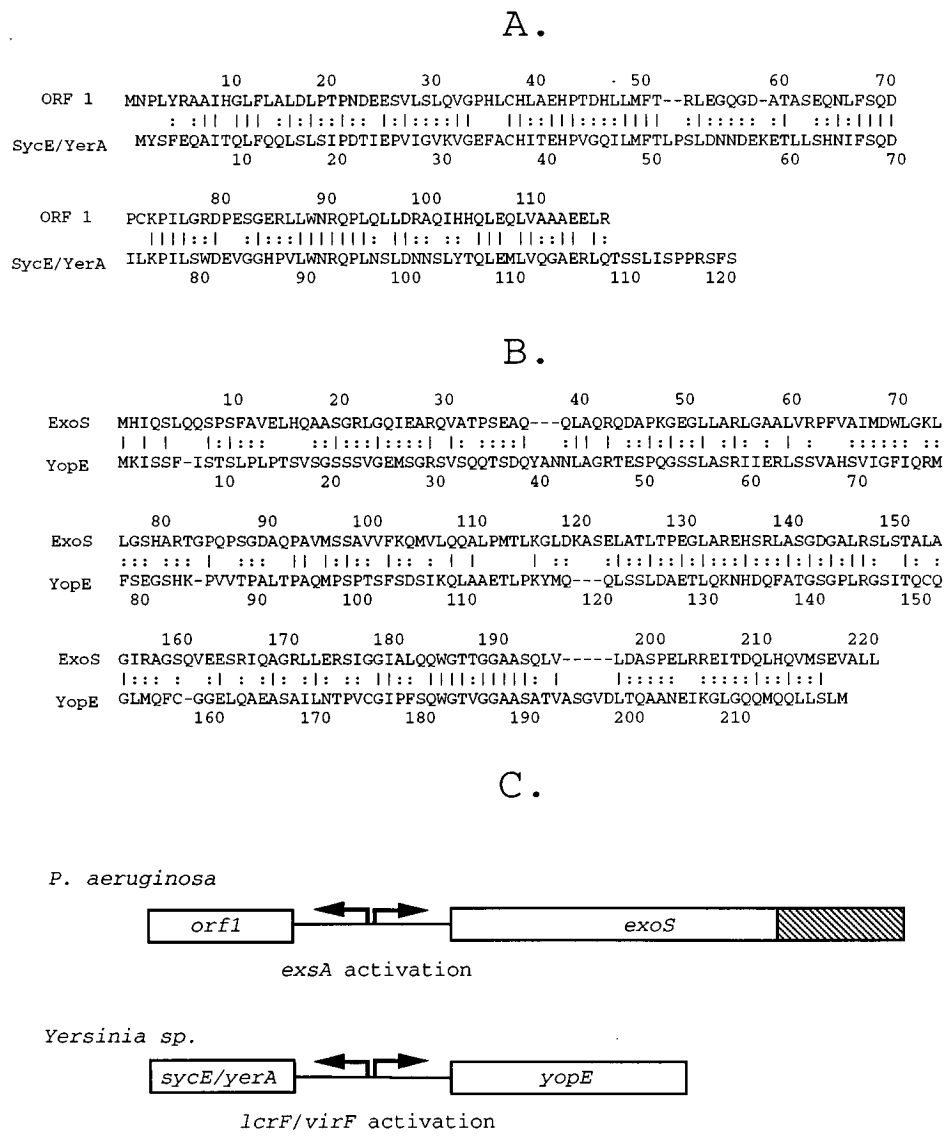

FIG. 8. Comparison of the *exoS* and *yopE* loci. (A) FASTA alignment of amino acid sequences of ORF 1 and the *Yersinia pestis* YerA protein. The YerA protein of *Y. pestis* is approximately 98% identical to SycE of *Yersinia pseudotuberculosis*. (B) BESTFIT alignment of the amino terminus of ExoS with YopE. (C) Genetic organization and transcriptional regulation of the *exoS* and *yopE* loci. The hatched area of the *exoS* gene represents the predicted catalytic domain (18).

