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A Specific PulD Homolog Is Required for the Secretion of Paracrystalline Surface Array Subunits in *Aeromonas hydrophila*

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Aeromonas hydrophila **is an important pathogen of fish, and its high-virulence strains display a two-dimensional paracrystalline layer (S-layer) on their outermost surfaces. The nucleotide sequence of a 4.1-kb region located 700 bp upstream of the *A. hydrophila* TF7 S-layer protein gene (*ahsA*) has been determined. A sequence analysis of the region revealed the presence of three complete open reading frames ending in a gene encoding a 79.8-kDa polypeptide that shows high homology to the PulD family of secretion proteins. The sequenced region displays both organizational and sequence homology to the *Xanthomonas campestris* pv. campestris Xps secretory system. Insertional inactivation of the *spsD* (S-protein secretion D) gene showed that the loss of expression of the PulD homolog coincided with the localization of the S-protein in the periplasm and the loss of the S-layer from the surface of the bacterium. However, the secretion of the enzymes hemolysin, amylase, and protease was unaffected in the mutant with the nonfunctional *spsD* gene, as was the export of flagella and fimbrial proteins. Southern blot analysis showed that the *spsD* gene was not conserved among all strains of S-protein-producing *A. hydrophila* or *Aeromonas veronii* biotype sobria. Use of the promoterless chloramphenicol acetyltransferase gene showed that unlike *pulD* and its homologs, *spsD* contains its own promoter. *A. hydrophila* has been shown to contain the *exe* operon, which is responsible for the secretion of a number of extracellular enzymes in this bacterium. A fragment of DNA was generated from the *exeD* gene of *A. hydrophila* Ah65 by PCR and was subsequently used in hybridization studies to probe the chromosome of *A. hydrophila* TF7. The presence of an *exeD* homolog in *A. hydrophila* TF7 was found; therefore, the *spsD* gene encodes a second *pulD* homolog that displays a high specificity for the secretion of the S-protein. This gene appears to be part of a second terminal branch of the general secretory pathway in *A. hydrophila*.**

The secretion of bacterial products is of considerable interest because of the restrictions imposed on them during their passage across cell membranes. In the case of gram-negative bacteria, extracellular secreted proteins must cross two barriers, the cytoplasmic membrane and the outer membrane, prior to extracellular release. The system responsible for the transport of most secreted polypeptides in bacteria is the general secretory pathway (GSP), the first step of which is a signal peptide-dependent mechanism requiring products of the *sec* genes to enable the exported product to cross the cytoplasmic membrane and then undergo processing and release into the periplasm. The movement of the extracellular secreted protein across the outer membrane is then accomplished by a more specific terminal branch of the GSP that recognizes its particular substrate(s). Previously characterized two-step secretory systems in gram-negative bacteria have recently been reviewed (33).

S-layers are two-dimensional paracrystalline arrays located on the outermost surfaces of many archaeae and eubacteria and are composed of regularly arranged protein or glycoprotein monomers which self-assemble into a supramolecular structure of precise ultrastructural morphology (for reviews, see references 18, 31, 32, 38, and 39). S-layers typically consist of up to 10% of the total cellular protein, and it has been estimated that for a bacterial cell with a doubling time of 20 min, approximately 400 S-protein monomers per s must be synthesized, secreted, and self-assembled on the cell surface in order to maintain a full paracrystalline layer (32, 40). A number of S-protein genes have now been cloned and sequenced, and surprisingly little homology is found at the genetic level, either among related species or indeed among strains of the same species (41).

Aeromonas hydrophila is a gram-negative pathogen that expresses a tetragonally arranged surface protein array on its cell surface that is composed of a single species of protein with an apparent subunit M_r of 52,000 (8, 9). The gene for this AhsA protein has been cloned and sequenced, and structural analysis has shown that the protein contains posttranslationally modified tyrosine amino acid residues (41). In addition to the AhsA protein, *A. hydrophila* secretes a number of extracellular enzymes, including a glycero-phospholipid-cholesterol-acyltransferase, an amylase, an alpha and beta hemolysin, a cytotonic enterotoxin, and both thermostable and thermolabile proteases (1, 5, 14, 27, 28, 34, 42). Recently, a main terminal branch of the GSP, the so-called *exe* operon, has been characterized in *A. hydrophila* (19, 23, 24). The *exe* operon shows high homology over its entire length to the pullulanase general secretory operon of *Klebsiella oxytoca*. The products of the *exe* operon are required for both the secretion of the extracellular enzymes and the normal outer membrane structure of *A. hydrophila*.

We have now cloned and sequenced a 4.1-kb region of DNA located upstream of the *A. hydrophila* TF7 S-protein structural gene *ahsA*. This DNA contains a gene encoding a polypeptide that shows homology to the PulD protein of *Klebsiella* spp. This

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties or source	Reference or source
A. hydrophila		
TF7	Trout lesion, Quebec	R. Lallier
Ah274	Sloth septicemia, Australia	H. M. Atkinson
Ah423	Human diarrheal feces	This laboratory
Ah598	Human diarrheal feces	This laboratory
Ah77-115	Otary lung, Quebec	R. Lallier
Ah80-140	Aborted piglet liver, Quebec	R. Lallier
Ah80-160	Bovine brain, Quebec	R. Lallier
Ah300	Human diarrheal feces	H. M. Atkinson
Ah65	Trout lesion	This laboratory
TF7-D2	Marker exchange mutant (*spsD*$^-$)	This study
TF7-D2D	Complemented mutant TF7-D2	This study
TF7-DS	Double mutant *spsD*$^-$ *ahsA*$^-$	This study
A. veronii biotype sobria		
As701	Human septicemia, United States	J. M. Janda
As702	Human septicemia, United States	J. M. Janda
A. salmonicida		
A450	Brown trout, Tarn, France	C. Michel
E. coli		
DH5α	*supE44* Δ*lacU169*(ϕ80*lacZ*ΔM15)	17
S17-1	Containing Mob site in the chromosome	37
Plasmids		
pBluescript (KS and SK)	High-copy-number cloning vector; Amp^r	Stratagene
pCAT40	Promoterless CAT gene	10
pUC18	High-copy-number cloning vector; Amp^r	46
pUC19	High-copy-number cloning vector; Amp^r	46
pSUP205	Broad-host-range cloning vector; Cm^r Tet^r	37
pAT19	Broad-host-range cloning vector; Er^r	43
pGK2003	pUC18 *oriT*; Amp^r	15
pUC4KNOT	Kanamycin resistance gene cassette	2

