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Isolation and Analysis of Eight *exe* Genes and Their Involvement in Extracellular Protein Secretion and Outer Membrane Assembly in *Aeromonas hydrophila*

S. PETER HOWARD,* JEFF CRITCH, AND ANIL BEDI

University of Regina, Regina, Saskatchewan, Canada S4S 0A2

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The *exeE* gene of *Aeromonas hydrophila* has been shown to be required for the secretion of most if not all of the extracellular proteins produced by this bacterium. In addition, an *exeE*::Tn*5*-751 insertion mutant of *A. hydrophila* was found to be deficient in the amounts of a number of its major outer membrane proteins (B. Jiang and S. P. Howard, J. Bacteriol. 173:1241–1249, 1991). The *exeE* gene and the *exeF* gene were previously isolated as part of a fragment which complemented this mutant. In this study, we have isolated and sequenced a further eight *exe* genes, *exeG* through *exeN*, which constitute the 3′ end of the *exe* operon. These genes have a high degree of similarity with the extracellular secretion operons of a number of different gram-negative bacteria. Marker exchange mutagenesis was used to insert kanamycin resistance cassettes into three different regions of the *exe* operon. The phenotypes of these mutants showed that in *A. hydrophila* this operon is required not only for extracellular protein secretion but also for normal assembly of the outer membrane, in particular with respect to the quantities of the major porins. Five of the Exe proteins contain type IV prepilin signal sequences, although the prepilin peptidase gene does not appear to form part of the *exe* operon. Limited processing of the ExeG protein was observed when it was expressed in *Escherichia coli*, and this processing was greatly accelerated in the presence of the prepilin peptidase of *Pseudomonas aeruginosa*.

Aeromonas hydrophila, like many other gram-negative pathogens, secretes a number of extracellular proteins which play important roles in the pathogenesis of infections by this organism (4, 26). Using the hemolysin aerolysin as a model for the secretion of these proteins, we have shown that *A. hydrophila* possesses a specific pathway for the secretion of a group of signal sequence-containing proteins across the outer membrane, such that mutations in this pathway cause accumulation of the proteins in the periplasm (16, 18, 22). Other studies have shown that this two-step pathway is held in common with a number of other gram-negative genera and that in members of the family *Enterobacteriaceae* it is dependent on the *sec* gene products for translocation of the preproteins across the inner membrane and removal of the signal sequence. The stepwise transfer of the protein across the two membranes in this manner has been termed the general secretion pathway (see reference 34 for a review). It can be distinguished from another pathway shared by many prokaryotes and eukaryotes in which extracellular proteins that do not contain classical signal sequences are translocated across the envelope without entering the periplasm (15).

During our previous studies of aerolysin secretion, we isolated a number of Tn*5*-751 insertion mutants of the *A. hydrophila* strain Ah65 which were unable to secrete extracellular proteins from the cell (22). One of these mutants, L1.97, was also shown to be deficient in the quantities of several of its major outer membrane proteins. We have since demonstrated that in this mutant, the Tn*5*-751 had inserted into the *exeE* gene, which encodes part of the second step of the general secretion pathway in this bacterium (23). The *exeF* gene was also isolated, as part of an *exeE*-containing 4.1-kb fragment of DNA. This fragment complemented the L1.97 strain only in the presence of an exogenous promoter; furthermore, no terminator-like sequences could be identified during our sequence analysis, suggesting that it was an internal section of an operon.

In this study, we have isolated and sequenced the 3′ end of the *exe* operon, composed of eight additional genes, *exeG* to *exeN*, followed by a terminator. We used marker exchange mutagenesis to show that inactivation of genes in the upstream, central, or downstream region of the *exe* operon not only prevented extracellular secretion but also resulted in a pronounced deficiency in the quantities of the major porins in the outer membranes of the mutants. These results demonstrate that in contrast to other bacteria in which it has been found, a functioning extracellular secretion operon is required for the normal assembly of the outer membrane in this bacterium.

The *exe* genes are similar to those contained in other secretion operons, such as those of *Klebsiella oxytoca* (36); however, wide variations in the extent of that similarity are observed. The most highly conserved of the newly isolated genes is *exeG*, which, along with *exeH*, *exeI*, *exeJ*, and *exeK*, is predicted to encode a protein which contains a prepilin signal peptide. These peptides would be expected to be cleaved by a prepilin peptidase similar to PulO of the pullulanase secretion operon of *K. oxytoca* (37). The *exe* operon, however, does not appear to contain a homolog of the prepilin peptidase gene, suggesting that, as in *Pseudomonas aeruginosa*, the prepilin peptidase is encoded by a gene unlinked to the secretion operon (1, 29). Processing of the ExeG protein was observed in a T7 RNA polymerase-dependent expression system in *Escherichia coli* in the presence of a plasmid containing the cloned prepilin peptidase of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *A. hydrophila* Ah65 and its derivatives were grown at 30°C in brain heart infusion supplemented with the appropriate antibiotics.

* Corresponding author.

