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Isolation and Characterization of a Second *exe* Operon Required for Extracellular Protein Secretion in *Aeromonas hydrophila*

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Strain C5.84 is a Tn*5*-751 insertion mutant of *Aeromonas hydrophila* which is unable to secrete extracellular proteins, instead accumulating them in the periplasm (B. Jiang and S. P. Howard, J. Bacteriol. 173:1241–1249, 1991). A 3.5-kb *Bgl*II fragment which complements this mutation was isolated from the chromosome of the parent strain. Analysis of this fragment revealed an operon-like structure with two complete genes, *exeA* and *exeB*, a functional promoter 5′ to the *exeA* gene, and a 13-bp inverted repeat immediately 3′ to the *exeB* gene. Although the transposon had inserted in *exeA*, provision of a wild-type copy of this gene alone in *trans* did not restore competence for export to C5.84. Complementation required the presence of both *exeA* and *exeB*, and marker exchange mutagenesis confirmed the requirement for both gene products for secretion. In vitro expression as well as analysis of the deduced amino acid sequence of ExeA indicated that it is a hydrophilic 60-kDa protein with a consensus ATP binding site. ExeB is a 25-kDa basic protein which shares limited homology with PulB, a protein of unknown function associated with the maltose regulon of *Klebsiella oxytoca*, and OutB, a protein which has been shown to be required for efficient secretion in *Erwinia chrysanthemi*. The hydrophilic character of these proteins and preliminary localization studies suggested that they are anchored to the inner membrane. These results demonstrate the involvement of a second operon encoding a putative ATP-binding protein in the secretion of extracellular proteins from gram-negative bacteria and further suggest that the cytoplasmic compartment may play a greater role in protein translocation across the outer membrane from the periplasm than previously thought.

A number of distinct modes of extracellular protein secretion exist in gram-negative bacteria (24, 29). One of the better-understood pathways involves secretion of proteins across both inner and outer membranes in a single step, possibly through membrane adhesion sites, thereby bypassing the periplasm, as exemplified by the hemolysin of *Escherichia coli* (9). It is more common, however, for secretion to involve translocation first across the inner membrane and then, in a separate step, across the outer membrane. Numerous genetic and biochemical studies have characterized the translocation of proteins across the inner membrane; this complex machinery is termed the Sec-dependent or general export pathway (reviewed in reference 24). The general export pathway consists of the translocase SecA, SecY, SecE, SecD, and SecF proteins and the chaperone SecB (41). More recently, secretion across the outer membrane has become the subject of intensive study and has been shown in a number of cases to involve a host of additional proteins comprising a secretion machinery which is known as the general secretion pathway. The components of this pathway have been best characterized in *Klebsiella oxytoca*, in which they are involved in the secretion of pullulanase, and the pathway has also been identified in a growing number of other families of gram-negative bacteria (24, 25). Since the proteins exported by this pathway contain typical amino-terminal signal sequences and accumulate in the periplasmic space in mutants, it is generally considered to be an extension of the *sec* gene pathway for translocation across the inner membrane and to function only after the proteins have arrived at the periplasm via this route. This is despite the fact that one of the proteins encoded by this operon (PulE and its homologs) contains a consensus ATP binding site which is essential for its function (23, 35).

During our earlier studies into the mechanism of extracellular secretion in *Aeromonas hydrophila*, we isolated transposon Tn*5*-751 insertion mutants which were defective in secretion of extracellular proteins (14). One of these, L1.97, led to the isolation of the 12-gene operon *exeC-N*, which is highly homologous to the *pul* operon and which is required for secretion of a large group of toxins by this bacterium (13). It is also required, however, for correct assembly of the outer membrane, since mutations in any one of three different genes of the operon were shown to cause a marked reduction in the quantity of the OmpF homolog protein II in the outer membrane, an effect which renders the bacteria osmotically fragile (13, 14). In the same mutagenesis experiment, we isolated another mutant, C5.84, which like L1.97 produces all of the extracellular proteins assayed but accumulates them in the periplasmic space. Southern hybridization experiments demonstrated that in this mutant the transposon had not inserted in the *exeC-N* operon. Furthermore, these mutants differ in possessing an outer membrane protein profile identical to that of the wild type (14). Together, these results suggested that the mutation in C5.84 had affected an aspect or step in extracellular secretion which is separate from that controlled by the *exeC-N* operon.

In the studies reported here, we have isolated and sequenced a 3.5-kb *Bgl*II fragment which complemented the mutation in C5.84. By subcloning, complementation, analysis of expression in vitro, and marker exchange mutagenesis, we show that this locus consists of an operon composed of two genes, each of which is required for extracellular secretion.

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One of these genes encodes a hydrophilic 60-kDa protein which contains a putative ATP binding site, while the other encodes a 25-kDa basic protein which shares limited homology with PulB, a protein of unknown function in the maltose regulon of *K. oxytoca*, and OutB, which is required for efficient secretion in *Erwinia chrysanthemi*. The fact that mutations in these genes give rise to a phenotype clearly separable from those in the *exeC-N* operon indicates that extracellular secretion is more complex than previously thought. Furthermore, the fact that they encode hydrophilic proteins, one of which may bind and/or hydrolyze ATP, may be an indication that extracellular proteins either are recognized as such while still in the cytoplasm or require continued interaction with this compartment for their translocation across the outer membrane.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The wild-type strain used in this study was *A. hydrophila* Ah65, and all other *Aeromonas* strains were derived from it. These bacteria were grown at 30°C in brain heart infusion medium or Riddle's medium (28). *E. coli* XLBlue1 and S17-1 were grown at 37°C in LB medium (17). Plasmid pBluescript SKII/KSII$^+$ was used for subcloning and in vitro expression experiments, and the wide-host-range vectors pMMB67EH/HEcam (15) and pMMB207/208 (18) were used for transferring the inserts into *A. hydrophila*. Antibiotics were used at the following concentrations (micrograms per milliliter): ampicillin, 100; choramphenicol, 5; gentamicin, 30; kanamycin, 50; nalidixic acid, 10; streptomycin, 100; and tetracycline, 10.

