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Identification of Type III Secreted Products of the *Pseudomonas aeruginosa* Exoenzyme S Regulon

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Extracellular protein profiles from wild-type and regulatory or secretory isogenic mutants of the *Pseudomonas aeruginosa* exoenzyme S regulon were compared to identify proteins coordinately secreted with ExoS. Data from amino-terminal sequence analysis of purified extracellular proteins were combined with data from nucleotide sequence analysis of loci linked to exoenzyme S production. We report the identification of *P. aeruginosa* homologs to proteins of *Yersinia* spp. that function as regulators of the low calcium response, regulators of secretion, and mediators of the type III translocation mechanism.

Exoenzyme S (ExoS) and ExoT are related extracellular ADP-ribosyltransferases secreted by a type III pathway in *Pseudomonas aeruginosa* (42, 43). In previous studies we identified genes required for the regulation and secretion of ExoS (14, 43). ExsA, the central regulator of the exoenzyme S regulon, controls transcription of structural, regulatory, and secretory loci (19, 41, 43, 44). Loci, including *pscB-L* and *pscN*, encode homologs of type III secretion components (33, 43). Mutations in either regulatory or secretory loci result in a phenotype characterized by a defect in the production of ExoS, ExoT, and several additional extracellular proteins (15, 43). We postulated that proteins, coordinately regulated and secreted with ExoS, may represent additional virulence determinants.

Identification of proteins coordinately secreted with ExoS and ExoT. To identify the proteins coordinately secreted with ExoS and ExoT, wild-type (PAK, PA103, and 388) and isogenic mutant (PAK*exsA*::Ω, PA103*exsA*::Ω, and 388*exs1*::Tn*1*[*pscC*]) strains of *P. aeruginosa* were grown under inducing conditions for exoenzyme S synthesis (medium containing 10 mM nitrilotriacetic acid) (38). Extracellular fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15, 24). Major proteins that were common among the three wild-type strains included polypeptides that possessed molecular masses of 32.2, 32.8, 34, and 39 kDa and ExoT (53 kDa) (Fig. 1; Table 1). ExoS (49 kDa) and a 42-kDa protein were absent from strain PA103. A 72-kDa protein (ExoU) was not produced in strains 388 and PAK. Mutations in either regulatory (*exsA*::Ω) or secretory (*exs1*::Tn*1*[*pscC*]) loci resulted in a general defect in the production of the set of proteins specific for each strain. Hybridization studies with probes for *exoS* and *exoU* confirm that the lack of ExoS synthesis in strain PA103 and the lack of ExoU synthesis in strains 388 and PAK are due to the absence of the corresponding genes (9–11).

To determine if the extracellular proteins are related to previously cloned and sequenced members of the ExoS regulon, a protein sequencing approach was used. Proteins in concentrated culture supernatants were separated by SDS-PAGE, transferred to polyvinyl difluoride membranes, and stained with amido black (2). Stained bands corresponding to proteins of 32.2, 32.8, 34, 39 (isolated from the PA103 supernatant), and 42 (isolated from the PAK supernatant) kDa were excised and subjected to amino acid sequence analysis (2). Sequence analysis demonstrated that the 32.2-, 32.8-, 34-, 39-, and 42-kDa proteins were not breakdown products of ExoS, ExoT, or ExoU. FastA and BLAST homology searches indicated that each protein was unique (1, 27). These data suggested that a family of proteins (ExoU, ExoS, ExoT, and the 42-, 39-, 34-, 32.8-, and 32.2-kDa proteins) is secreted by the type III pathway of *P. aeruginosa.*

Nucleotide sequence analysis. One clue as to the identity of the extracellular proteins came from the analysis of the exoenzyme S *trans*-regulatory locus. Translation of an open reading frame immediately upstream of *exsC* (Fig. 2) identified a short peptide of 23 amino acids which exhibited a high degree of homology (64% identity) to the carboxy terminus of YopD of *Yersinia* spp. (12). YopD is encoded by the last gene of an operon consisting of *lcrGVH-yopBD* (Fig. 2) (3, 22, 31). Three of the proteins (LcrV, YopB, and YopD) encoded by the *lcrGVH-yopBD* locus are secreted by the type III system of *Yersinia* spp. and possess molecular masses similar to those of