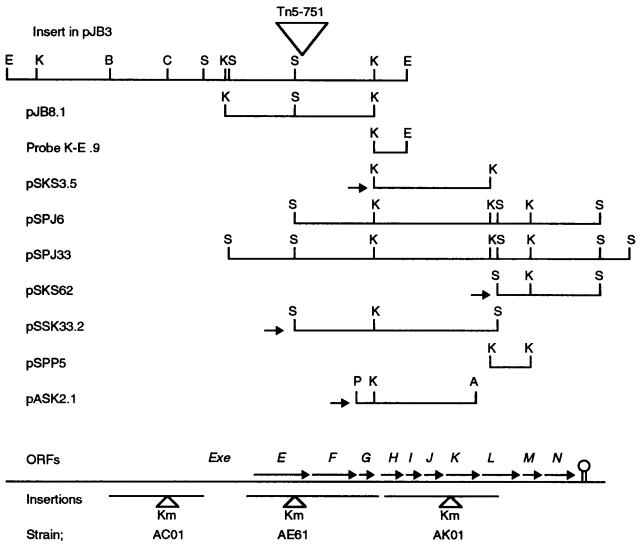


FIG. 1. Isolation of *exe* genes. The site of insertion of Tn*5*-751 in the *A. hydrophila* L1.97 mutant, the original complementing fragment in pJB3, and the previously sequenced pJB8.1 fragment are shown at the top of the figure. The 0.9-kb *Kpn*I-*Eco*RI probe was used to isolate pSKS3.5 and the cosmid clones pSPJ6 and pSPJ33. pSKS3.5, pSKS62, pSSK33.2, and pSPP5 are pBluescript clones, used for the sequence analysis and in vitro transcription-translation reactions described in the text. pASK2.1 contains the indicated fragment in the pETK5 vector and was used in the T7 RNA polymerase-directed expression studies. The positions of the sequenced *exe* ORFs are shown at the bottom of the figure, as are those of the kanamycin resistance cassettes exchanged into the Ah65 chromosome. A, *Aat*II; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sal*I.

E. coli HB101, XLBlue, S-17, and BL21(DE3) were grown in Luria-Bertani or M9 medium. Antibiotics were used at the following concentrations (in micrograms per milliliter): ampicillin, 100; kanamycin, 50; chloramphenicol, 20; and tetracycline, 20. An *A. hydrophila* gene bank in the wide-host-range cosmid pVK102 with HB101 as the host has been previously described (19). Subcloning and sequence analysis were done with the phagemids pBluescript-II SK^+ and KS^+ in XLBlue, while the vector pETK5 in BL21(DE3) was used as the vector for expression of genes under the control of the T7 RNA polymerase promoter (43). pETK5 is a derivative of pET5, in which the kanamycin resistance cassette of pUC4K (45) has been inserted into the unique *Sca*I site, allowing selection for maintenance with kanamycin. The mobilizable suicide vector pSUP202 was used for marker exchange mutagenesis and was conjugated into *A. hydrophila* from S-17 (39). Plasmid pJE4, containing the prepilin peptidase gene *xcpA* (*pilD*) under the control of the *lac* promoter, was kindly provided by Andrée Lazdunski (1). The plasmids and strains constructed and used in this study are diagrammed in Fig. 1.

DNA manipulations and analyses. DNA sequence analysis was performed on nested deletions (13) by using T7 DNA polymerase (U.S. Biochemical Corp.) and the dideoxynucleotide chain termination method (38). Two complete series of unidirectional deletions, one from each end, were prepared for each of the fragments sequenced (Fig. 1), and overlapping deletions were identified by using minipreps or the polymerase chain reaction as previously described (12, 23). All sequence ambiguities between the two strands were resolved by using dITP in the sequencing reaction. Marker exchange mutagenesis with pSUP202 as the delivery plasmid was performed as described elsewhere (39). In each case, a kanamycin resistance cassette (45) was first inserted into a pBluescript subclone of the region to be exchanged (Fig. 1), and the fragment was then ligated into pSUP202. Following conjugation from *E. coli* S-17 into *A. hydrophila* Ah65, the exconjugants were selected with kanamycin and further screened for the chloramphenicol or tetracycline resistance markers of the plasmid. Presumptive marker exchange mutants were then analyzed by Southern hybridization (40), as shown in Fig. 5.

TABLE 1. Characteristics of the *exe* ORFs

Gene	Ribosome binding site	Start codon	Length (bp)	Mass (Da)	% Identity[a]
exeE	TC**AGGG**TAACAAGTA	ATG	501	55,847	63
exeF	CGCC**AGAAG**CAGGGG	GTG	388	43,404	49
exeG	GTT**AGGGAG**TAAGTC	ATG	143	15,892	71
exeH	GGT**GGGG**TGGGACCA	ATG	183	21,056	27
exeI	CG**AGGAGG**AAGAGGA	ATG	119	13,330	50
exeJ	GCC**AA**C**GA**CACCCAA	ATG	207	23,439	43
exeK	AAGC**GGA**CCGGCAGC	ATG	331	36,670	38
exeL	GGGATGC**GGA**CGGGA	ATG	389	42,752	29
exeM	G**AAGGG**TAAATCATC	ATG	163	18,559	26
exeN	ATC**GAG**TCGTTTTCT	ATG	252	27,810	29

[a] Percent amino acid identity between the Exe protein and the corresponding Pul protein.

The cosmids pSPJ6 and pSPJ33 were identified by a colony hybridization procedure as previously described (11). All hybridizations employed probes labeled with digoxigenin, and the probes were detected with an anti-digoxigenin antibody-alkaline phosphatase conjugate and the Lumiphos substrate (Boehringer Mannheim).

