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Mapping of Export Signals of *Pseudomonas aeruginosa* Pilin with Alkaline Phosphatase Fusions

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Pili of *Pseudomonas aeruginosa* are assembled from monomers of the structural subunit, pilin, after secretion of this protein across the bacterial membrane. These subunits are initially synthesized as precursors (prepilin) with a six-amino-acid leader peptide that is cleaved off during or after membrane traversal, followed by methylation of the amino-terminal phenylalanine residue. This report demonstrates that additional sequences from the N terminus of the mature protein are necessary for membrane translocation. Gene fusions were made between amino-terminal coding sequences of the cloned pilin gene (*pilA*) and the structural gene for *Escherichia coli* alkaline phosphatase (*phoA*) devoid of a signal sequence. Fusions between at least 45 amino acid residues of the mature pilin and alkaline phosphatase resulted in translocation of the fusion proteins across the cytoplasmic membranes of both *P. aeruginosa* and *E. coli* strains carrying recombinant plasmids, as measured by alkaline phosphatase activity and Western blotting. Fusion proteins constructed with the first 10 amino acids of prepilin (including the 6-amino-acid leader peptide) were not secreted, although they were detected in the cytoplasm. Therefore, unlike that of the majority of secreted proteins that are synthesized with transient signal sequences, the membrane traversal of pilin across the bacterial membrane requires the transient six-amino-acid leader peptide as well as sequences contained in the N-terminal region of the mature pilin protein.

The majority of bacterial polypeptides that are localized to the exterior of the cytoplasmic membrane are synthesized with N-terminal peptide extensions that function as export signals directing the membrane translocation process (30, 34). These signal peptides are cleaved during or immediately following membrane traversal by specific proteases called signal (leader) peptidases (35). Typical signal peptides are 20 to 30 amino acids long and contain three recognizable regions: (i) a polar and often positively charged sequence at the amino terminus, (ii) a core region of 9 to 15 hydrophobic amino acids, and (iii) a carboxyl-terminal sequence of 3 to 5 amino acids that specifies the recognition site for signal peptidase.

In addition to polypeptides that are translocated across or incorporated into membranes, many procaryotic organisms export proteins that are components of surface structures. Pili represent one class of surface appendages. Pili of gram-negative bacteria are hollow tubular structures assembled from a pool of homogeneous pilin subunits. Sequence analysis of various *Escherichia coli* pilin genes revealed that they are all synthesized with N-terminal amino acid extensions that resemble classical signal sequences in size and distribution of polar, charged, and hydrophobic amino acids (2, 11, 15, 16, 28, 31, 32, 36). These precursors (prepilin) are most likely secreted via the same export pathway used by periplasmic or membrane proteins. Studies by Dodd and Eisenstein (13) support this suggestion, since in *E. coli*, the export and processing of type 1 pili (fimbriae) were shown to be dependent on the presence of the *secA* gene product, one of the essential components of the bacterial export apparatus (30). The final assembly involves the interaction of several additional gene products, presumably forming a firm anchor to a component of the cell wall.

Pili of *Pseudomonas aeruginosa* possibly represent yet another distinct class of secreted polypeptides. Pilin monomers are synthesized as precursors, containing a short six-amino-acid leader peptide, Met-Lys-Ala-Gln-Lys-Gly (21, 33, 38), which is different from signal sequences found on secreted polypeptides, including those of *P. aeruginosa*. Moreover, a similar leader peptide is also found on the N termini of prepilin from several other microorganisms, including *Neisseria gonnorhoeae* (26), *Moraxella bovis* (24), and *Bacteroides nodosus* (1). An even more striking conservation of amino acid sequence among the pilin polypeptides from these four microorganisms is found in the first 30-amino-acid region of the mature pilin, with greater than 80% sequence homology (17, 25, 39). Following removal of the leader peptide from the precursor molecule, the amino-terminal phenylalanine is N-methylated, and monomers are assembled into an organelle. It is highly probable that the basic mechanism of export and assembly of the methylphenylalanine class of pilin is also conserved among the same microorganisms.

To better understand the process of pilus assembly, we attempted to determine the location of the export signal within the pilin polypeptide chain. Utilizing an alkaline phosphatase fusion vector (18), we located the export signal on prepilin. Unlike that of the majority of secreted proteins, the traversal of pilin monomers across the bacterial cytoplasmic membrane is directed by a sequence consisting of the six-amino-acid transient leader peptide and an amino-terminal domain of the mature polypeptide.