so-called *spsD* (S-protein secretion D) gene ends approximately 700 bp upstream of *ahsA*. Here, we provide evidence that this gene is part of an S-protein-specific secretion pathway which is distinct from and in addition to the *exe* secretory pathway, and we report on the organization of this S-protein-specific secretion system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study and their relevant properties are listed in Table 1. *A. hydrophila* and *Aeromonas veronii* biotype sobria strains were grown on Luria broth (LB) agar or LB either overnight or for 18 h at 37°C unless otherwise stated. All vector- and plasmid-containing strains were grown on LB media, and antibiotics were used in the following concentrations: ampicillin (Boehringer, Mannheim, Germany) (50 μg/ml), chloramphenicol (25 μg/ml for vector-containing strains and 10 μg/ml for transconjugants), erythromycin (150 μg/ml), and kanamycin (Sigma Chemical Co., St. Louis, Mo.) (50 μg/ml for vector-containing strains and 25 μg/ml for transconjugants).

Cloning and sequencing of the *spsD* region. The generation and selection of the EMBL 3 clone, EMBL 3S, containing *A. hydrophila* chromosomal DNA have been described elsewhere, as have the generation and selection of plasmid pST107 (41). In order to obtain the region upstream of pST107, the fragment between the endonuclease sites of *Sal*I and *Sac*I (Fig. 1) was excised from an agarose gel and labeled with the nonradioactive digoxigenin DNA labeling and detection system of Boehringer as suggested by the manufacturer. The labeled product was used for probing a *Cla*I-*Sac*I double digest of EMBL 3S, and the reacting band was excised from a 0.8% agarose gel. Following isolation of the correct band with the Quiex gel extraction system supplied by Promega (Promega Corp., Madison, Wis.), it was ligated into pBluescript(KS) (Stratagene Cloning Systems, La Jolla, Calif.) to generate pBSS50.

Figure 1 contains a restriction enzyme map of the *sps* operon which shows all of the sites used in the generation of subclones used for the sequencing of the region. To obtain complete nucleotide sequences in both directions, oligonucleotide primers synthesized with an Applied Biosystems 391 DNA (Applied Biosystems, Inc.) synthesizer as recommended by the suppliers were used. Sequencing was performed with an Applied Biosystems 373A DNA sequencer using dye primers. The Wizard mini-prep system supplied by Promega was used for the isolation of all plasmid DNA according to the manufacturer's instructions.

Detection of promoter activity from the 5′ region of *spsD*. To determine whether the *spsD* gene displayed any autonomous promoter activity, construct pBSS250 was generated by removing the *Kpn*I-*Sac*I fragment from pBSS100 and ligating it into pUC19. This fragment was then removed from pUC19 on a *Bam*HI-*Pst*I fragment and ligated into pBluescript(KS), generating pBSS250-1. For the insertion of the promoterless chloramphenicol acetyltransferase (CAT) gene into pBSS250-1 to generate pBSSC250-1, a *Pst*I-*Sal*I double digest was performed on vector pCAT40, and the resulting fragment containing the CAT gene was inserted into the similarly digested pBSS250-1. Selection of transformants containing the pBSSC250-1 construct was performed on LB agar containing ampicillin and chloramphenicol. The insert was also subcloned into pBluescript(SK) to check that the expression of CAT was indeed from the *spsD* promoter.

Southern blotting of chromosomal DNA. Southern blots were performed to (i) examine the conservation of *spsD* among *Aeromonas* species, (ii) ensure that a correct double crossover event had occurred in the generation of the marker exchange mutants, and (iii) to detect an *exeD* homolog in *A. hydrophila* TF7. Chromosomal DNA of *A. hydrophila* TF7 was isolated by the method of Manning et al. (29). Approximately 5 μg of chromosomal DNA was digested for 2 h at 37°C with *Kpn*I for the *spsD* conservation and *exe* homolog studies and with *Kpn*I and *Bam*HI in order to check that a double crossover event had occurred in the marker exchange mutagenesis protocol. Samples were separated on a 0.8% agarose gel at 90 V in Tris-acetate buffer and were visualized with ethidium bromide (0.5 μg/ml) for 15 min. The transfer and probing of immobilized DNA were performed as described previously (41).

PCR generation of a fragment of the *exeD* gene. To determine the presence of a homolog of the *A. hydrophila* Ah65 *exeD* gene in *A. hydrophila* TF7, two primers, TTCTATCAAGGTAACTGGGCCATG (Ex1) and GGATATCCCC GAGCAGCGGCTC (Ex2), were synthesized on the basis of the previously published gene sequence (24) and used to amplify a fragment of *exeD* from *A. hydrophila* Ah65 chromosomal DNA by PCR. The PCR product was purified from an agarose gel, labeled with ^{32}P by random priming, and used to probe a *Kpn*I chromosomal digest of *A. hydrophila* TF7 as described under the Southern blotting paragraph above. PCR was performed as described by Gustafson et al. (16).

Marker exchange mutagenesis and gene complementation studies. To generate mutant TF7-D2 lacking expression of the *spsD* gene, the kanamycin resistance cassette from pUC4KNOT was inserted into the unique *Xho*I site in the *spsD* gene of plasmid pBSS100-3, giving pBSS100-3K (Fig. 1). Plasmid pBSS100-3 carrying the complete *spsD* gene was constructed in order to ensure enough flanking DNA on the sides of the *Xho*I site for a favorable double crossover event and for the complementation studies. The construction of pBSS100-3 was accomplished as follows. The *Bam*HI-*Sac*I fragment was removed from pBSS50 and ligated into pUC18. The insert was then inserted back into pBluescript(KS) on a *Bam*HI-*Eco*RI fragment. Once in pBluescript(KS), the fragment could be removed on a *Bam*HI-*Sal*I fragment and inserted into pUC19 incorporating an additional *Cla*I site. The *Xho*I-*Cla*I fragment from pST107 was inserted into the *Xho*I-*Cla*I site, completing the *spsD* gene and generating plasmid pBSS100-3.