Cell fractionation and enzyme assays. For the assays of extracellular secretion, *A. hydrophila* cells were grown in brain heart infusion to late log phase and centrifuged to recover the cells and culture supernatant. The cells were osmotically shocked by the method described by Willis et al. (47). The shocked cells were then ruptured by treatment with lysozyme in the presence of EDTA as previously described (22). All fractions, including the 25% sucrose–33 mM Tris (pH 8.0)–1 mM EDTA wash solution used to prepare the cells for osmotic shock, were assayed for enzyme activities as shown in Table 1. Aerolysin was assayed in microtiter plates with a 0.8% (vol/vol) solution of human erythrocytes (17). The activities are expressed as titers, equal to the inverse of the highest dilution at which 100% lysis of the erythrocytes was observed after incubation for 1 h at 37°C. Protease activity was measured with resorufin-conjugated casein (Boehringer Mannheim) as the substrate, by a protocol described by Twining (44). One unit is defined as a change in optical density at 574 nm of 0.01/h at 37°C. β-Lactamase was assayed with the chromogenic substrate PADAC (18). Units are nanomoles of substrate hydrolyzed per minute at 25°C.

To prepare outer membrane samples, cells were grown in brain heart infusion to late log phase, treated with lysozyme and EDTA, and ruptured by dilution in cold water. Cell membranes were collected by centrifugation of the lysate for 1 h at 35,000 rpm in a Beckman 60 Ti rotor. The membranes were resuspended in 20 mM Tris-HCl, pH 8.0, to which sodium lauryl sarcosinate was added to a final concentration of 0.5% to solubilize the inner membrane (10, 22). The outer membranes were recovered by a further centrifugation as described above and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

General procedures. SDS-PAGE was performed on 10 or

12% acrylamide gels in the buffer system described by Laemmli (25). The pBluescript subclones indicated in Fig. 4 were used in a cell-free transcription-translation system to identify the polypeptides encoded by the *exe* genes (50). The proteins synthesized by the extract were labeled with [^{35}S]methionine (1,200 Ci/mmol) at a concentration of 200 μCi/ml and were analyzed by SDS-PAGE and autoradiography. For expression under the control of the T7 RNA polymerase, BL21(DE3) cells containing the plasmids indicated in Fig. 7 were grown at 37°C in M9 medium and induced by the addition of isopropyl-β-D-thiogalactopyranoside to 0.4 mM. After 30 min of incubation, rifampin was added to 200 μg/ml to inhibit endogenous RNA synthesis. After a further 15 min, [^{35}S]methionine was added to a final concentration of 100 μCi/ml and chased after 1 min with 0.25 mg of unlabeled methionine per ml. Samples were taken after 1, 15, 30, and 60 min of chase and were analyzed by SDS-PAGE and autoradiography.

Nucleotide sequence accession number. The sequence presented in this report has been submitted to the EMBL and GenBank data bases under accession number X66504.

RESULTS

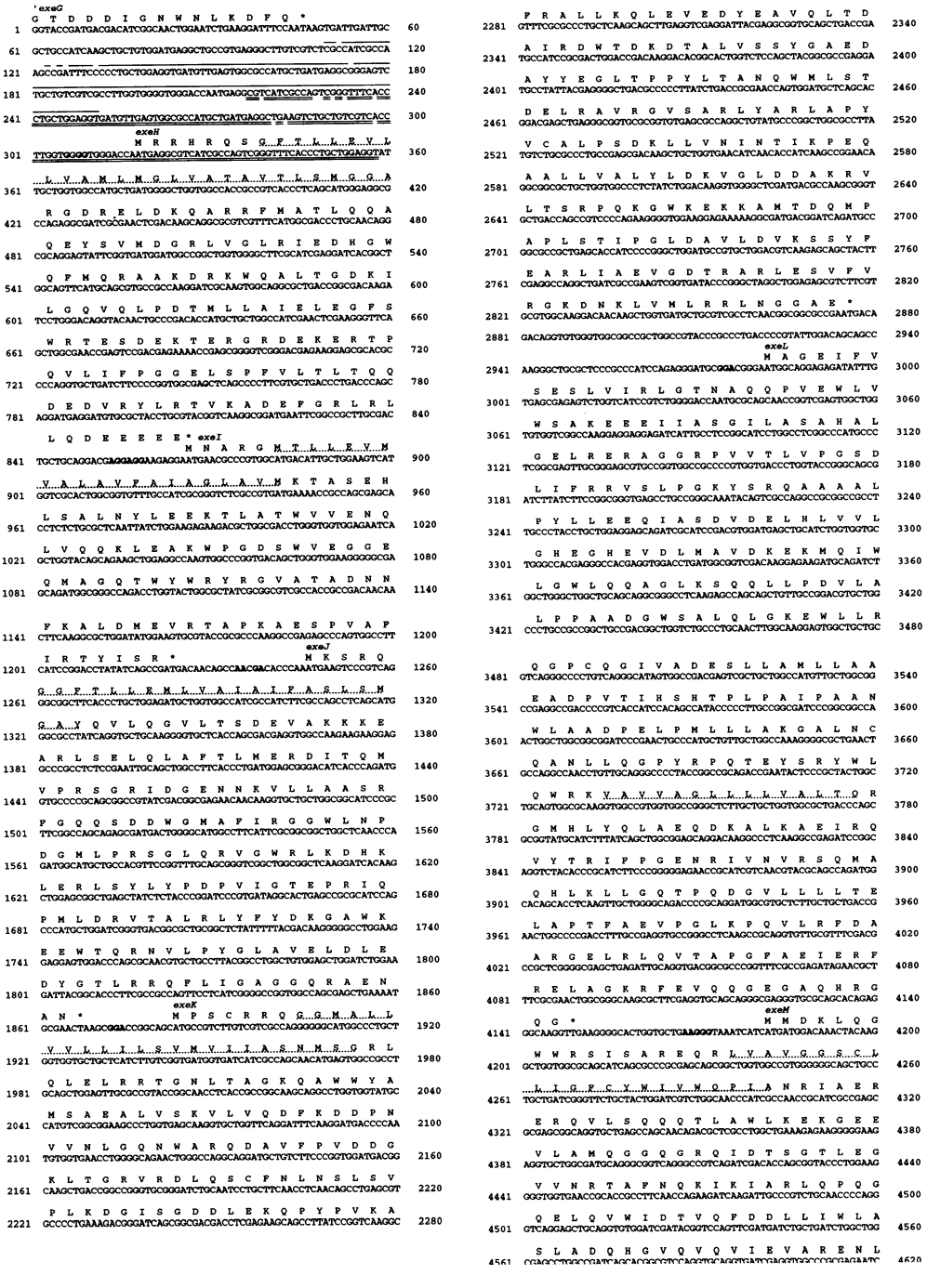
Isolation and analysis of eight *exe* genes 3′ to *exeF*. We have previously sequenced a 4.1-kb *Kpn*I fragment containing the *exeE* and *exeF* genes, isolated from the 3′ side of an 11-kb fragment which complemented the Tn*5*-751 mutant L1.97 (22, 23). The analysis of this sequence had indicated that a partial open reading frame (ORF) containing the beginning of the *exeG* gene was present and would continue into the 0.9-kb *Kpn*I-*Eco*RI fragment which composed the rest of the original 11-kb fragment cloned in pJB3 (Fig. 1). We purified this fragment and used it as a probe in the isolation of the 3.2-kb *Kpn*I fragment 3′ adjacent to the 4.1-kb *Kpn*I. In addition, the 0.9-kb fragment was used to isolate the overlapping cosmids shown in Fig. 1 from a library of *A. hydrophila* DNA constructed in pVK102. The 3.2-kb *Kpn*I fragment and the 3.1-kb *Sal*I and 1.1-kb *Kpn*I fragments shown in Fig. 1 were subcloned from pSPJ6 and pSPJ33 and sequenced. The sequence, which starts at the 3′ end of the *exeG* ORF, is shown in Fig. 2. Sequence analysis revealed the presence of seven additional complete ORFs, shown in translation on the figure, which appear to constitute the 3′ side of the *exe* operon. In addition, a 142-bp direct repeat which is overlapped by 35 bp is present between the *exeG* and *exeH* ORFs (Fig. 2). The last complete ORF, *exeN*, is followed by a 10-bp inverted repeat which was predicted to be a rho-independent terminator by the algorithm described by Brendel and Trifonov (3). A promoter-like sequence could be identified 260 bp further downstream of this, followed by a truncated ORF which FASTA analysis showed was 86% identical in amino acid sequence to the equivalent portion of the *celA* gene of *E. coli*. This gene, whose function is unknown, is the first ORF of the cryptic cellobiose utilization operon (30).