DNA preparation, manipulation, and analyses. Small-scale plasmid DNA preparation was done by the alkaline lysis protocol (2). CsCl-ethidium bromide density gradient centrifugation was used to prepare large-scale plasmid DNA (30). Restriction mapping, blunting of overhanging single-stranded ends by using the Klenow fragment of *E. coli* DNA polymerase or T4 DNA polymerase, ligations, vector dephosphorylation by calf intestinal alkaline phosphatase, and agarose gel electrophoresis were performed as described previously (30). Restriction fragments were purified by using Geneclean (Bio 101, Inc). Plasmid DNA ligation mixtures were transformed by electroporation into *E. coli* strains and selected by plating on media containing appropriate antibiotics. Wide-host-range plasmids were introduced into *A. hydrophila* by conjugation from *E. coli* S-17 (32).

Marker exchange mutagenesis. Marker exchange mutagenesis was performed by using the suicide plasmids pSUP202 (32) for *exeA* and pJQ200SK (27) for *exeB*. In both cases, a kanamycin resistance cassette from pUC4K (37) was first introduced into the appropriate region of subclone pSRJ5.2 (see Fig. 5A), and the fragment was then ligated into the suicide vector. The constructs were then conjugated from *E. coli* S17-1 into *A. hydrophila* Ah65 with selection for kanamycin resistance and further screened for the plasmid chloramphenicol resistance marker in the case of pSUP202. For pJQ200SK, the transconjugants were selected on kanamycin plus 5% sucrose to select against the presence of the entire plasmid and further screened for gentamicin resistance marker of the plasmid. The putative mutants thus obtained were analyzed by Southern hybridization (33) as shown in Fig. 5B. The probes used for the analysis were labeled with digoxigenin-11-dUTP, and an antidigoxigenin antibody-alkaline phosphatase conjugate and the substrate Lumiphos (Boehringer Mannheim) were used to detect the probes following the hybridization.

Nucleotide sequence determination. The 3.5-kb *Bgl*II fragment was cloned in both orientations into the *Bam*HI site of the pBluescript vector SKII$^+$. Two sets of unidirectional nested deletions were obtained by exonuclease III and mung-bean nuclease, starting from either end of the fragment (8). The deletion derivatives were screened by PCR amplification of the insert DNA, using bacterial cells from the transformation plates as previously described (7). The appropriate deletion clones were superinfected with VCSM13 helper phage for single-stranded DNA template isolation (38). Sequencing was performed by the dideoxy-chain termination method of Sanger et al. (31). Ambiguities in the sequence were resolved by using dITP in place of dGTP in the sequencing reaction mixtures to inhibit the formation of secondary structure.

In vitro transcription-translation reaction. Proteins encoded by plasmids pSRJ5.2, pKRJ20.2, pSRJ46.1, pKRJ58.1, pSRJ57.1, pKRJ50.3, and pKRJ50.2 were identified by using an S30 cell extract of *E. coli* in a coupled transcription-translation system (43). [^{35}S]methionine (1,200 Ci/mmol, 400 μCi/ml) was used to label the proteins, and reactions were carried out under the conditions recommended by the supplier (Promega). The reaction mixtures were electrophoresed on sodium dodecyl sulfate (SDS)–12% polyacrylamide gels and autoradiographed.

Enzyme assays. Aerolysin activity was measured as previously described in microtiter plates with 0.8% (vol/vol) human erythrocytes, after conversion of all proaerolysin to aerolysin using trypsin (11). The activity of the samples is measured as the inverse of the highest dilution in which 100% erythrocyte lysis has occurred after 1 h of incubation at 37°C. Protease activity was measured by the method of Twining (36), using resorufin-conjugated casein (Boehringer Mannheim) as the substrate. A change in the optical density at 574 nm of 0.01/h at 37°C is defined as 1 unit.

Nucleotide sequence accession number. The sequence presented in this report has been submitted to the EMBL and GenBank databases under accession number X81473.

RESULTS

Subcloning of the C5.84 Tn*5*-751 insertion site. *A. hydrophila* C5.84 was isolated as a Tn*5*-751 insertion mutant which is unable to secrete the hemolysin aerolysin or any other extracellular protein that we assayed (14). We have previously cloned the site of transposon insertion on a 15-kb *Eco*RI fragment isolated from the wild-type genome and found that it complemented the mutation in C5.84 (14). Using this fragment (in pJB4) as the starting point, we subcloned the complementing gene, in each case placing the fragment into a wide-host-range vector and assaying it for the ability to complement C5.84 (Fig. 1). A 6.6-kb *Xho*I-*Sal*I fragment which contained the transposon insertion site was cloned into pMMB67EH/HEcam in both orientations (pRJ3.1 and pRJ4.1) with respect to the *tac* promoter of the vector. In both cases, complementation was observed, as scored by the presence of a halo of hemolysis around the transconjugants on blood agar plates that was similar to that of the wild type. Similar results were obtained when a 3.5-kb *Bgl*II fragment was cloned in either orientation in this vector (pRJ12.1 and pRJ13.1; Fig. 1) except that the zone of hemolysis around the transconjugants was noticeably larger for pRJ12.1. The aerolysin titers of broth culture supernatants of these transconjugants were 256 for the wild type and 8 for C5.84, while C5.84(pRJ12.1) had a titer of 256 and C5.84(pRJ13.1) had a titer of 128. The addition of isopropylthiogalactopyranoside (IPTG) to the cultures did not have any effect on the titers. The results of the broth culture