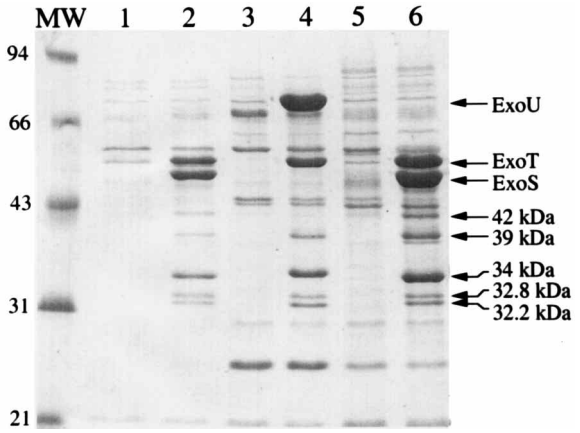

FIG. 1. Extracellular protein profiles of wild-type and mutant strains deficient in type III secreted proteins. Ammonium sulfate-concentrated culture supernatants were separated by SDS–11% PAGE and stained with Coomassie blue. Supernatants from 388, PA103, and PAK are shown in lanes 2, 4, and 6, respectively, while supernatants from isogenic mutants 388*exs1*::Tn*1*[*pscC*], PA103*exsA*::Ω, and PAK*exsA*::Ω are shown in lanes 1, 3, and 5, respectively. Molecular weight (MW) standards (in thousands) are labeled on the left side of the figure. The relative mobilities of ExoU (72 kDa), ExoS (49 kDa), ExoT (53 kDa), and proteins of 42, 39, 34, 32.8, and 32.2 kDa are indicated.

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TABLE 1. Extracellular proteins of *P. aeruginosa* strains

Strain	Presence of extracellular protein[a]:							
	32.2	32.8	34	39	42	ExoS	ExoT	ExoU
388	+	+	+	+	+	+	+	ND
PA103	+	+	+	+	ND	−	+	+
PAK	+	+	+	+	+	+	+	ND
exsA mutants	−	−	−	−	−	−	−	−
pscC mutant[b]	−	−	−	−	−	−	−	ND

[a] Numbers are molecular masses of extracellular proteins in kilodaltons. ND, not detected.
[b] *exs1*::Tn*1* has been mapped as a transcriptionally nonpolar mutation in *pscC*.

TABLE 2. Properties of *P. aeruginosa* proteins

P. aeruginosa protein	No. of residues	Molecular mass (Da)	Isoelectric point
PopN	288	31,319	5.3
Pcr1	92	10,441	4.6
Pcr2	123	13,682	7.3
Pcr3	121	13,722	7.2
Pcr4	109	12,222	7.9
PcrD	706	77,009	5.2
PcrR	144	16,266	11.4
PcrG	98	11,029	4.6
PcrV	294	32,283	4.9
PcrH	168	18,482	4.4
PopB	392	40,277	6.0
PopD	295	31,279	6.0

the major type III secreted proteins of *P. aeruginosa* (Table 1) (3, 35, 36). For these reasons, an 8.6-kb region upstream of the exoenzyme S *trans*-regulatory locus was subcloned from cosmid clone pDF100 (strain 388) into M13 vectors and subjected to nucleotide sequence analysis with an ALF automated DNA sequencer and reagents (Pharmacia Biotech Inc., Piscataway, N.J.) (14). Twelve open reading frames encoding homologs to *Yersinia* proteins (Tables 2 and 3; Fig. 2) were identified by these analyses. Potential protein products included homologs of the *Yersinia* type III secretion apparatus, translocation machinery, and regulatory system of the Yop regulon (Table 3). Five proteins (LcrE or YopN, LcrG, LcrV, YopB, and YopD) are secreted by the type III system of *Yersinia* when the bacteria are grown in vitro.

Analysis of the nucleotide sequence identified two putative promoter regions, pPOP and pPCR, located upstream of *popN* and *pcrG*, respectively (Fig. 2). The pPOP promoter possesses a perfect consensus ExsA binding site, while the ExsA binding site in pPCR diverges from the consensus binding site at two positions (19, 20). Both ExsA binding sites are centered 14 to 15 bp upstream from consensus −35 hexamers. No additional sequences matching ExsA binding sites or −35 and −10 hexamers with spacing characteristics of active promoters were identified. These data suggested that the *P. aeruginosa* homologs may be transcriptionally regulated by ExsA and organized into two operons consisting of *pcrG-popD* and *popN-pcrR* (Fig. 2).