MATERIALS AND METHODS

Bacterial strains, plasmid vectors, and growth media. The bacterial strains and plasmid vectors are listed in Table 1. All cultures were routinely grown on L medium (27) or in minimal A salts (10) at 37°C. Ampicillin and tetracycline (Sigma Chemical Co., St. Louis, Mo.) were used at 100 and

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TABLE 1. Strains, vectors, and plasmids

Strain, vector, or plasmid	Relevant genotype or properties	Source or reference
Strains		
Escherichia coli		
HB101	F⁻ *hsdS20 recA13 proA2 lacY1 galK2* λ⁻	6, 7
JC2926(pRK2013)	Mobilizer or helper plasmid	14
Pseudomonas aeruginosa PAK	Source of cloned pilin gene; host for recombinant plasmids carrying *pilA-phoA* fusions	David Bradley
Cloning vectors		
pUC7	*E. coli* cloning vector with polylinker sequence; Ap^r	42
pUC18	*E. coli* cloning vector with polylinker sequence; Ap^r	29
pRK404	Broad-host-range cloning vector with polylinker sequence; Tc^r	12
pUCH218	*phoA* fusion cloning vector with polylinker sequence replacing signal sequence; Ap^r	This study
Recombinant plasmids		
pCH2	Recombinant plasmid carrying *phoA* fused to *bla* signal sequence; Tc^r	18
pMS27	pUC18 carrying a 1.2-kilobase-pair insert that contains the pilin structural gene from *P. aeruginosa* PAK; Ap^r	40

20 μg/ml, respectively, for *E. coli*. *P. aeruginosa* was grown on 200 μg of tetracycline per ml for recombinant plasmid selection.

Enzymes and chemicals. All restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. IgGsorb (fixed *Staphylococcus aureus* cells) was from The Enzyme Center, Malden, Mass., while 5-bromo-4-chloro-3-indolyl phosphate (XP) and *p*-nitrophenyl phosphate were purchased from Sigma. Rabbit antisera against *E. coli* alkaline phosphatase were a gift from C. Gardell, Harvard Medical School, Cambridge, Mass.

Construction of a signal sequence-deficient alkaline phosphatase cloning vector. The vector plasmid pCH2, which contains a fusion between the β-lactamase (*bla*) gene signal sequence and the alkaline phosphatase (*phoA*) structural gene, was a gift from A. Wright and is described elsewhere (18). Figure 1 shows the construction of pUCH218, which contains the *phoA* structural gene in the polylinker of pUC18 without the *bla* gene signal sequence.

Construction of *pilA-phoA* fusions. Plasmid pMS27 (40) carrying the PAK pilin (*pilA*) structural gene was linearized with *Eco*RV, which cuts at position 765 of the 1,220-base-pair insert, a point just after amino acid residue 103 of the prepilin (unprocessed) protein (21). This fragment was then digested at 30°C with *Bal*31 exonuclease (Boehringer GmbH, Mannheim, Federal Republic of Germany) at a ratio of 0.25 U of enzyme per μg of DNA. Aliquots were removed at 30-s intervals over a 3.5-min period, and the reaction was terminated by phenol and chloroform extractions. After ethanol precipitation, the *Bal*31-digested DNA was then cloned into pUCH218 as described in Fig. 1. Routine plasmid screening was performed by using the alkaline sodium dodecyl sulfate (SDS) lysis procedure (3).

For transfer of the *pilA-phoA* fusions into *P. aeruginosa* PAK, recombinant plasmids pMS2B6, pMS2B1, and pMS27ss were digested with *Hin*dIII, and the inserts were ligated into the *Hin*dIII site of pRK404 (12). As a positive control for alkaline phosphatase secretion, pCH2 was also ligated as a *Hin*dIII fragment into pRK404. The resulting plasmids (pRK2B6, pRK2B1, pRK27ss, and pRKCH2) were transferred into PAK via conjugation by using *E. coli* JC2926(pRK2013) (14) as the mobilizer in triparental matings.