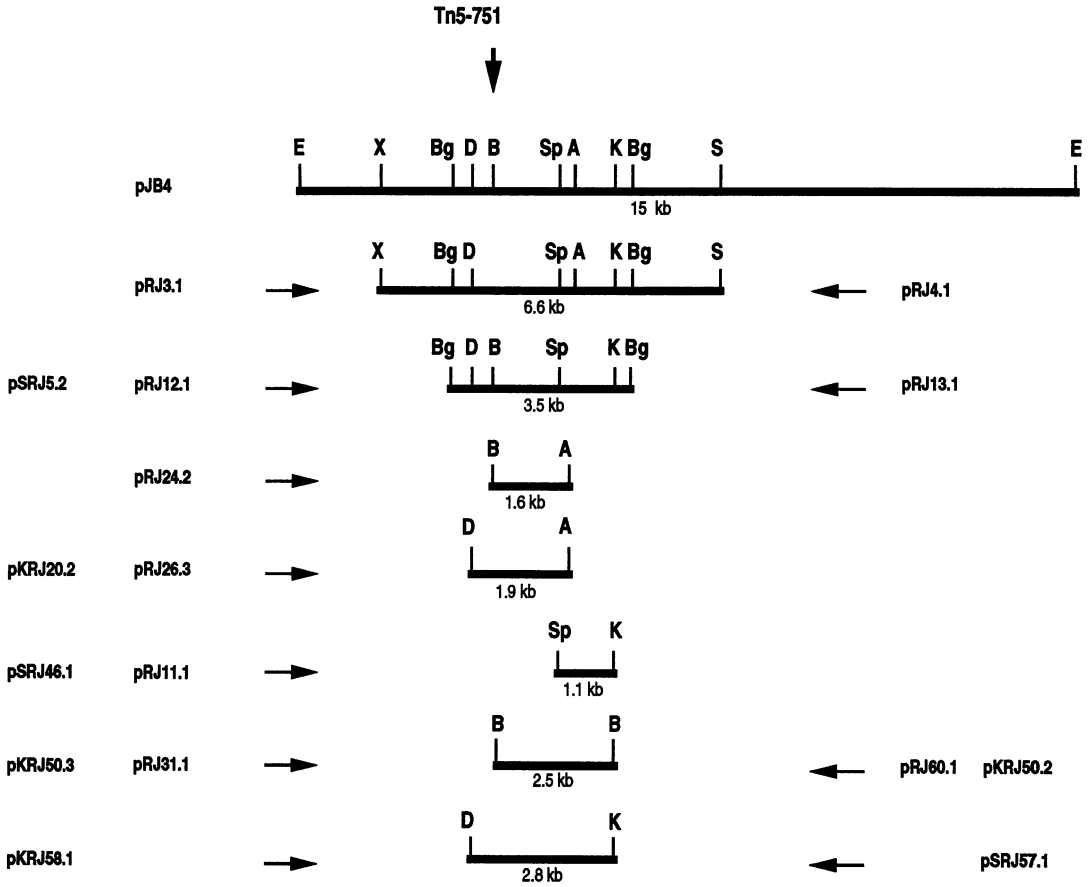

FIG. 1. Subcloning of a region complementing C5.84 from pJB4. The *A. hydrophila* Ah65 insert in pJB4 and the fragments subcloned from it are shown. The vertical arrow indicates the site of Tn*5*-751 insertion in the C5.84 mutant, and the horizontal arrows indicate the direction of the vector promoter for each clone. All pRJ clones were constructed in wide-host-range vectors and used in the complementation studies; the pKRJ and pSRJ clones were constructed in pBluescript KSII$^+$ and pSKII$^+$, respectively, and used in the in vitro transcription-translation reactions. A, *Acc*I; B, *Bst*XI; Bg, *Bgl*II; D, *Dra*III; E, *Eco*RI; K, *Kpn*I; S, *Sal*I; Sp, *Sph*I; X, *Xho*I.

experiments suggested that the 3.5-kb *Bgl*II fragment contains its own promoter in addition to the complementing gene and also that the direction of transcription for the complementing function was from left to right of the fragment as it is depicted in Fig. 1.