PcrV, PopN, PopD, and PopB are type III secreted exoproducts. Several of the proteins encoded within the putative *popN-pcrR* and *pcrG-popD* operons were similar in predicted molecular weight to the Yop proteins of *Yersinia*. This suggested that the unique extracellular proteins identified by their amino acid sequences may correspond to the cloned and sequenced open reading frames of these postulated ExsA-regulated loci. BestFit alignments between the deduced amino acid sequences and the amino-terminal sequences of the extracellular proteins were performed (8). Alignment of the sequences confirmed that the 32.2-, 32.8-, 34-, and 39-kDa proteins are PcrV, PopN, PopD, and PopB, respectively (Fig. 3). None of the deduced amino acid sequences matched the amino-terminal sequence obtained from the 42-kDa protein. The discrepancies between the amino-terminal sequences of PcrV and PopB and the de-

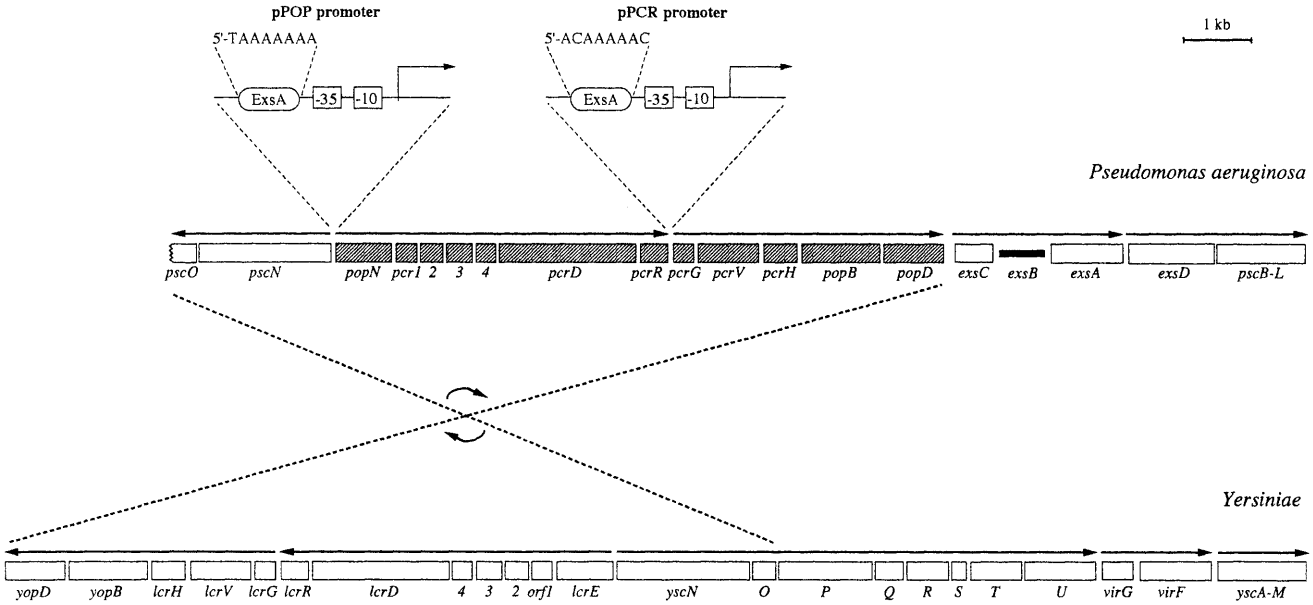

FIG. 2. Genetic organizations of the *popN-pcrR* and *pcrG-popD* loci. The boxes indicating the genes identified in this study are hatched, and the genes are located at 55 min on the *P. aeruginosa* chromosome. The structures of the pPOP and pPCR promoters are indicated. Straight arrows indicate proposed transcriptional units based on the consensus sequences which are illustrated above each operon. ExsA-regulated promoters are also located upstream of *exsC* and *exsD*. Dashed lines and curved arrows indicate the inversion of the *P. aeruginosa pscO-popD* genes with respect to the *Yersinia* homologs.