For conjugation studies, plasmids pBSS100-3K and pBSS100-3 were transformed into *Escherichia coli* S17-1, which served as the conjugal donor, and *A. hydrophila* TF7 was the conjugal recipient. Conjugation was achieved with logarithmic-phase cultures which were mixed on an LB agar plate and incubated for 3 h at 37°C prior to the plating of the cultures on selective media. For the selection of *spsD* mutants, a small loop of the conjugation culture was passaged into LB containing ampicillin and kanamycin and was placed under stationary conditions at 15°C for 48 h. An aliquot (100 μl) of the top phase was subcultured into fresh LB containing ampicillin and kanamycin and was grown for a further 48 h under conditions similar to those for the *spsD* mutants prior to the selection of the *A. hydrophila* transconjugants on LB plates containing ampicillin and kanamycin at room temperature. Transconjugants displaying kanamycin resistance and chloramphenicol sensitivity at room temperature were selected for further study.

The marker exchange mutants were tested for the loss of the extracellular secretion of the S-protein, i.e., the inability to form an S-layer, by measuring any decrease in their ability to autoaggregate in static broth and by 0.2 M glycine extraction of the S-layer from whole cells for 1 min at pH 3.00, as described below (9). Isolates showing a marked decrease in the quantity of surface-extractable S-protein in the low-pH samples were subcultured for further study.

The insert from plasmid pBSS100-3 was subcloned into broad-host-range vector pAT19 on a *Sal*I-*Bam*HI fragment for complementation analysis of mutant TF7-D2. Conjugation was achieved as described above, but at 30°C rather

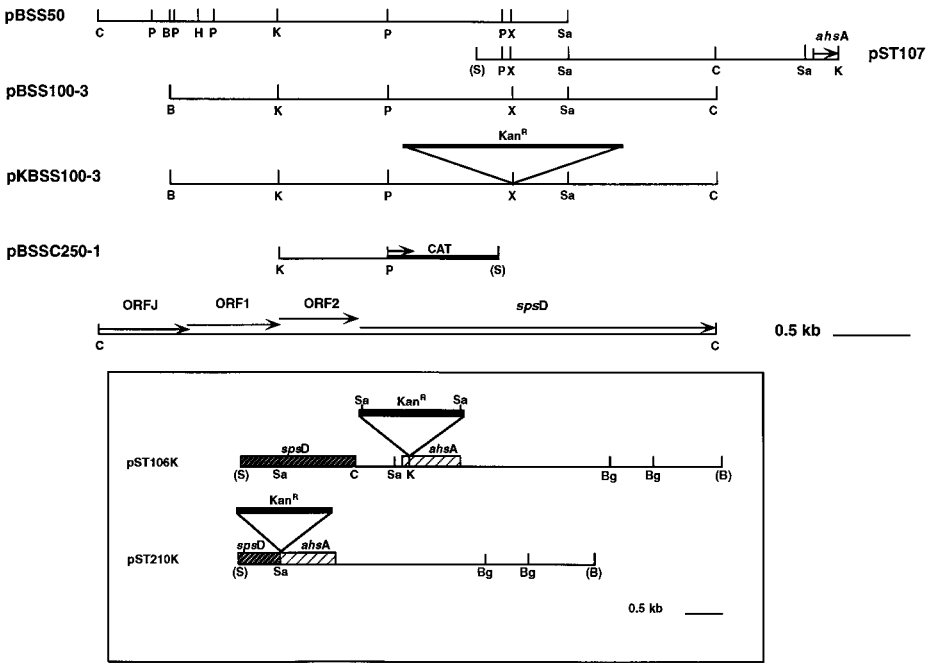

FIG. 1. Maps of constructs used in the cloning, nucleotide sequencing, and mutagenesis of the *A. hydrophila* TF7 *spsD* gene. The position of the *spsD* gene is indicated relative to that of the S-protein *ahsA* gene. Plasmid pKBSS100-3 shows the insertion of the kanamycin resistance cassette into pBSS100-3, which was used for the generation of the marker exchange mutant TF7-D2, and plasmid pBSSC250-1 indicates the construct containing the promoterless CAT gene used for showing endogenous promoter activity from the 5′ region of the *spsD* gene. Also included is a representation of the *sps* operon showing the three complete and one incomplete ORFs found in the region. The inset shows the previously described pST106K plasmid (41), which was used for the generation of an S-protein-negative mutant of *A. hydrophila* TF7, and pST210K, which has a deletion of the *Sac*I fragment from pST106K and which was used for the generation of a double *spsD ahsA* marker exchange mutant, TF7-DS. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sa, *Sac*I; S, *Sal*I; X, *Xho*I. Restriction sites contained within the multiple cloning sites of the vectors are indicated by parentheses.

than 37°C. The selection of transconjugants was achieved on LB plates containing ampicillin, erythromycin, and kanamycin. *A. hydrophila* colonies were tested for the presence of the S-layer by glycine extraction and restoration of the phenotypes of autoaggregation and growth at 37°C.

Localization of S-protein in TF7, TF7-D2, TF7-D2D, and TF7-DS. Cells were collected from logarithmic-phase cultures at an A_{650} of 0.5, washed three times in 30 mM Tris-HCl (pH 7.5), and pelleted at 10,000 × *g* for 10 min. The periplasmic fraction was collected by the osmotic shock method of Willis et al. (45), and the shocked fraction was centrifuged at 200,000 × *g* for 1 h to remove possible contaminating small membrane particles. Once the periplasmic fraction had been removed, the cells were lysed by passage through a French pressure cell, and the cytoplasmic, cytoplasmic membrane, and outer membrane fractions were isolated as described previously (3, 12). Other samples examined included supernatants and cells following a 1-min treatment in 0.2 M glycine HCl, pH 3.00, and one wash in 30 mM Tris-HCl (pH 7.5). Culture supernatants were also collected to ensure that loss of the S-protein from the cell surface of the mutant TF7-D2 was not occurring during growth. For the TF7-DS mutant, the complete loss of expression of S-protein was determined by analysis of whole cells.