Sequence analysis. To determine the gene structure of the 3.5-kb *Bgl*II fragment, it was cloned into the vector pBluescript SKII$^+$ and sequenced. The 3,537 bp of this sequence are shown in Fig. 2. In the direction of transcription indicated by the complementation studies, two complete open reading frames (ORFs) were present. The first of these (hereafter called *exeA*), in which the Tn*5*-751 had inserted to create the mutant C5.84, has a number of potential ATG start codons beginning at nucleotide (nt) 743 and ends with a TGA codon at nt 2456. The third of these ATG codons, at nt 815, is preceded by a putative ribosome binding site, GGAA, at nt 806. The calculated molecular mass of this 547-amino-acid protein would be 60,170 Da. A second ORF (*exeB*) follows this immediately with an overlapping start codon at nt 2458 and ends at a TGA codon at nt 3136, for a 226-amino-acid protein with a calculated molecular mass of 24,860 Da. A possible ribosome binding site, GGAGG, precedes the start codon of this ORF by 7 nt. Two further partial ORFs are on the sequenced fragment. ORF3, in the same direction as *exeA* and *exeB*, starts with a GTG codon at nt 3240 and extends beyond the 3′ region of the 3.5-kb *Bgl*II fragment. ORF4, in the opposite direction to the three discussed above, starts with a GTG codon at nt 551 and extends beyond the 5′ end of the sequence, as shown in Fig. 2. Two regions which may play a role in transcriptional control were observed flanking the *exeA* and *exeB* ORFs. A putative promoter region is located at nt 758 to 787 5′ to *exeA* and *exeB* is followed immediately by a 13-bp inverted repeat.

Polypeptides encoded by the 3.5-kb *Bgl*II fragment. To validate the ORF assignments made on the basis of the sequence analysis, the 3.5-kb *Bgl*II fragment was cloned in the pBluescript SKII$^+$ vector (pSRJ5.2; Fig. 1) and expressed in an in vitro transcription-translation system (Fig. 3). In addition to the β-lactamase encoded by the vector (compare lanes 7 and 8), four polypeptides with apparent molecular masses 60, 30, 29, and 27 kDa were produced. Subclones of this fragment were constructed in pBluescript SKII$^+$ or KSII$^+$ and similarly analyzed to identify these individual polypeptides. Construct pKRJ20.2 (*Dra*III-*Acc*I), which contains only the *exeA* ORF, produced only the 60-kDa protein, while pSRJ46.1, which contains only the *exeB* ORF and 334 bp 5′ to its putative start codon, produced only the 29-kDa protein (Fig. 3, lanes 1 and 2). This protein apparently migrates anomalously on SDS-polyacrylamide gel electrophoresis since as noted earlier, its calculated molecular mass is 24,860 Da.