All of the ORFs were analyzed by the methods described by Kyte and Doolittle (24) and Engelman et al. (9) to identify possible transmembrane regions. Seven of the eight *exe* ORFs sequenced in the present study would encode a protein which contains at least one stretch of >20 hydrophobic amino acids, as shown in Fig. 2. In ExeG, ExeH, ExeI, ExeJ, ExeK, ExeM, and ExeN, the single strongly hydrophobic region is very close to the amino terminus of the protein, which is otherwise hydrophilic. The ExeL protein also has one highly hydrophobic sequence, of 15 amino acids, located between residues 252 and 266. Although the amino-terminal sequences of ExeG, ExeH, ExeI, ExeJ, and ExeK resemble classical signal sequences, they more closely resemble the consensus cleavage sequence for the recently discovered prepilin peptidase (Fig. 3; also, see below). The amino-terminal hydrophobic region of the ExeN protein contains the structural features of a signal sequence cleaved by signal peptidase I, although at 14 residues it would be among the shortest of signal peptides (5). The rest of the sequence of this protein is quite hydrophilic. However, hydrophobic moment analysis by the algorithm described by Eisenberg et al. (8) revealed the presence of a number of sequences which, if found in a β-sheet conformation, would be strongly amphiphilic (Fig. 2).

FASTA analysis also showed that the sequence of the ORFs from *exeG* to *exeN*, like those of *exeE* and *exeF*, showed strong sequence similarity to the *pul* genes which have been shown to be required for pullulanase secretion in *K. oxytoca* (6), as well as the *xcp* genes of *P. aeruginosa* (2). The lengths, possible ribosomal binding sites, start codons, and molecular masses of the predicted proteins and the similarities to the corresponding Pul protein of each of the sequenced *exe* ORFs, from *exeE* to *exeN*, are listed in Table 1.