TABLE 3. Comparison between predicted *P. aeruginosa* proteins and *Yersinia* homologs

P. aeruginosa protein	*Yersinia* homolog	Function(s) (reference[s])[a]	% Identity	% Similarity
PopN	YopN	Regulation of translocation, calcium sensor (5, 13, 28, 34)	47	56
Pcr1	Orf1	Unknown (39)	47	66
Pcr2	Orf2	Unknown (39)	48	55
Pcr3	Orf3	Unknown (39)	48	55
Pcr4	Orf4	Unknown (39)	49	56
PcrD	LcrD	Type III secretion apparatus (29, 30)	80	85
PcrR	LcrR	Unknown (36)	53	60
PcrG	LcrG	Regulation of the LCR (36)	43	53
PcrV	LcrV	Regulation of the LCR, suppressor of tumor necrosis factor alpha and gamma interferon, protective antigen (21, 23, 37)	41	63
PcrH	LcrH or SycD	Regulation of the LCR, chaperone for YopD secretion (32, 40)	57	75
PopB	YopB	Translocation of Yop effector proteins, suppressor of tumor necrosis factor alpha (4, 16–18)	40	51
PopD	YopD	Translocation of Yop effector proteins (5, 16)	43	52

[a] LCR, low calcium response.

duced amino acid sequences most likely represent differences between the strains used as sources of DNA (388) and protein (PA103).

Concluding remarks. Our goal was to identify the major proteins secreted by the type III pathway of *P. aeruginosa*. Using a combination of protein and nucleic acid sequence analyses, we identified 12 proteins possessing high homology to proteins of the *Yersinia* Yop virulon (7). The *Yersinia* homologs are involved in the regulation of Yop effector proteins and their delivery into eukaryotic cells. As with the *Yersinia* proteins, four of the *P. aeruginosa* proteins, PopN, PcrV, PopB, and PopD, were shown to be localized extracellularly. The extracellular localization of each was dependent upon an intact copy of *exsA* and a functional type III secretion apparatus, suggesting that these proteins are coordinately regulated by *exsA* and secreted by the type III pathway. The identification of ExsA-binding sequences in the corresponding promoter regions supports this hypothesis.

In vitro production of the extracellular proteins of the exoenzyme S regulon requires growth in a medium containing nitrilotriacetic acid (a chelator of calcium and zinc). Previous studies have shown that induction of synthesis and secretion occurs at the transcriptional level of the *trans*-regulatory locus (41). These data implied that a positive signal for *exsA* transcription was required to activate the entire pathway. A negative regulatory pathway was postulated when mutations in genes encoding the secretory apparatus were found to result in the down-regulation of ExoS production (43). The association between the regulation of production and secretion of members of the exoenzyme S regulon may be clarified as the functions of the *P. aeruginosa* low-calcium-response homologs (PcrG, PcrV, and PcrH) and PopN are examined.

Unlike many of the classic ADP-ribosyltransferase toxins possessing A-B structure-function, ExoS does not appear to possess any apparent cytotoxicity when it is applied directly to eukaryotic cells (6, 25, 26). When wild-type and ExoS-deficient strains were compared in infection experiments, however, significant differences in eukaryotic cell viabilities and levels of DNA synthesis were observed, suggesting that the bacterium was required for ExoS delivery (26). The discovery of PopB and PopD that are homologous to the *Yersinia* translocation proteins and the coordinate regulation of structural, regulatory, and secretory components support the notion that ExoS, ExoT, ExoU, and perhaps other virulence determinants are delivered into eukaryotic cells by a contact-dependent mechanism.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been submitted to the GenBank database under the accession no. AF010149 and AF010150.

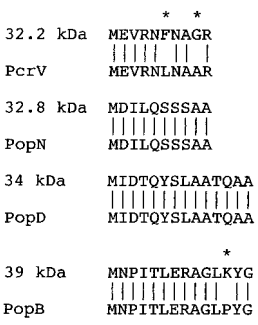

FIG. 3. Identification of *P. aeruginosa* extracellular proteins. Amino-terminal amino acid sequences derived from the major extracellular proteins coordinately secreted with ExoS and ExoT are labeled with the approximate molecular masses. The second line of each pair is an alignment with the deduced amino acid sequences from nucleotide sequence analysis of loci linked to exoenzyme S production. BestFit alignments indicate that the *P. aeruginosa* extracellular proteins correspond to PcrV, PopN, PopD, and PopB. Asterisks indicate discrepancies between the amino-terminal sequences and amino acid sequences deduced from the nucleotide sequences.

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