Assay of alkaline phosphatase activity. Alkaline phosphatase activity was assayed by the production of *p*-nitrophenol from *p*-nitrophenyl phosphate as described elsewhere (8). Cultures were grown in L broth prior to the assay, since the transcription of recombinant plasmids carrying the *phoA* fusions was not regulated by the phosphate concentration.

Cell fraction and Western transfer. Periplasmic, cytoplasmic, and membrane fractions of *E. coli* were prepared by a modification of the method of Ito et al. (20) as described previously (40). A similar procedure was used to obtain the subcellular fractions from *P. aeruginosa*, except that the procedure of Hoshino and Kageyama (19) was used to release the periplasmic contents. Samples were boiled for 10 min in a running buffer containing 2% SDS, 1% 2-mercaptoethanol, and 50 mM Tris hydrochloride (pH 7.5) before being loaded on 12.5% polyacrylamide gels with 0.1% SDS by the method of Laemmli (23). Prestained protein standards (Bethesda Research Laboratories) were used to determine molecular mass. Western transfer to nitrocellulose was accomplished by the method of Towbin et al. (41). Filters were probed with rabbit anti-alkaline phosphatase antisera and ^{125}I-protein A (New England Nuclear Corp., Boston, Mass.), followed by autoradiography.

Protein labeling and immunoprecipitation. Cells were grown in enriched medium A (10) containing the appropriate antibiotic and supplemented with 0.2% glycerol, 10 mM $MgCl_2$, and a 0.2 mM concentration of each amino acid, except for methionine. During the logarithmic phase of growth, the cells were labeled for 3 min with 10 μCi of [^{35}S]methionine (New England Nuclear) per 0.2 ml of cells. Samples were then boiled immediately in 0.5% SDS, followed by dilution in immunoprecipitation buffer (50 mM Tris hydrochloride buffer [pH 7.6], 5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100) to lower the SDS concentration to 0.1%.

Insoluble material was removed by brief centrifugation, and an excess of rabbit anti-alkaline phosphatase antisera was added. After 30 min at 4°C, 25 μl of a 10% suspension of IgGsorb was added, and incubation was continued for 20 min. The precipitate was collected by centrifugation and washed three times in immunoprecipitation buffer, and bound antigen was dissociated by boiling in 2% SDS. Samples were electrophoresed on 12.5% SDS–polyacrylamide gels as described above, after which the gels were treated with En^{3}Hance, and the bands were visualized by fluorography.

DNA sequence analysis of *pilA-phoA* fusion junctions. Plasmid DNA was isolated from cells and purified on CsCl gradients prior to Sanger sequence analysis (37) of the supercoiled plasmid DNA by a Pharmacia, Inc. (Piscataway, N.J.), adaptation of the method originally described by Chen and Seeburg (9) with avian myeloblastosis virus reverse transcriptase (Pharmacia) and [α-^{32}P]dATP. A 17-mer oligonucleotide corresponding to nucleotide residues 390 to 406 of the 3′-to-5′ strand of the published sequence of *phoA* (22) was synthesized on a Biosearch 8600 DNA synthesizer and used as a primer for the sequencing reaction.