Expression of the *exe* genes in vitro. To validate the assignment of ORFs in the sequenced region, a number of fragments were subcloned downstream of the *lac* promoter in the pBluescript vectors, and in vitro transcription-translation reactions were carried out (50). The 3.2-kb *Kpn*I fragment in pSKS3.5 produced proteins of the expected molecular masses corresponding to ExeH, ExeI, ExeJ, and ExeK, while the 3.1-kb *Sal*I fragment in pSKS62 expressed proteins of the expected sizes from the *exeM* and *exeN* ORFs (Fig. 4; Table 1). Because the entire *exeG* ORF is not present in the 4.1-kb *Kpn*I fragment in pJB8.1, which was shown to contain the genes encoding ExeE and ExeF (23), the 5.1-kb *Sal*I fragment containing *exeF*, *exeG*, *exeH*, *exeI*, *exeJ*, and most of *exeK* was also subcloned as SSK33.2 and similarly analyzed. This fragment produced a protein identical to that previously identified as ExeF (23) and, in addition, a very strongly expressed protein with an apparent molecular mass of 18 kDa (Fig. 4). This protein is presumably the *exeG* gene product. If it is, however, it must migrate anomalously on SDS-PAGE gels, since its calculated molecular mass is 15,892 Da. A truncated fragment of this gene present in the 4.1-kb *Kpn*I fragment which contains *exeE* and *exeF* produced a polypeptide with a molecular mass of 16.5 kDa, despite a calculated molecular mass of 14,547 Da, supporting this interpretation (23). Surprisingly, this construct did not produce appreciable amounts of the peptides identified as ExeH, ExeI, ExeJ, and ExeK, despite the presence of the corresponding genes on the fragment (Fig. 4; compare lanes 1 and 3).

Marker exchange mutagenesis of *exe* genes. To determine the involvement of the *exe* genes and their products in extracellular secretion and to examine their possible roles in outer membrane protein assembly in *A. hydrophila*, three different regions of the *exe* operon in Ah65 were interrupted by the insertion of a kanamycin resistance gene cassette using marker exchange mutagenesis. The *exeE* gene, into which Tn*5*-751 had inserted in the original secretory mutant L1.97 (23), was inactivated, as was the *exeK* gene sequenced in the present study. In addition, a kanamycin resistance cassette was inserted 1.6 kb upstream of the 5′ end of the 4.1-kb *Kpn*I fragment (Fig. 1), which contains the *exeE* and *exeF* genes, to determine whether additional genes 5′ to the sequenced region are involved in extracellular protein secretion. Each of the insertionally inactivated regions was subcloned into the mobilizable suicide vector pSUP202 and conjugated into Ah65. Exconjugates from these crosses were examined by Southern analysis. In each case, strains in which a double crossover had resulted



in the replacement of the wild-type gene by the insertionally inactivated one were obtained (Fig. 5).

The mutants constructed were grown in broth culture, and the extracellular proteins aerolysin and protease were assayed. As the results in Table 2 show, the mutants were completely unable to secrete these proteins, which instead were accumulated in the periplasmic space, as indicated by their release from the mutant cells by osmotic shock. Envelope and outer membrane samples were also prepared from the wild-type and mutant strains and examined by SDS-PAGE (Fig. 6). The previously identified major outer membrane proteins I, II, IV, V, and VI (21) could be observed in the outer membrane fractions isolated from the wild type. However, in all three of the mutants, proteins I and II were almost completely absent from the outer membranes. Examination of the whole-envelope preparations by SDS-PAGE indicated that the total membrane fractions of these cells were also deficient in these proteins (Fig. 6), and they could not be observed in SDS-PAGE gels of cytoplasmic or periplasmic fractions either (data not shown).

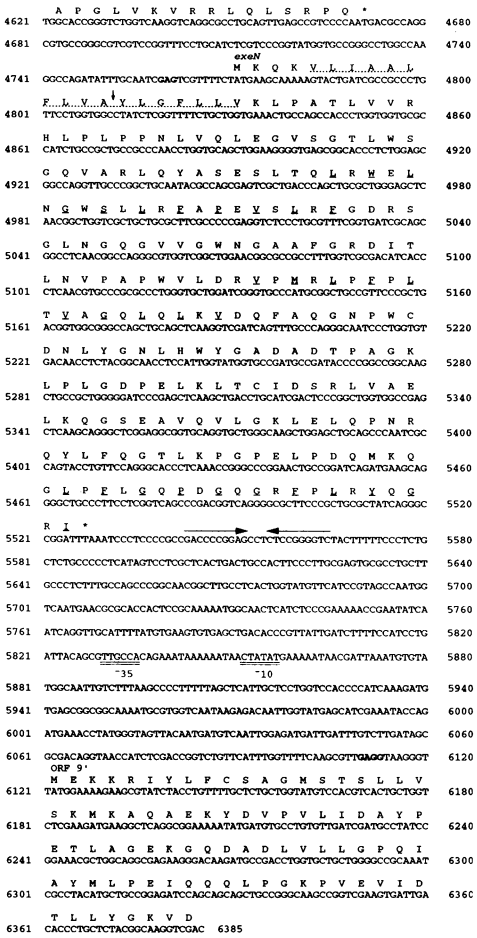


FIG. 2. Sequence of the *exe* genes. The sequence of the noncoding strand of the region sequenced in this study is given, along with its translation. Potential ribosome-binding sites are in boldface type. The first of the 142-bp direct repeats between *exeG* and *exeH* is overlined, while the second is doubly underlined. The inverted repeat of a sequence predicted to be a rho-independent terminator following *exeN* is overlined with arrows, and −35 and −10 regions of a promoter-like sequence which precedes ORF9 are doubly underlined. Regions of pronounced hydrophobicity in the protein sequences are dotted, while the residues on the hydrophobic face of ExeN regions predicted to be amphiphilic if in a β-sheet conformation are individually underlined. A potential signal peptidase I cleavage site following the hydrophobic sequence at the amino terminus of ExeN is denoted by a vertical arrow.