Expression of cloned *exoS* in strain PA103 required a functional *exsA* gene. CAT activity of promoter fusions was undetectable in host backgrounds containing an inactive *exsA* gene (PAK*exsA*::Ω) or not containing *exsA* (*E. coli*). A purified fusion derivative of ExsA, MALA3A2, specifically bound to the postulated promoter region for exoenzyme S. DNase I protection studies localized the MALA3A2 binding site(s) to an intergenic region which lies directly upstream of the RNA polymerase binding sites postulated to direct transcription of ORF 1 and *exoS*. In previous studies, we demonstrated that transcriptional initiation of two additional loci, encoding proteins required for exoenzyme S synthesis (ExsC, ExsB, and ExsA) and postulated to be important for exoenzyme S secretion (ExsD), were coordinately regulated with exoenzyme S production and required a functional *exsA* gene. Both promoter regions, pC and pD, respectively, have been subsequently shown to bind both native and fusion derivatives of ExsA (14). These data support the model that ExsA functions as a transcriptional activator that directly binds to specific DNA sequences to initiate transcription of operons involved in exoenzyme S synthesis, regulation, and perhaps secretion.

Several pieces of evidence indicated that the *exoS* promoter region consisted of two, divergent promoters that were coordinately controlled by ExsA. In promoter fusion experiments, both orientations of the DNA fragment were able to initiate reporter gene expression in an NTA- and *exsA*-dependent manner. Northern blot analysis of total RNA with an upstream probe (*Apa*I fragment) and a probe internal to *exoS* (*Eag*I fragment) suggested that different mRNAs were being detected. Northern blot analysis and transcriptional start site mapping demonstrated that different messages were induced when cells were grown in the presence of NTA and downregulated when cells were grown in the absence of NTA. MALA3A2 protected a large region from DNase I cleavage

which was located between the −35 boxes of *exoS* and ORF 1. From these data, we concluded that a second coordinately regulated locus was located upstream of *exoS*.

The region upstream of *exoS* was subjected to nucleotide sequence and codon usage analyses. Several potential ORFs were found. ORF 1 demonstrated significant homology (44% identity over a 115-amino-acid overlap) to the SycE/YerA proteins of *Yersinia* sp. (Fig. 8A) (9, 41). Recent studies indicate that SycE binds to the amino-terminal portion of the YopE protein to facilitate secretion (41). As ORF 1 is predicted to encode a cytoplasmic protein similar in size and pI to SycE, it is possible that ORF 1 serves as a specific chaperone for exoenzyme S. This hypothesis is currently being investigated.

Comparison of the *yopE* and *exoS* loci suggests a similar organization and regulatory pattern and significant overall homology (Fig. 8). Both loci are controlled by a divergent promoter which requires homologous activators for transcription, *exsA* for *exoS* and *lcrF*/*virF* for *yopE*. Alignment of YopE and the amino terminus of ExoS reveals a fairly high degree of similarity (54%) but a relatively low identity score (27%) (Fig. 8B). One sequence of similar amino acids was found between ExoS (180 to 192) and YopE (181 to 193); however, this region appeared unimportant for SycE binding or for secretion, as truncated forms of YopE which lacked this region were secretion competent (24, 41). YopE lacks the domain of ExoS which aligns with active-site residues of other ADP-ribosyltransferases (18) and appears not to possess ADP-ribosyltransferase activity (31).

YopE is a cytotoxin that disrupts actin filaments and appears to be nontoxic unless directly injected into susceptible cells (31). ExoS which possesses ADP-ribosyltransferase activity has been shown to covalently modify vimentin, an intermediate filament, in vitro. The toxicity of purified exoenzyme S has not been demonstrated (4). The lack of toxicity may be due to the conditions used for deaggregation of exoenzyme S. Some of these treatments include exposure to urea (16), SDS (27), and phenol (6). These conditions may remove a binding subunit required for toxicity or may denature the molecule, leaving enzyme activity intact but altering a domain required for interaction with cell receptors. Alternatively, intoxication by ExoS may require direct bacterial cell to eukaryotic cell contact as has been postulated for the YopE protein (32).

In summary, the regulation of *exoS* transcriptional initiation was shown to require a direct interaction with the transcriptional activator protein ExsA. A divergent promoter region was mapped 5′ of the ExoS ORF. Nucleotide sequence, transcriptional start site mapping, Northern blot, and ORF analyses indicated that a set of coordinately regulated genes was located 5′ of *exoS*. The first ORF of the set encoded a gene with significant homology to YerA/SycE, a postulated chaperone for the *Yersinia* sp. YopE protein. Comparison of the *yopE* and *exoS* loci suggested significant similarity in genetic organization, protein composition, and regulation. These data suggest that *P. aeruginosa* may express a set of protective genes that enhance survival in an extracellular eukaryotic environment (36).

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