Protein analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of proteins was performed with a mini-slab gel apparatus by the method of Laemmli (26). Samples were boiled in solubilization buffer, stacked in 4.5% acrylamide, and separated with either 10.0% or 12.5% acrylamide at 100 V of constant voltage. The staining of the proteins was achieved with Coomassie brilliant blue.

Detection of secreted enzymes in *A. hydrophila* TF7. Extracellular secreted enzymes were detected by culturing bacteria on plates at room temperature and comparing colony sizes and zones of clearing for wild-type *A. hydrophila* TF7 and the TF7-D2 mutant. The presence of aerolysin was detected after growth on LB plates supplemented with 0.6% horse blood. For protease detection, bacteria were subcultured on casein agar (2 g of sodium caseinate [ICN Biochemicals] per liter, 1.0 g of glucose per liter, 0.2 g of K_2HPO_4 per liter, 0.2 g of $MgSO_4$ per liter, 0.001 g of $FeSO_4$ per liter, and 15 g of agar per liter), while secreted amylase was detected on iodine-flooded LB agar containing 2 g of starch per liter.

Nucleotide sequence accession number. The nucleotide sequence described in this communication has been deposited in the GenBank database under accession number L41682.

RESULTS

Primary sequence of the region containing the *spsD* gene. The endonuclease sites used for the subcloning of small fragments from the original pBSS50 and pST107 plasmids for the sequencing of the *sps* region upstream of *ahsA* are shown in Fig. 1. Synthetic oligonucleotides were used for complete sequencing in both directions. Nucleotide sequence analysis of the approximately 4.1-kb region of DNA bounded by the *Cla*I endonuclease sites revealed the presence of three open reading frames (ORFs), with a fourth incomplete ORF, ORFJ, extending from upstream (Fig. 2). ORF1 contains 594 nucleotides and has a GC content of 52%, ORF2 contains 498 nucleotides and has a GC content of 55%, and the ORF describing the *spsD* gene contains 2,211 nucleotides and has a GC content of 51.4%. The incomplete ORFJ originating from upstream contains 615 nucleotides. The start site for ORF1 overlaps the final amino acid codon of the ORF extending from upstream, the initiation codon for ORF2 overlaps the terminal amino acid codon for ORF1, and the initiation codon for the *spsD* gene is separated from the stop codon of ORF2 by 24 nucleotides.

Each of the potential coding regions shows a possible ribosome binding site at the expected distance upstream of the respective ATG start codons of the regions (Fig. 2). The Shine-Dalgarno site for the *spsD* gene is located in the 24 nucleotides between its initiation codon and ORF2, while the ribosome binding sites for ORF1 and ORF2 are contained within the coding region of the preceding gene. At position 308, there is a palindromic sequence 22 bp in length, with a 2-bp mismatch that resides within the coding region of the ORFJ sequence.