Processing of *exeG* by prepilin peptidase. Examination of the sequences of the *exe* ORFs showed that four of them, *exeG*, *exeH*, *exeI*, and *exeJ*, would encode proteins with amino-terminal sequences which are highly similar to those of type IV pilin precursors. In addition, ExeK would contain an amino-terminal sequence of significant homology to a new consensus sequence which includes these and other newly identified prepilin-like precursors (27) (Fig. 3). It has been shown that type IV prepilin precursors are processed by a novel signal peptidase, called prepilin peptidase, which removes only the first five to seven amino acids of the precursor, usually resulting in a phenylalanine at the new amino terminus which is methylated in the mature subunit (29, 42). A fragment containing the complete *exeG*, *exeH*, *exeI*, and *exeJ* genes was cloned behind the T7 RNA polymerase promoter in pETK5 to

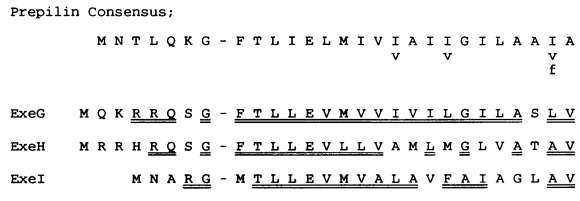


FIG. 3. Prepilin peptidase cleavage sites at the amino termini of the ExeG, ExeH, ExeI, ExeJ, and ExeK proteins. The consensus sequence for prepilin proteins is given at the top of the figure, while a new consensus sequence which includes the sequenced prepilin-like proteins of the general secretion pathway is given at the bottom. Residues which are present in 50% or more of the protein sequences examined (n = 26) are capitalized, while residues present in 25 to 50% of the proteins are included as lowercase letters. Amino acid residues of the Exe proteins which match the comprehensive consensus sequence are doubly underlined. The *A. hydrophila* Ae6 W pilin sequence is given at the bottom of the figure for comparison (14).

examine the possible processing of these proteins in vivo. When this construct was induced in *E. coli* under selective labeling conditions, however, only a protein with a molecular mass of 18 kDa was strongly expressed (Fig. 7). This protein, indicated by the in vitro transcription-translation analysis to be ExeG, appeared to be very slowly processed to a form that was 1 to 2 kDa smaller in these cells. The extent of this processing was increased dramatically but still took place relatively slowly when the cells containing this plasmid were also transformed with pJE4, containing the prepilin peptidase gene of *P. aeruginosa* (1).

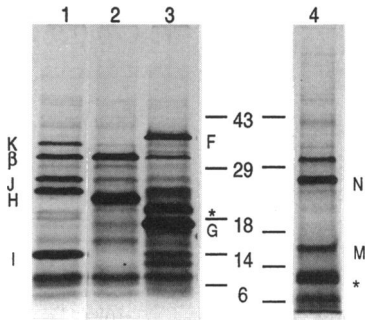


FIG. 4. In vitro transcription-translation of *exe* genes. An autoradiograph of [^{35}S]methionine-labeled proteins produced by pSKS3.5 (lane 1), pKSII$^+$ (lane 2), pSSK33.2 (lane 3), and pSKS62 is shown. The bands which correspond to the molecular masses expected for the Exe proteins encoded by the plasmids are indicated by the appropriate letters (see Table 1), while fusion proteins which would be encoded by the plasmids are denoted by asterisks (a 21-kDa fusion protein encoded by *lacZ* and *exeE* in lane 3 and a 8.6-kDa fusion protein composed of *lacZ* and an out-of-frame portion of *exeL* in lane 4). Molecular mass standards (in kilodaltons) are shown between the panels.

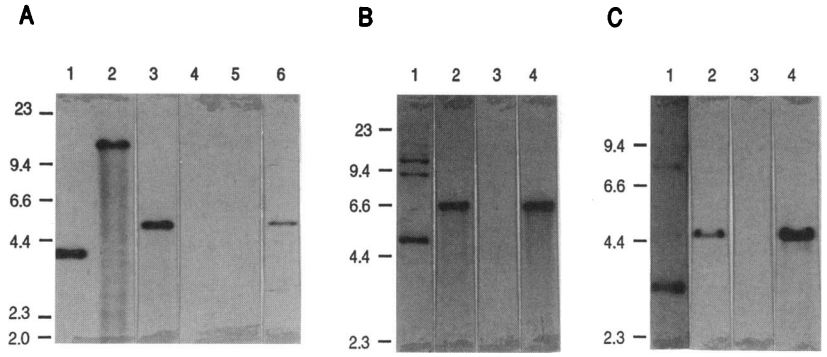


FIG. 5. Southern analysis of marker exchange mutants of *A. hydrophila* Ah65. Fluorographs of the Southern blots are shown. (A) Ah65 (lanes 1 and 4), L1.97 (lanes 2 and 5), and AE61 (lanes 3 and 6) were probed with the insert from pJB8.1 (lanes 1 to 3) and the *aph* gene encoding kanamycin resistance from pUC4K (lanes 4 to 6); (B) Ah65 (lanes 1 and 3) and AC01 (lanes 2 and 4) were probed with the 4.8-kb *Kpn*I fragment from pJB3 (lanes 1 and 2) and the *aph* gene encoding kanamycin resistance from pUC4K (lanes 3 and 4); (C) Ah65 (lanes 1 and 3) and AK01 (lanes 2 and 4) were probed with the insert from pSKS3.5 (lanes 1 and 2) and the *aph* gene from pUC4K (lanes 3 and 4).