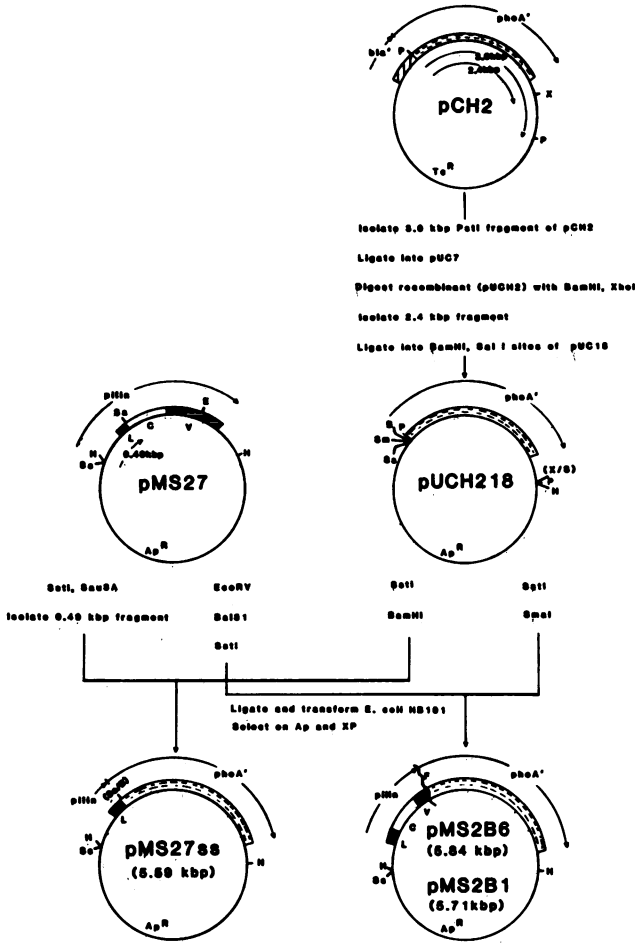

FIG. 1. Summary of cloning strategy of *pilA-phoA* fusions. Abbreviations for restriction endonuclease sites are as follows: B, *Bam*HI; E, *Eco*RV; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sa, *Sau*3A; Sm, *Sma*I; Ss, *Sst*I; and X, *Xho*I. L, C, V, and F stand for the pilin leader sequence, constant and variable regions, and fusion junction between *pilA* and *phoA*, respectively. Other abbreviations are as follows: kbp, kilobase pairs; and Tc^R and Ap^R, tetracycline and ampicillin resistances, respectively.

	PILIN	POLYLINKER SEQUENCES	PHO A
pMS27ss	...Thr Leu Ile (+10)	Arg Arg Pro	(+11) Ala Ala Gln...
pMS2B1	...Leu Lys Thr (+51)	Gly Ile Arg Arg Pro	(+11) Ala Ala Gln...
pMS2B6	...Pro Asp Pro (+97)	Gly Ile Arg Arg Pro	(+11) Ala Ala Gln...

FIG. 2. Amino acid sequences spanning the *pilA-phoA* fusion junctions. The last three amino acids of the pilin part of the fusion are shown, with the numbers referring to the positions of these residues on the prepilin (unprocessed) molecule (21). The first three amino acids of the alkaline phosphatase (PHO A) part of the fusion are shown, with the numbers referring to the positions of these residues on the mature (processed) molecule (18, 22). The intervening residues are derived from the polylinker sequences of the cloning vector.

RESULTS

Construction and selection of pilin-alkaline phosphatase fusion proteins. In-frame fusions between pilin and alkaline phosphatase genes were generated by the procedure outlined in Fig. 1. Various portions of the *pilA* gene encoding the carboxy terminus of the polypeptide were removed by limited digestion with exonuclease *Bal*31, followed by ligation of these DNA fragments to the cloned *phoA* gene in vector pUCH218, which lacks its promoter and signal sequence. The orientation of the fusion was determined by choice of restriction digests of the plasmids, placing the truncated *pilA* gene in front of the *phoA* gene. The plasmids from transformants were analyzed for insert size and the ability to express alkaline phosphatase by the appearance of blue colonies on plates containing XP. Approximately 10% of the recombinants were alkaline phosphatase positive (PhoA$^+$), provided the exonuclease digestion did not remove more than approximately 150 nucleotides of the pilin-coding sequence. No PhoA$^+$ colonies were recovered when additional nucleotides of the pilin-coding sequence were removed. Two PhoA$^+$ recombinants (pMS2B6 and pMS2B1), carrying differently sized deletions in the pilin gene, were chosen for further studies.

In addition, pMS27 was completely digested with *Sau*3A and then *Sst*I, and the 482-base-pair fragment was isolated and ligated into pUCH218 (Fig. 1). The location of the *Sau*3A site in the pilin-coding sequence allows an in-frame fusion of the coding region for the first 10 amino acids of the *pilA* gene (the 6-amino-acid leader sequence and the first 4 amino acids of the mature pilin) to the *phoA* gene in pUCH218 digested with *Sst*I and *Bam*HI. The resulting clone (pMS27ss) was alkaline phosphatase negative (PhoA$^-$) when grown on a medium containing XP.