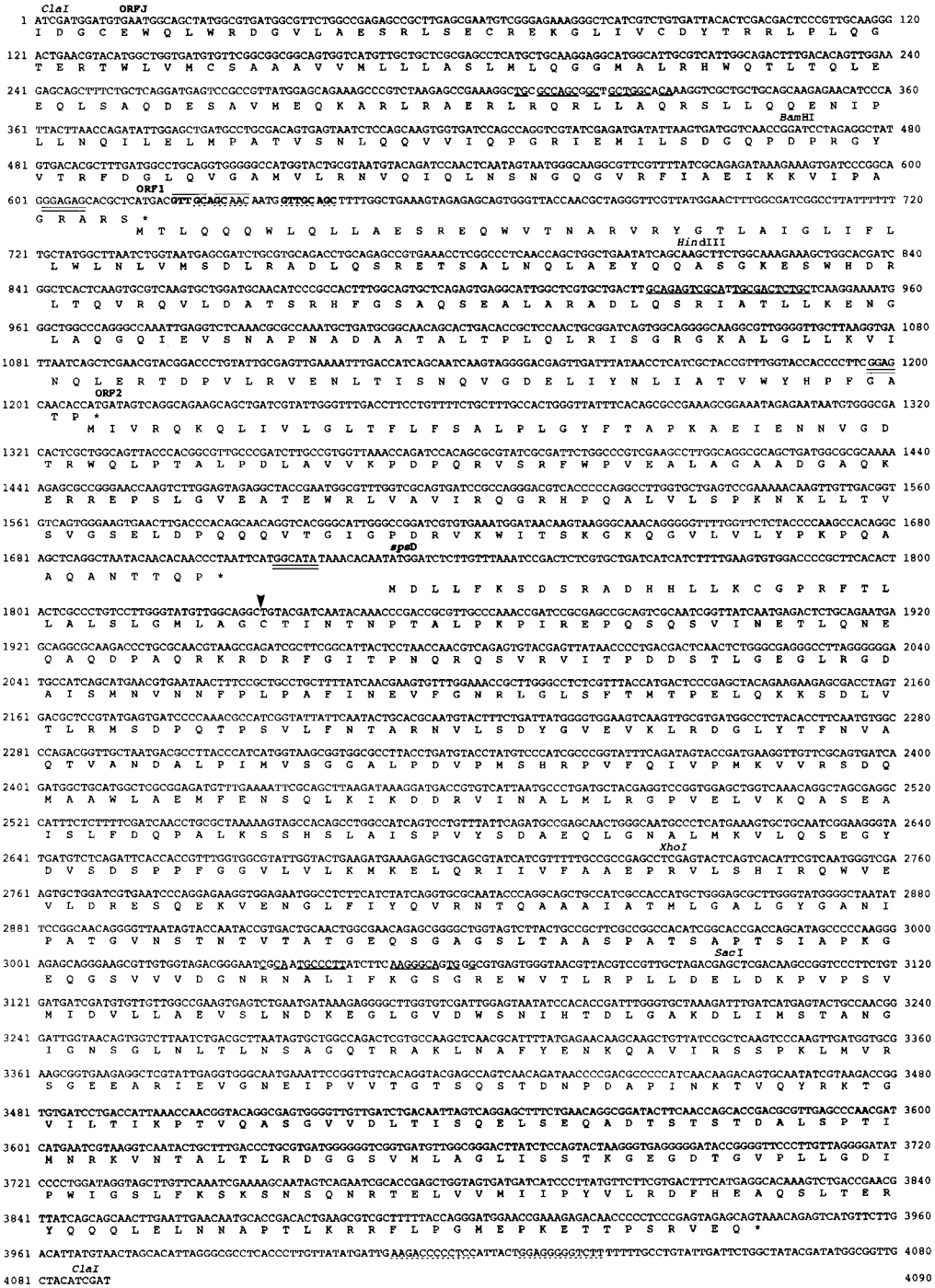

FIG. 2. Nucleotide sequence of a portion of the putative *sps* operon from *A. hydrophila* TF7. Stop codons for each ORF are represented by an asterisk, and various palindromes contained within the coding sequences are underlined. Putative Shine-Dalgarno ribosomal binding sites are double underlined, and a potential rho-independent terminator palindrome of the *spsD* gene is underscored with dots. Also indicated is the conserved signal peptidase II cleavage site (▼) in the SpsD protein. Following the incomplete ORFs from upstream and beginning at position 620 are a further palindrome (overlined) that overlaps a possible rho-independent terminator and a direct repeat sequence (bold). Included are the endonuclease restriction sites for the enzymes *Cla*I, *Bam*HI, *Hin*dIII, *Kpn*I, *Sac*I, and *Xho*I. The deduced amino acid sequence is shown below the nucleotide sequence.

Directly following the stop codon for this ORF and overlapping the start of the ORF1 reading frame (spanning 23 bp and beginning at position 620), there is a direct repeat sequence (GTTGCAGCXXXXXXXGTTGCAGC) and two palindromes, GTTGCAGCAAC and GCAGCAACAATGGTTG CAGC (Fig. 2). This final palindrome, which is followed by a poly T sequence of four nucleotides, could act as a weak transcriptional terminator if the central AT bases were part of a loop structure. Within the coding sequence of ORF1 at bp 926, there is a third palindrome of 23 bp with a 1-bp mismatch

at the center (GCAGAGTCGCATTGCGACTCTGC). Also beginning at position 3032 and falling within the *spsD* gene, there is a possible stem-loop structure with a stem length of 12 bp, a 2-bp mismatch, and a loop size of 6 bp. 3′ to the *spsD* gene is a predicted rho-independent terminator followed by a pyrimidine-rich region with a stem length of 13 bp, a loop size of 4 bp, and a GC content of 62% displaying a free energy of −32 kJ/mol.

The *spsD* gene contains endogenous promoter activity. The presence of the palindrome at the end of the incomplete ORFJ prompted us to determine whether or not the *spsD* gene contained its own promoter. The insertion of the promoterless CAT gene into the *Pst*I site beginning at position 1910 (Fig. 2) immediately downstream of the initiation site for the *spsD* gene and the subsequent growth of *E. coli* containing the construct in selective media demonstrated that the *spsD* gene did indeed contain its own promoter. Because of the complexity of the DNA sequence in the region upstream of the *spsD* gene, where there are a number of palindromes, especially around the possible ORFJ terminator itself, regulated transcriptional read-through from upstream may be occurring. This would be in addition to a basic constitutive level of expression from the promoter specific to the *spsD* gene. Unfortunately, subcloning of the *spsD* gene with transcription in the same direction as the β-galactosidase promoter was not possible because the subclones were unstable, presumably because of the increase in the expression of SpsD and subsequent localization of the polypeptide into the outer membrane of *E. coli*.

We initiated a study based on this observation (data not shown) in order to determine whether or not the palindrome at the end of ORFJ was functional. The plasmid pBSS100-3 was subcloned into the suicide vector pGK2003 (15), which contains the *oriT* sequence inserted into the *Eco*RI site, thereby halting any effect of the β-galactosidase promoter contained in pUC18 on the transcription of *spsD*. This allowed us to (i) selectively remove the *oriT* fragment by *Eco*RI digestion followed by intramolecular ligation, leaving the palindrome intact, and (ii) remove the palindrome on a *Kpn*I fragment by using the site for this enzyme contained in the vector and the site located upstream of the *spsD* gene in the insert. Following the removal of each of these fragments, the effect of the β-galactosidase promoter on the resulting construct could be monitored by the loss of the integrity of the *E. coli* cells containing the plasmids. We found that removal of *oriT* alone led to unstable growth, presumably because of the added effect from the exogenous β-galactosidase promoter; however, the removal of the palindrome on the *Kpn*I fragment resulted in extremely weak growth for *E. coli* and the complete inability to reisolate the plasmid containing the *spsD* gene from these cells. This effect was presumably due to the loss of the function of the terminator followed by an increase in the expression of the SpsD protein, leading to cell death.

Primary amino acid sequence of the *sps* region. Four polypeptides are predicted to be coded for by the cloned DNA (Fig. 1), including the carboxy terminus of the incomplete ORFJ extending from upstream. A search of the databases of both EMBL and GenBank showed a significant sequence similarity among portions of the proteins coded for by this region and proteins contained in the *Xanthomonas campestris* pv. campestris Xps secretory system. The latter pathway is responsible for the export of a number of enzymes involved in the pathogenesis of this plant pathogen (11, 20). The protein coded for by *sps* ORFJ (205 C-terminal amino acid residues) displays some similarity to XpsJ (out of 54 amino acid residues, 35% are identical and 30% are similar at the amino-terminal end of XpsJ). In addition, the 198-amino-acid residue, *sps* ORF1-encoded protein shows a 28% identity with and a 51% similarity to the Xps ORF1 polypeptide over its entire length. However, the *sps* ORF2 166-residue protein shows no sequence similarity to the ORF2-encoded polypeptide of the Xps system. The protein coded for by the final ORF (737 amino acid residues) in the *sps* region, SpsD, displays the greatest similarity overall to known polypeptides (see below), including the carboxy terminus of PulD and its homologs, with the highest homology over the entire length of the SpsD polypeptide being with the *X. campestris* XpsD amino acid sequence.