DISCUSSION

A. hydrophila secretes a large number of proteins completely across the two-membrane barrier of its envelope, and, as in many other gram-negative bacteria, these proteins play important roles as toxins during infections by this bacterium. It has been demonstrated, for example, that the hemolysin aerolysin is an important virulence determinant in a mouse model of pathogenicity, and protease-negative mutants of *A. hydrophila* have been shown to be comparatively avirulent in infections of fish (4, 26). An increasingly large number of the genes for these extracellular proteins have been cloned and sequenced, and, in each case, a typical signal sequence can be identified at the amino terminus of the protein. Indeed, in the case of aerolysin, it has been shown that the signal sequence is removed during energy-dependent translocation of the protein across the inner membrane, before it is translocated across the outer membrane in a second step separable from inner membrane translocation by either mutation or specific growth conditions (22, 48). We have shown that transposon insertion into the *exeE* gene interrupts this second step of the pathway, resulting in the accumulation of the normally extracellular proteins in the periplasmic space (23). In the present study, we have isolated and sequenced an additional eight genes, *exeG* to *exeN*, from the 3′ end of the *exe* operon. The operon appears to terminate at *exeN*, since a rho-independent terminator-like sequence follows this ORF and is then followed by a promoter sequence and the beginning of an unrelated ORF. We used marker exchange mutagenesis to insert a kanamycin resistance cassette into *exeE*, *exeK*, and the upstream region of the operon. Each of the resulting mutant strains displayed a nonsecretory phenotype identical to that of the original Tn*5*-751 insertion mutant L1.97, indicating that the integrity of the entire operon is required for extracellular secretion. In addition, each of these mutants also displayed the same protein deficiencies in the outer membrane as L1.97. This demonstrates conclusively that both phenotypes were due to the inactivation of *exeE* by Tn*5*-751 in the original mutant and further suggests that, at least in *A. hydrophila*, the products of this entire operon play a fundamental role not only in extracellular secretion but also in normal assembly of the outer

TABLE 2. Enzyme activities in Ah65 and insertion mutants[a]

Fraction	Aerolysin in HU	Protease in U/ml (%)	β-Lactamase in U/ml (%)
Ah65			
Supernatant	512	147 (70)	0.53 (4)
Wash Fluid	0	27 (13)	0.47 (3)
Shock Fluid	4	27 (13)	11.35 (83)
Shocked Cells	0	9 (4)	1.42 (10)
AC01			
Supernatant	0	7 (3)	0.45 (9)
Wash Fluid	0	1 (1)	0.51 (10)
Shock Fluid	128	217 (90)	3.55 (69)
Shocked Cells	0	15 (6)	0.67 (13)
AE61			
Supernatant	0	8 (5)	0.51 (6)
Wash Fluid	0	2 (1)	0.51 (6)
Shock Fluid	128	162 (93)	6.27 (79)
Shocked Cells	0	3 (2)	0.65 (8)
AK01			
Supernatant	0	9 (4)	0.59 (10)
Wash Fluid	0	2 (1)	0.55 (10)
Shock Fluid	128	214 (95)	3.67 (65)
Shocked Cells	0	1 (1)	0.85 (15)

[a] The hemolytic activities of aerolysin are given as the titers, while protease and β-lactamase activities are given as units per milliliter of the original culture. The results are typical of those of a number of similar experiments. HU, hemolytic units.

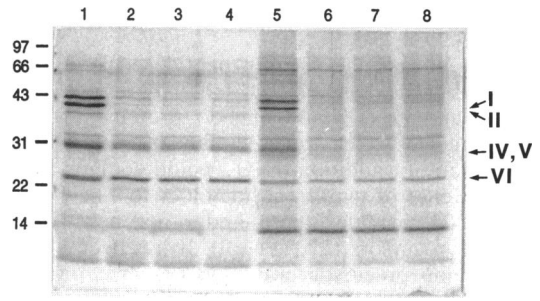


FIG. 6. Membrane fractions isolated from wild type and insertion mutants. A Coomassie blue-stained SDS-PAGE gel of outer membrane (lanes 1 to 4) and envelope (lanes 5 to 8) fractions is shown. Lanes 1 and 5, Ah65 membranes; lanes 2 and 6, AE61; lanes 3 and 7, AC01; lanes 4 and 8, AK01. The positions of the major outer membrane proteins I, II, IV, V, and VI are indicated.

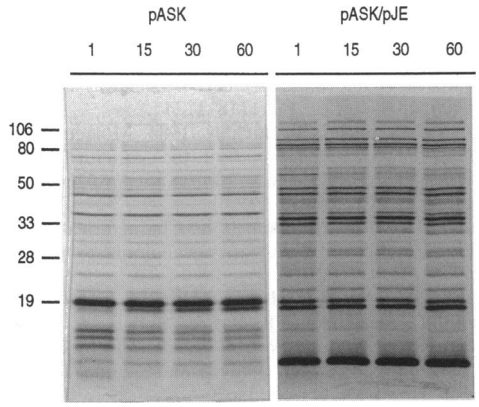


FIG. 7. Proteins expressed under the control of the T7 RNA polymerase promoter. BL21(DE3) cells containing pASK2.1 (left) or pASK2.1 and pJE4 (right) were induced as described in Materials and Methods, labeled with 100 μCi of [^{35}S]methionine per ml for 1 min, and chased with unlabeled methionine for 1, 15, 30, and 60 min, as indicated. An autoradiograph of the SDS-PAGE gel is shown.