When the fragment in pSRJ46.1 was inverted with respect to

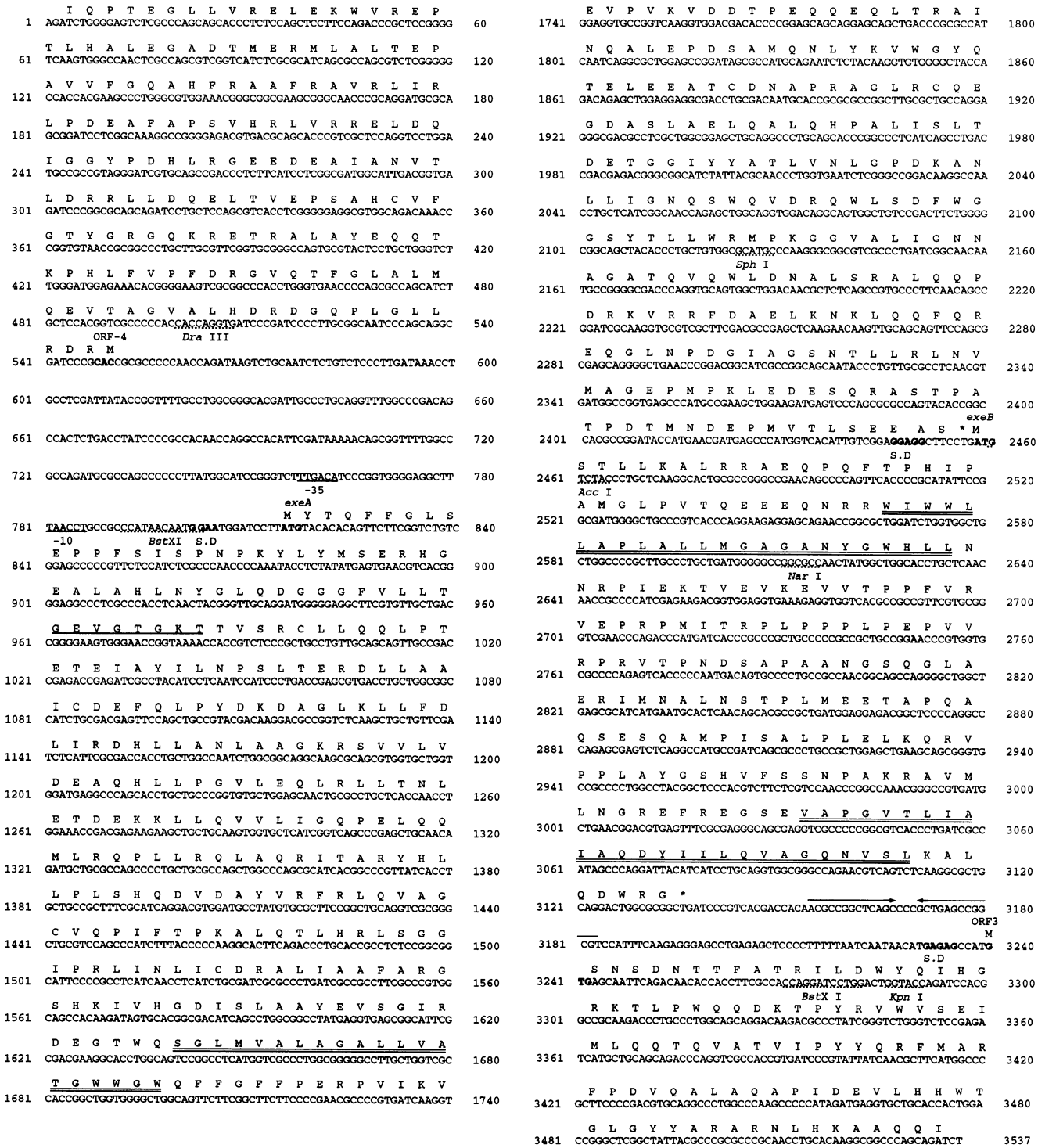

FIG. 2. Sequence of the 3.5-kb *Bgl*II fragment. The sequence of the noncoding strand along with the translation of the *exeA* and *exeB* ORFs are shown. Potential ribosome binding sites and the start codons are in boldface. The putative −35 and −10 promoter sequences upstream of *exeA* are underlined. A consensus ATP binding site in ExeA is overlined; the regions of pronounced hydrophobicity are doubly overlined. The 13-bp inverted repeat following *exeB* is overlined with arrows. Stop codons are denoted by asterisks, and the restriction endonuclease cleavage sites used in the construction of the subclones described in the text are dotted. The truncated ORFs at the boundaries of the fragment which have high sequence similarity to the *cca* and *micA* genes of *E. coli* are also indicated. S.D., Shine-Dalgarno sequence.

the *lac* promoter of the vector, no protein was produced (data not shown). Constructs pKRJ58.1 and pSRJ57.1 contained a *Dra*III-*Kpn*I fragment encompassing the complete *exeA* and *exeB* ORFs plus 309 bp upstream of the putative *exeA* start codon at nt 815 in both orientations with respect to the vector promoter. Each of these constructs produced the 60- and 29-kDa proteins (Fig. 3, lanes 3 and 4). In contrast, pKRJ50.3 (*Bst*XI-*Bst*XI), containing these ORFs but beginning just 15

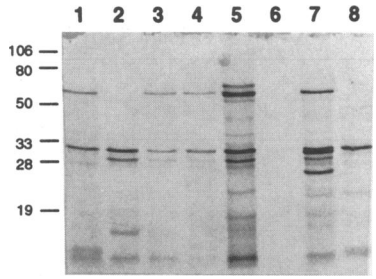

FIG. 3. In vitro transcription-translation reactions of the *exeA* and *exeB* genes. An autoradiograph of an SDS–12% polyacrylamide gel of the [^{35}S]methionine-labeled proteins produced in subclones pKRJ20.2 (lane 1), pSRJ46.1 (lane 2), pKRJ58.1.1 (lane 3), pSRJ57.1 (lane 4), pKRJ50.3 (lane 5), pKRJ50.2 (lane 6), pSRJ5.2 (lane 7), and pBluescript SKII$^+$ (lane 8) is shown. The molecular masses of radiolabeled protein standards (in kilodaltons) are shown on the left.

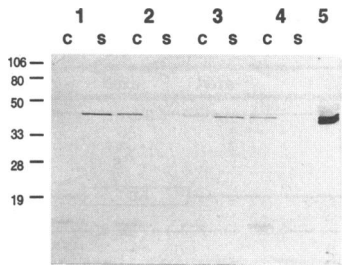

FIG. 4. Immunoblot analysis of aerolysin secretion. Cell samples (lanes C) and culture supernatants (lanes S) representing equal volumes (2.5 μl) of freshly grown brain heart infusion cultures were electrophoresed, transferred to nitrocellulose, and immunoblotted with an antiaerolysin monoclonal antibody. Lane 1, Ah65; lane 2, C5.84; lane 3, C5.84(pRJ31.1); lane 4, C5.84(pRJ60.1); lane 5, purified aerolysin. The upper and lower bands in lane 5 are proaerolysin and aerolysin, respectively. Molecular masses of standards are given on the left in kilodaltons.

bases 5′ of the *exeA* ORF as shown in Fig. 2, produced the 60- and 29-kDa proteins, whereas pKRJ50.2, with the same fragment in opposite orientation with respect to the vector promoter, produced neither protein (Fig. 3, lanes 5 and 6).

These analyses allowed us to conclude that ORFs *exeA* and *exeB* as shown in Fig. 2 produce the expected proteins and that a promoter which is functional in *E. coli* lies in the 309 bp between the *Dra*III and *Bst*XI sites 5′ to *exeA*. The absence of a functional promoter in the 334 bp upstream of *exeB* and the overlap between the termination codon of *exeA* and the start codon of *exeB* further suggested that these two genes form an operon.

Complementation analysis. The site of insertion of the Tn*5*-751 which resulted in the creation of the C5.84 mutant was mapped to a location about 250 bases downstream of the *Bst*XI site in the *exeA* ORF (Fig. 1). We therefore subcloned this ORF only on a *Bst*XI-*Acc*I fragment (pRJ24.2), as shown in Fig. 1, and assayed the secretion of aerolysin from C5.84 cells containing the plasmid. This fragment, however, failed to complement the mutant, whether or not it was placed under the control of the *tac* promoter of the vector. Another construct containing the *Dra*III-*Acc*I fragment (pRJ26.3) which contained the additional 309 bp upstream of the *Bst*XI site similarly failed to complement the mutant. The *Bst*XI-*Acc*I fragment was therefore extended to include the *exeB* ORF as well, in plasmids pRJ31.1 and pRJ60.1 (*Bst*XI-*Bst*XI). The immunoblot of the transconjugants using antiaerolysin antibody shown in Fig. 4 demonstrates that this fragment complemented the mutation, but only when the two *exe* ORFs were in the same orientation as the vector promoter (compare lanes 3C and 3S with lanes 4C and 4S). For the *Dra*III-*Kpn*I fragment, which also contains the 309 bases upstream of the *exeA* ORF, complementation was observed when the fragment was placed in either orientation with respect to the vector promoter (not shown). Finally, to rule out the possibility that only the second ORF, *exeB*, was required to complement the mutant, a *Sph*I-*Kpn*I fragment (pRJ11.1) which contained this ORF alone was also cloned and conjugated into C5.84. This fragment failed to complement when it was placed in either orientation with respect to the vector promoter (data not shown).