A possible lipoprotein signal sequence exists at the amino-terminal end of the SpsD polypeptide, with the first 34 amino acid residues terminating with a typical LAGC motif characteristic of an LspA or signal peptidase II processing site. Lipoprotein signal sequences, however, are normally shorter than those of signal peptides recognized by signal peptidase I, with a more hydrophobic helical region. In the case of SpsD, the hydrophilic amino end is 21 residues long, with a net positive charge of 3 and a hydrophobic segment consisting of 11 residues including the leucine at position −3. Processing at this predicted site would leave a mature SpsD protein with a size of 76.1 kDa. Interestingly, the *xpsD* gene of *X. campestris* also contains a lipoprotein processing site, but its signal sequence is somewhat shorter (20).

The *spsD* gene encodes an unprocessed protein with an M_r of 79,811, an α-helical content of 28%, a β-strand content of 21.3%, and a loop content of 50.7%, as was predicted by the secondary structure prediction program developed by Rost and Sander (35). This is in contrast to such outer membrane proteins as the porins, for which the predominant form of secondary structure is β-strand at a content of 65% ± 8% (4, 6, 22, 25, 44). SpsD does, however, have other properties consistent with a membrane-associated protein. For example, the average length of the antiparallel pleated strands required to traverse the membrane is thought to be between 10 and 12 residues, and there are potentially five regions capable of forming β-strands of this length in the SpsD protein. Also, as is typical for outer membrane-located proteins, SpsD contains very little α-helical content capable of anchoring the polypeptide in the cytoplasmic membrane during its movement to its final position.

Homology of SpsD with other outer membrane PulD proteins. A superfamily of proteins grouped on the basis of their homology to PulD has previously been identified (13). The amino acid sequence homology of the SpsD protein to the PulD polypeptide of *K. oxytoca* is limited to a region of 181 residues in the C-terminal end, with a 39% identity and a 24% similarity. The *A. hydrophila* ExeD protein (GenBank accession number X66504) shows homology with SpsD over a region of 291 amino acid residues, with an approximate identity of 34% and a similarity of 55%, the majority of which lies at the C-terminal end. The greatest similarity that SpsD shows to known PulD homologs is to the *X. campestris* XpsD protein. In this case, the similarity spans various regions covering a total of 420 residues over the entire polypeptide, with an approximately 30% overall identity and a 55% similarity. Within the C-terminal region of SpsD, there is a sequence, VPLLGDIP WIGSLF, that conforms closely to a secretion protein motif common to all the PulD homologs, VP(L/F)LXXIPXIGXL (F/L) (21). There are also many other regions of homology to this family of protein secretors that match closely with those published by Genin and Boucher (13).

Conservation of the *spsD* gene among *Aeromonas* species. All strains of *A. hydrophila* displaying high virulence for fish contain S-layers, and we were therefore interested in determining the extent of conservation of the *spsD* gene among a number of

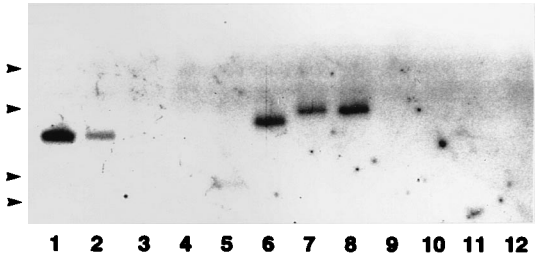

FIG. 3. Southern blot analysis of *Kpn*I chromosomal DNA digests showing the presence of the *spsD* gene among various *A. hydrophila*, *A. veronii* biotype sobria, and *A. salmonicida* strains. Lane 1, EMBL-3S; lane 2, TF7; lane 3, Ah 274; lane 4, Ah 423; lane 5, Ah 598; lane 6, Ah 77-115; lane 7, Ah 80-140; lane 8, Ah 80-160; lane 9, As 701; lane 10, As 702; lane 11, Ah 300; lane 12, *A. salmonicida* A450. The internal *Pst*I fragment from the *spsD* gene was isolated and labeled with ^{32}P by nick translation. This fragment was then used to probe the immobilized DNA. The arrowheads on the left indicate the lambda standards in kilobases (from the top, 6.68, 4.36, 2.32, and 2.03).

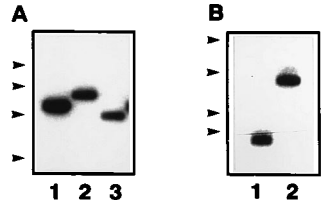

FIG. 4. (A) Southern blot of a *Kpn*I-*Bam*HI double digest of chromosomal DNA showing the correct insertion of the kanamycin resistance cassette during the generation of marker exchange mutants. The *spsD* mutant TF7-D2 (lane 2) shows an increase in size compared with that of the TF7 parent (lane 1) because of the addition of the 1.3-kb insert. The double mutant TF7-DS (lane 3) shows the overall decrease in size by comparison with that of TF7 that resulted from the addition of the 1.3-kb kanamycin resistance cassette and the deletion of a 2.44-kb fragment that included the 3′ end of the *spsD* gene and the 5′ end of the *ahsA* gene. Detection of the immobilized DNA was achieved with the same probe described in the legend to Fig. 3. (B) Southern blot of a *Kpn*I digest showing the presence of a second *pulD* homolog in *A. hydrophila* TF7 (lane 1) and the positive control containing chromosomal DNA from *A. hydrophila* Ah65 (lane 2). A fragment from the *exeD* gene of *A. hydrophila* Ah65 was generated by PCR amplification and labeled by nick translation (see text). The arrowheads on the left indicate the lambda standards in kilobases (from the top, 9.42, 6.68, 4.36, and 2.32 [A] and 6.68, 4.36, 2.32, and 2.03 [B]).

strains. We have shown previously (41) that the S-protein gene *ahsA* is not conserved among all S-layer-producing strains of *A. hydrophila* and *A. veronii* biotype sobria. Using the *Pst*I fragment from the *spsD* gene (Fig. 1) as a probe, we found that unlike the *ahsA* gene, which is conserved at least partially for strains Ah598, Ah77-115, Ah80-140, Ah80-160, As701, and As702 (41), the *spsD* gene was only conserved in strains Ah77-115, Ah80-140, and Ah80-160 (Fig. 3, lanes 6 to 8). Weak hybridization was also obtained with DNA from strain Ah598 (Fig. 3, lane 5), and no hybridization was found for *spsD* and DNA of the related S-layer-producing organism *Aeromonas salmonicida* A450 (lane 12) or for *spsD* and DNA from an S-protein-negative strain of *A. hydrophila* (Ah300) (lane 11).