membrane. The outer membrane proteins most affected by these mutations, I and II, have been shown to be homologs of the LamB and OmpF porins of *E. coli* (21). It is possible that the structure of the envelope in these mutants is disturbed in such a way that these porins cannot properly assemble in its modified architecture; however, an alternative explanation would be that some part of the assembly pathway for the outer membrane porins is shared by the extracellular proteins, which jam this point in the absence of a functional route outside the cell. In this context, it must be noted that another Tn*5*-751 insertion mutant which accumulates extracellular proteins in the periplasm, C5.84, does not display any deficiency in the amounts of these porins in its outer membrane (22). We have isolated this locus, and sequence analysis indicates that it bears no relationship to any of the identified members of the PulC to PulO extracellular secretion pathway, including the prepilin peptidase (20a). This suggests that it represents a hitherto unidentified additional step in the general secretion pathway. If this step is earlier than that shared by the outer membrane and extracellular proteins, the mutation may prevent the extracellular proteins from jamming the shared machinery. We are currently constructing a double mutation for these two loci in an attempt to test this hypothesis. Another possibility is that the inability to secrete extracellular proteins in the *exe* mutants in some way interferes with the cells' ability to receive environmental signals necessary for the expression of the OmpF homolog. An example of this kind of secondary effect is that the transcription of *ompF* is reduced in *E. coli* cells which have been grown in dithiothreitol or which are unable to synthesize DsbA, a periplasmic protein which catalyzes the formation of disulfide bonds (35). Interestingly, the *dsbA* mutation has also been shown to inhibit the secretion of a number of extracellular proteins (32, 33, 49). The amounts of none of the major outer membrane proteins are altered in *A. hydrophila* cells grown in the presence of dithiothreitol (data not shown), suggesting that a similar link between disulfide bond formation and expression of outer membrane proteins does not exist in this bacterium.

The *exe* operon, like each of the other extracellular operons characterized to date, encodes a number of proteins which contain prepilin signal sequences. One of these prepilin-like proteins is ExeK, for which the homology with the consensus sequence is the weakest of the group (Fig. 3). However, it does conform to the consensus sequence for 7 of 11 residues in the vicinity of the cleavage site and includes the invariant Gly at the −1 position which mutational analysis has shown is required for cleavage (41). The other PulK homologs identified to date also show similarity to the consensus cleavage sequence, albeit not as extensive as that for ExeK (2, 27, 34). The most notable deviation from the consensus sequence for these proteins is the lack of a Glu residue at the +5 position. Two different mutations at this position (one of which was Glu→Val, as is found here) have been shown to block methylation of the +1 residue of the mature pilin but not processing of the prepilin (31, 41).

We were able to selectively label only the ExeG protein in vivo in this study. The ExeG protein was very slowly processed in *E. coli*, which may reflect the partial functioning of remnants of this secretion pathway in this bacterium (7, 46). However, this processing was greatly increased in the presence of the prepilin peptidase of *P. aeruginosa*, making it likely that these proteins are also processed in vivo in *A. hydrophila*. A similar construct which was used to examine the processing of the *xcp* prepilin-like genes involved in extracellular secretion in *P. aeruginosa* also strongly expressed only one of these four proteins; however, on the basis of its apparent molecular weight, it was concluded that it was the XcpU protein, the homolog of ExeH, rather than the ExeG homolog XcpT (2). However, our in vitro transcription-translation results suggest that ExeG migrates at an anomalously high apparent M_r in SDS-PAGE gels; if a similar situation exists in *P. aeruginosa*, it would appear that in both systems some mechanism operates to strongly attenuate the expression of the genes downstream of *exeG* and *xcpT*. Although no terminator-like structure was observed during our sequence analysis of this region, there is a large overlapped direct repeat in the *exeG-exeH* intergenic region (Fig. 2). Expression studies in constructs in which this region is deleted may shed light on the mechanism of this apparent attenuation. In any case, the processing of the ExeG protein in the presence of the *P. aeruginosa* prepilin peptidase indicates that, as in the other bacteria in which this secretion system has been identified, a prepilin peptidase is required for the assembly of the secretion apparatus itself rather than for extracellular secretion proper. In the *pul* operon of *K. oxytoca* and the *out* operon of *Erwinia chrysanthemi*, the prepilin peptidases (PulO and OutO, respectively) are encoded by the last ORF of the secretion operon itself (27, 37), whereas the *exe* operon appears to end with *exeN*. While we have not yet completed our analysis of the 5′ end of the operon, this suggests that in *A. hydrophila*, the prepilin peptidase gene is located elsewhere on the chromosome. In *P. aeruginosa*, the prepilin peptidase gene was isolated as the extracellular protein secretion gene *xcpA* and as the pilin assembly gene *pilD*, and this enzyme is found in the pilin assembly operon *pilBCD* rather than the *xcp* operon (28). *A. hydrophila* elaborates a number of pili, and examination of the reported sequences of three of the wavy or flexible pilins indicates that they are in fact type IV pilins, suggesting that *A. hydrophila* may also contain a pilin assembly operon which includes the prepilin peptidase (14, 20).

All of the evidence accumulated so far suggests that most if not all of the extracellular proteins of *A. hydrophila* are secreted via the general secretion pathway, in which proteins homologous to the *sec* gene products of *E. coli* are responsible for recognition of the signal sequence and translocation across the inner membrane, while the *exe* gene products are responsible for translocation across the outer membrane and also play some role in outer membrane assembly. This second step of

the pathway has now been identified in a great many different gram-negative bacteria, including members of the families *Enterobacteriaceae*, *Vibrionaceae*, and *Psuedomonaceae* (34). This operon is thus of fundamental significance with respect to the physiology of the gram-negative bacteria, and especially of the exotoxin-producing pathogens.

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