Marker exchange mutagenesis of *exeA* and *exeB*. The in vitro transcription-translation reactions and the complementation analysis both indicated that there is a promoter upstream of *exeA*, and the complementation analysis strongly suggested that *exeA* and *exeB* form an operon, both members of which are required for secretion in *A. hydrophila*. If the promoter sequence between the *Dra*III and *Bst*XI sites shown in Fig. 2 is used to initiate a transcript which terminates in the vicinity of the 13-bp inverted repeat which follows *exeB*, an mRNA of about 2,378 bases would result. A Northern (RNA) blot of RNA isolated from the wild type and C5.84 hybridized to a probe composed of the 2.5-kb *Bst*XI-*Bst*XI fragment showed that the wild-type cells indeed contained a message of approximately 2,500 nt which was lacking in the mutant (data not shown).

To confirm that both genes of this operon are required for secretion, we used marker exchange mutagenesis to recreate the original insertion mutation of *exeA* and also to create an insertion mutation in *exeB*, as shown in Fig. 5A. For each gene, a kanamycin resistance cassette was inserted into the cloned copy of the gene, and the suicide vector containing the construct was conjugated into *A. hydrophila* Ah65 for exchange with the wild type *exe* gene. In each case, the structure of the mutated *exe* gene was confirmed by Southern blotting of a chromosomal digest of the selected exconjugant (Fig. 5B). Each of these two mutants as well as the wild type and C5.84 were then grown in broth culture, and cell and supernatant fractions were assayed for aerolysin and protease activities. As can be seen in Table 1, both of the mutants displayed the same inability to secrete these extracellular enzymes as did the original mutant C5.84.

Characteristics of the Exe proteins. The amino acid sequences of the ExeA and ExeB proteins were deduced from the nucleotide sequence and analyzed by computer. Hydropathy analysis was done by the method of Kyte and Doolittle (16). ExeA is quite hydrophilic, with an average hydrophobicity of −1.9, and it did not have a sequence resembling a signal sequence. ExeA has one very hydrophobic segment, however, between residues 276 and 295, as shown in Fig. 2, and a calculated isoelectric point of 5.06. A search of the EMBO protein sequence motif database of Fuchs (6) revealed a consensus nucleotide binding site ([A/G] X (4) GK [S/T] (39)) at residues 50 to 57, as shown in Fig. 2. The average hydrophobicity for the product of *exeB* was −2.4; it too appeared to have no signal sequence, and it is quite basic, with a calculated isoelectric point of 9.58. It contains a highly hydrophobic stretch of 17 amino acids at residues 37 to 51 and another region at residues 193 to 218 which is quite hydrophobic but which contains an Asp residue at position 205 (Fig. 2). The presence of the consensus ATP binding site in ExeA and the

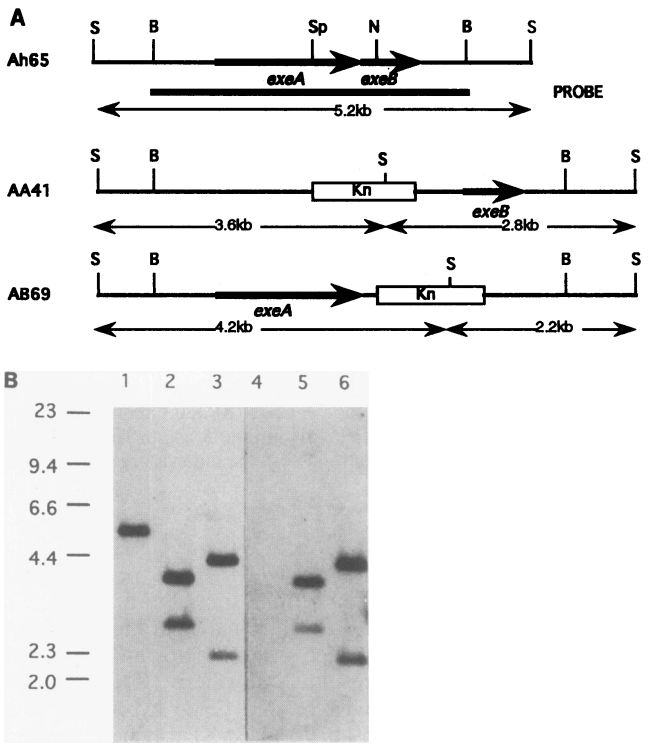

FIG. 5. Southern analysis of marker exchange mutants of Ah65. (A) Physical maps of the probed regions of Ah65, *exeA* mutant AA41, and *exeB* mutant AB69. (B) Genomic DNAs were digested to completion with *Sma*I. Lanes 1 to 3 were probed with the 3.5-kb *Bgl*II insert from pSRJ5.2, and lanes 4 to 6 were probed with the *aph* gene from pUC4K. Lanes 1 and 4, Ah65; lanes 2 and 5, AA41; lanes 3 and 6, AB69. Fluorographs of the Southern blots are shown. S, *Sma*I; B, *Bgl*II; Sp, *Sph*I; N, *Nar*I. Kn, kanamycin resistance cassette.

hydrophilic profiles of both ExeA and ExeB thus suggested that these proteins would be either cytoplasmic or at best anchored to the inner membrane by a single hydrophobic hairpin.