Isolation and localization of the S-protein in the marker exchange mutant TF7-D2. The isolation of a marker exchange mutant of *spsD* proved to be difficult, even though the phenotypic alteration due to the loss of the S-layer from the cell surface (loss of autoaggregation in static broth) allows for a strong enrichment process. Eventually, SpsD mutant TF7-D2, which was autoaggregating negative, was isolated. Compared with that of the parent TF7, the growth of mutant TF7-D2 was restricted at 37°C. We predicted that the difficulty experienced in isolating this SpsD mutant was because of the high expression level of the S-protein. To confirm that this was the case, a two-gene mutation was constructed by deleting the 3′ region of the *spsD* gene and the 5′ region of the *ahsA* gene, and we tested to determine if such two-gene mutants were more easily isolated. Following the enrichment process of serially subculturing the upper phase from stationary growth cells three times into fresh broth, 100% of the colonies tested proved to be S-protein negative. Southern blotting of a representative double mutant TF7-DS (Fig. 4A) showed that a correct recombinational event had occurred, deleting regions of both genes and effectively halting expression of both SpsD and AhsA.

SDS-PAGE analysis of parent TF7 and marker exchange mutant TF7-D2 showed no differences in the quantities of S-protein present in whole-cell lysates (data not shown). In the case of S-layer-producing aeromonads, the majority of S-protein normally copurifies with the outer membrane fraction (Fig. 5A, lane 1) and can be extracted from the cell surface by treatment with 0.2 M glycine HCl, pH 3.0 (Fig. 5A, lane 7). However, when the outer membrane fraction was isolated from mutant TF7-D2, S-protein was absent (Fig. 5A, lane 2), and S-protein could not be extracted from the cell surface with glycine HCl (Fig. 5A, lane 8). Further fractionation of the cells of mutant TF7-D2 showed that the majority of S-protein was located in the periplasmic fraction (Fig. 5A, lane 6). This finding was similar to those of previous studies with certain A-protein secretion mutants of *A. salmonicida* (3). Figure 5A also shows the absence of AhsA in the outer membrane and glycine extract fractions isolated from double mutant TF7-DS (Fig. 5A, lanes 4 and 10).

The presence of a functional extracellular enzyme secretory system in the TF7-D2 S-protein secretion mutant. The mutational analysis of the *spsD* gene was especially interesting not only because of the gene's location immediately upstream of the *ahsA* S-protein gene but also because of the previous finding of *exeD*, a further *pulD* gene homolog in *A. hydrophila* (24). The *exeD* gene has been shown to be part of an operon with high overall homology to the general secretory system and is required for the export of a number of extracellular enzymes in *A. hydrophila*. In the marker exchange mutant TF7-D2, a number of other secreted proteins were therefore examined in order to ensure that their export had not been affected by the loss of the expression of the SpsD protein. Negative staining of whole cells of both TF7 and TF7-D2 followed by examination by electron microscopy showed no difference in the presence of

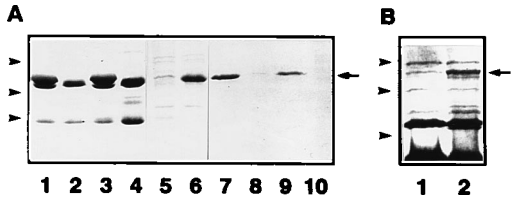

FIG. 5. Coomassie blue-stained SDS-PAGE gels showing the localization of the S-protein (arrows) in the marker exchange mutants TF7-D2 and TF7-DS compared with that in the parent strain TF7 and *spsD*-complemented TF7-D2D. (A) Outer-membrane-protein profiles of TF7, TF7-D2, TF7-D2D, and TF7-DS (lanes 1 to 4, respectively). Lanes 5 and 6 contain periplasmic fractions from TF7 and TF7-D2, respectively. Lanes 7 to 10 show glycine extractions from the same strains (in the same order) used for lanes 1 to 4. (B) Coomassie blue-stained outer membrane fractions from *E. coli* containing plasmid pBSS100-3 (lane 2) for which a stronger band (arrow) can be seen by comparison with lane 1, which contains *E. coli* harboring the vector alone. The samples shown in panel A were separated on an SDS–12% PAGE gel, and the samples in panel B were separated on an SDS–10% PAGE gel. Indicated on the left of the panels are the positions of the molecular weight markers (from the top, 66,200, 45,000, and 31,000 [A] and 97,400, 66,200, and 45,000 [B]).

pili and flagella in these strains (data not shown), indicating that there is no effect on the export and assembly of these structures by mutation of the *spsD* gene. Of more interest, however, was the observation that secretion of extracellular enzymes by *A. hydrophila* was not affected by this mutation. For cultures grown on agar plates containing the substrates for the enzymes aerolysin, protease, and amylase, there was no visual indication that the secretion of these proteins was impaired by the loss of the expression of *spsD* as determined by comparisons of colony sizes and zones of clearing (data not shown).

This ability of the *spsD* mutant to secrete extracellular enzymes was consistent with the presence of *exeD* in strain TF7. To confirm the presence of this second *pulD* homolog, PCR was used to generate a fragment (approximately 500 bp) from the *exeD* gene of *A. hydrophila* Ah65. This *exeD* DNA was radioactively labeled and used as a probe in Southern blot studies against a chromosomal digest of TF7. The results in Fig. 4B show the presence of a homolog of *exeD* in strain TF7 on a *Kpn*I fragment with a size of approximately 1.7 kb (Fig. 4B). By comparison, the *spsD* gene was present on a 3.2-kb *Kpn*I fragment (Fig. 3, lanes 1 and 2).

Complementation of the TF7-D2 marker exchange mutant with the *spsD* gene. Plasmid pBSS100-3 (Fig. 1) was initially transformed into *E. coli*, an event that led to a large amount of cell lysis and instability within this foreign host, and from this result, we concluded that the *spsD* gene was active. Complementation of the *A. hydrophila* marker exchange mutant TF7-D2 was then achieved by inserting the fragment from plasmid pBSS100-3 into the broad-host-range vector pAT19 (Table 1) and conjugating it back into the parent strain. Following conjugation of the *spsD* gene into the TF7-D2 mutant, phenotypic alterations included (i) a return to the parent autoaggregation-positive phenotype, (ii) reinstatement of normal growth at 37°C, and (iii) the presence of large amounts of S-protein in samples isolated by the low-pH glycine extraction of whole cells (Fig. 5A, lane 9) and in outer membrane fractions (Fig. 5A, lane 4). Figure 5B shows a Coomassie blue-stained SDS-PAGE gel of outer membrane fractions of both *E. coli* harboring the vector alone (lane 1) and the complete pBSS100-3 construct (lane 2). A protein band is present in both lanes 1 and 2 (Fig. 5B) at the same position, approximately 80.1 kDa, which is close to the M_r predicted by the nucleotide sequence of the *spsD* gene (79.8 kDa). However, a clear quantitative difference can be seen for lane 2, which contains the pBSS100-3 sample, and indeed, there are a number of lower-molecular-weight products that are apparent below the major band at 80.1 kDa and that are not present in lane 1. These lower-molecular-weight bands may represent degradation products from the overexpressed SpsD polypeptide.

DISCUSSION

This study has identified a gene encoding a PulD homolog which appears to be specific for the transport of S-layer protein subunits across the outer membrane of *A. hydrophila* TF7. Mutations of this gene result in the loss of the S-layer from the cell surface and periplasmic accumulation of S-protein but have no apparent effect on the outer-membrane-protein profile of the organism or the secretion of extracellular enzymes. The secretion of these enzymes in *A. hydrophila* and the presence of a normal outer-membrane-protein composition have previously been shown to require another PulD homolog, ExeD (23, 24). Southern blot analysis confirmed that *A. hydrophila* carried the *exeD* gene in addition to *spsD*. This finding is consistent with the notion of S-layer-producing strains having separate pathways for S-layer protein subunits, extracellular enzymes, and outer membrane proteins. Interestingly however, *spsD* was not conserved in all S-layer-producing strains of *A. hydrophila* and *A. veronii* biotype sobria, suggesting the presence of further PulD-encoding gene homologs in this group of bacteria.

In *A. hydrophila* TF7, *spsD* is located 700 bp upstream of *ahsA*, the structural gene for the S-layer protein, and on a chromosomal fragment different from that of *exeD*. The two genes show little similarity at the nucleotide level, and *spsD* also appears to possess its own promoter, making it unusual among genes encoding PulD homologs. *spsD* appears to be the final gene of a substrate-specific GSP. In this regard, the putative S-protein secretion operon of *A. hydrophila* TF7 shows an organizational similarity to the *xps* operon of *X. campestris* pv. campestris, in which *xpsD* is also the terminal ORF in the secretion operon (20). Two of the upstream genes, ORFJ and ORF1, in both systems also encode structurally related proteins (11, 20). In contrast, *exeD* and other genes encoding PulD homologs are located internally in their respective gene clusters (33).

Comparison of the predicted sequences of SpsD and ExeD shows that the proteins share significant identity at their C termini. This finding is typical for this family of proteins. Interestingly, other than a region of approximately 120 amino acid residues beginning at position 164, identity for XpsD and SpsD extends over the entire lengths of the two proteins. Within the PulD family of proteins, there are four highly conserved regions in the C-terminal domain (13, 30). The C terminus of SpsD contains only three of these regions, with the amino acid sequence contributing to region B being absent. The significance of this is not understood at the present time, especially as the B sequence is predicted to form a strongly amphipathic β-sheet and could therefore be anchored into or be a membrane-spanning region. However, SpsD does contain a number of other potential membrane-spanning sequences and carries the signature motif of a lipoprotein. Both of these facts argue for it being a membrane-associated protein. Also, consistent with this conclusion is the finding that the protein apparently expressed by *spsD* in an *E. coli* background cofractionates with the outer membrane.

How the PulD-like proteins function in their secretion of various substrates across the outer membrane is unknown. PulD contains seven stretches of 12 residues which are predicted to form strongly amphipathic β-sheets and which may form some type of β-barrel structure similar to that of the porins, although the size of the proteins to be transported across the membrane precludes the possibility of any constantly open pore (7). At least two hypotheses have been postulated regarding the likely method of passage of the secreted proteins across the outer membrane. Martin et al. have suggested that as fimbrae assemble in the periplasm of *Pseudomonas aeruginosa*, the PilQ protein may work as a porthole for the passage of the fimbrae through the outer membrane (30). In contrast, Russel has proposed the possibility that the PulD homolog pIV plays a role in the formation of a gated channel for the phage particle emerging from the cytoplasm (36). Given the high rate at which S-protein must be secreted and assembled to maintain a complete paracrystalline array in *A. hydrophila*, some preassembly might take place prior to secretion. If this is the case, then secretion of AhsA involving SpsD could be via either a gated channel or a porthole, as has been suggested for these other assembled proteins.

In summary, this study has shown that *A. hydrophila* TF7 has (at least) two PulD homologs, SpsD and a homolog of ExeD. SpsD appears to be specific for S-protein secretion and does not appear to be involved in exoenzyme secretion, in contrast

to ExeD. SpsD further appears to belong to an S-protein-specific terminal branch of the GSP, the genes of which are located immediately upstream of the structural gene for the S-protein subunit AhsA.

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