Comparison of the 3.5-kb *Bgl*II sequence with other DNA sequences. The FASTA and BLAST programs were used to search the EMBL and GenBank databases with the sequenced fragment and its translations (1, 21). No entries with significant similarity to ExeA were found; however, two proteins which had low levels of sequence similarity with ExeB were found. These were PulB from *K. oxytoca* (5) and OutB from *E. chrysanthemi* (3). An alignment of these three proteins is shown in Fig. 6. Significant homology was also found between ORF3 and the *E. coli micA* gene and between ORF4 and the *E. coli cca* gene. The *micA* gene encodes for the A/G-specific adenine glycosylase which is part of the methylation-independent mismatch repair pathway (34). *micA* has a nucleotide identity of 71% with the sequenced region of ORF3. The *cca* gene encodes the tRNA nucleotidyltransferase which incorporates AMP and CMP residues into the acceptor stem of tRNAs (4). The *cca* gene has a nucleotide identity level of 65% with the sequenced portion of ORF4.

DISCUSSION

A. hydrophila, like many other gram-negative bacteria, secretes a wide range of exoproteins. As shown for the hemolysin aerolysin, these exoproteins appear to be translocated across the envelope by a two-step process involving first, signal sequence-dependent translocation across the inner membrane, and second, traversal of the outer membrane (10, 12). It is now clear that this second step requires the products of a large operon, the prototype of which is the *pul* operon of *K. oxytoca* (25), and which in *A. hydrophila* is represented by the 12-gene *exeC-N* operon (13, 15). Since these gene products apparently function after the signal sequence-dependent transfer across the inner membrane, they have been considered to form an extension of the Sec pathway and therefore to be part of a general secretion pathway which encompasses the Sec proteins (24, 29). Little is yet known, however, about how these gene products function. In particular, there is as of yet no concrete information concerning the source of energy for the transfer of the exoproteins across the outer membrane, despite evidence that a proton gradient is required for the process in *A. hydrophila* (42). A prime candidate for an energy source has been PulE and its homologs (ExeE in *A. hydrophila* [15]). This protein is the most hydrophilic of the *pul* gene products and possesses an ATP binding site which has recently been shown to be essential for protein secretion (23, 35). In addition to the *xcpR* gene in the *pul* operon homolog *xcpP-Z*, *Pseudomonas aeruginosa* contains two other *pulE* homologs, *pilB* in the *pilBCD* operon, involved in type IV pilin assembly, and *pilT*, involved in twitching motility (19, 40). In addition, a number of the *pul* gene products have type IV prepilin signal sequences, and indeed the last gene of the *pul* operon is a prepilin peptidase (20, 26). On this basis, it has been speculated that PulE and its homologs provide energy not for secretion of proteins per se but for assembly of the secretion apparatus made up of the other Pul proteins (22, 35).

In this study, we have isolated and characterized a second *exe* operon composed of two genes which are required for extracellular secretion in *A. hydrophila*. One of these genes, *exeA*, encodes another hydrophilic protein which contains a consensus ATP binding site. The second protein is a hydrophilic, basic 25-kDa protein with homology to OutB and PulB, two proteins which have been reported to play little and no role, respectively, in secretion in *E. chrysanthemi* and *K. oxytoca*. Mutants of *pulB* had no phenotype with respect to the export of pullulanase, while mutants for *outB* secreted 70% of the normal amount of pectinases produced by wild-type *E. chrysanthemi* (3, 5). We have shown here and elsewhere (14), however, that both the original transposon insertion mutant of *exeA* and marker exchange mutants of either *exeA* or *exeB* secreted very little of the extracellular proteins that we assayed. This discrepancy suggests a substantial divergence in the function of this gene product between *A. hydrophila* and the

TABLE 1. Enzyme activities in *A. hydrophila* Ah65, C5.84, AA41, and AB69

Fraction	Aerolysin titer (hemolytic units)	Protease (U/ml of original culture [%])
Ah65		
Supernatant	128	199 (76)
Cells	8	64 (24)
C5.84		
Supernatant	8	106 (18)
Cells	128	488 (82)
AA41		
Supernatant	8	93 (15)
Cells	128	527 (85)
AB69		
Supernatant	0	14 (3)
Cells	128	618 (97)

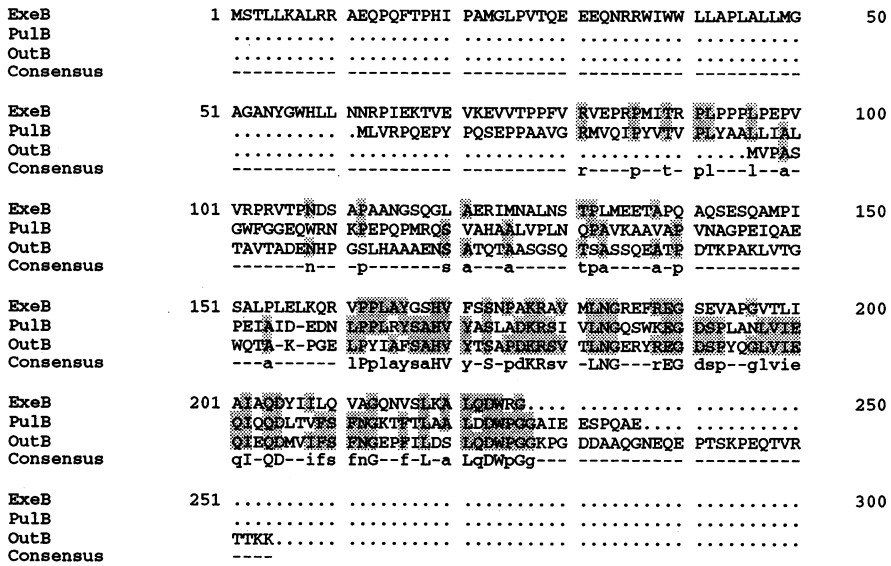

FIG. 6. Alignment of the predicted amino acid sequence of the *A. hydrophila exeB* gene product with the amino acid sequences of PulB and OutB. Dashes indicate gaps inserted to optimize the alignment. A consensus sequence is presented in uppercase when all three proteins share an amino acid and in lowercase when either of the two proteins share an amino acid.

other two bacteria, which may account for the low levels of homology observed (19.5% between ExeB and PulB and 19.7% between ExeB and OutB; Fig. 6) and also for the substantial variation in the size of these proteins, with PulB of 174 amino acids and OutB of 157 amino acids, compared with 226 amino acids for ExeB. Another difference of note, of course, is the association of *exeB* in an operon with *exeA*. Substantial sequence information from both sides of the *pulB* and *outB* genes has been reported and indicates that *exeA* homologs are not present, at least in an operon with the ExeB homolog (3, 5).

With respect to the location of ExeA and ExeB in the cell, both proteins are largely hydrophilic and lack signal sequences, but both also contain hydrophobic stretches which could serve to anchor them to the cytoplasmic membrane. In T7 expression experiments in *E. coli*, both proteins were pelleted by ultracentrifugation of cell lysates, suggesting that they are associated with the membranes (not shown here). These results must be taken as preliminary, however, given the possibility that the overproduction of proteins in this system or their synthesis in the heterologous host could lead to aggregation or aberrant localization. The localization of PulB and OutB has not been reported, although the PulB protein was predicted to be cytoplasmic on the basis of its structure (25). The amino-terminal region of ExeB which is absent in these two homologs contains the most hydrophobic of its two possible transmembrane regions (Fig. 6), so it is quite possible in any case that these proteins will not be found in the same compartment of their respective cells. We are attempting to obtain specific antisera to ExeA and ExeB which will allow a definitive determination of their localization in vivo.

Although we have demonstrated in this report that these two *exe* genes form an operon, both members of which are required for extracellular secretion, we have as yet little information on their role in this process. Two obvious possibilities, both of which are compatible with phosphorylation events that may be catalyzed by ExeA, are (i) that these proteins are involved in the regulation of the other (*exeC-N*) genes which make up the terminal segments of the general secretory pathway and (ii) that these proteins are directly involved in the secretion process. A strong argument against the first possibility is the clearly separable phenotypes which result from mutations in the two *exe* operons that we have now characterized. Mutations in either of the operons essentially abolish extracellular secretion and result in accumulation of the exoproteins in the periplasm (14). The Tn*5*-751 insertion into *exeE* which created strain L1.97, however, as well as three other marker exchange mutations created in the *exeC-N* operon, all resulted in an outer membrane which contained only trace quantities of the major porins and in addition rendered the cells exceedingly fragile during osmotic shock procedures (14). In contrast, the Tn*5*-751 insertion into *exeA* had no effect on the outer membrane protein profile or the fragility of the resulting mutant C5.84, nor did the marker exchange mutants of *exeA* and *exeB* created here (reference 14 and data not shown). This makes it unlikely that the effect of mutation in the *exeAB* operon is to prevent the expression of the *exeC-N* operon. Rather, we favor the hypothesis that the ExeA and ExeB proteins function directly in the secretion of the exoproteins across the outer membrane. Again, however, the differing phenotypes which result from mutation in the two operons both restrict and guide our speculations. It does not seem likely, for example, that ExeA and B are involved in the assembly of the ExeC-N secretion apparatus, since if this were the case we would expect the membrane effects typical of *exeC-N* mutations discussed above. We therefore assume that in the *exeAB* mutants, the ExeC-N apparatus is assembled but nonfunctional, either because the exoproteins cannot move through the apparatus or because they do not reach it in the absence of ExeAB. The absence of recognizable signal sequences in the latter two proteins, their largely hydrophilic character, and the presence of the consensus nucleotide binding site in ExeA all suggest that they perform their function on the cytoplasmic side of the inner membrane. In our view, this further suggests that the export of exotoxins either begins with a recognition step in the cytoplasm, with ExeA and B perhaps acting as chaperones, or requires the continuous function of these proteins in the cytoplasmic compartment. In either case, it would seem that

the role of cytoplasmic constituents in extracellular secretion by this pathway extends beyond the provision of a functioning Sec translocase and the assembly of the PulC-O apparatus. Further experiments will be aimed at determining at which step in the secretion pathway the ExeA and ExeB proteins act.

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