The exact location of the fusion junctions between *pilA* and *phoA* sequences was determined by DNA sequencing of recombinant plasmids (Fig. 2). The largest recombinant, pMS2B6, contains 97 amino acids from prepilin, 5 amino acids derived from the linker sequences, and the alkaline phosphatase-coding region beginning at the alanine at position 11. Similarly, pMS2B1 contains the same alkaline phosphatase- and linker-derived sequences, but prepilin contributes only 51 amino acids at the amino terminus of the hybrid protein. The smallest recombinant, pMS27ss, contains, in addition to the N-terminal 10 amino acids from prepilin, 3 amino acids from the polylinker fused to alkaline phosphatase at the same alanine.

TABLE 2. Alkaline phosphatase activity

Host	Plasmid	Alkaline phosphatase activity (U)
E. coli HB101	None	<5
	pRKCH2	2,100
	pMS2B6	8,000
	pMS2B1	2,800
	pMS27ss	<5
P. aeruginosa PAK	None	<5
	pRKCH2	1,100
	pRK2B6	4,900
	pRK2B1	2,500
	pRK27ss	<5

Alkaline phosphatase activity of fusion proteins. We assayed alkaline phosphatase activity in intact *P. aeruginosa* and *E. coli* hosts carrying the recombinant plasmids as an indication of the biosynthesis and export of alkaline phosphatase-containing secreted hybrid proteins. Since the total enzymatic activity of such proteins depends on the level of expression and stability as well as on export, the data presented in Table 2 are useful only for a qualitative evaluation of the ability of bacteria to translocate alkaline phosphatase-containing fusion proteins into their periplasm.

No alkaline phosphatase activity was measurable in either *E. coli* HB101 or *P. aeruginosa* PAK carrying no plasmid, despite the fact that both of these organisms contain genes for alkaline phosphatase (Table 2). This lack of activity was probably due to the presence of phosphate in the medium, which regulated the expression of the chromosomal *phoA* genes. No detectable activity was seen in either host carrying a plasmid containing a fusion with the N-terminal 10 amino acids of prepilin, which include the 6-amino-acid leader peptide and the first 4 amino acids of the mature pilin. These plasmids, pMS27ss in *E. coli* and pRK27ss in *P. aeruginosa*, directed the synthesis of significant amounts of hybrid proteins (see Fig. 3 and 4). Therefore, the lack of activity suggests the inability of the prepilin leader peptide to direct the export of alkaline phosphatase across the cytoplasmic membrane.

In contrast, fusions of alkaline phosphatase with longer regions of the mature pilin produced significant amounts of alkaline phosphatase activity, even exceeding that of a *bla-phoA* fusion (pRKCH2), in which export of the hybrid protein is directed by the β-lactamase signal sequence (18; this study). Fusion of at least 51 amino acids of prepilin to the amino terminus of alkaline phosphatase was sufficient to allow synthesis and secretion of active alkaline phosphatase in *E. coli* HB101 and *P. aeruginosa* PAK carrying plasmids pMS2B1 and pRK2B1, respectively. Similar results were obtained for hybrid proteins from pMS2B6 (in *E. coli*) and pRK2B6 (in *P. aeruginosa*), in which 97 amino acids of prepilin were fused to the C terminus of alkaline phosphatase.

Localization of fusion proteins. To determine whether the observed alkaline phosphatase activity of the fusion proteins correlated with their extracytoplasmic location, we fractionated both *E. coli* and *P. aeruginosa* containing the recombinant plasmids into periplasmic, membrane, and cytoplasmic fractions (19, 20) and analyzed these fractions by the Western blotting procedure. Rabbit immunoglobulin G raised against purified *E. coli* alkaline phosphatase was used to detect hybrid proteins.

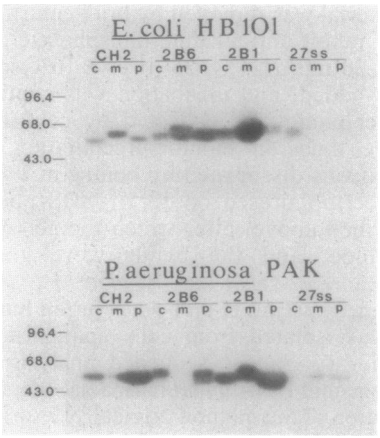

FIG. 3. Western blot analysis showing the cellular location of the pilin-alkaline phosphatase fusion proteins in both *E. coli* HB101 (containing pRKCH2, pMS2B6, pMS2B1, and pMS27ss) and *P. aeruginosa* PAK (containing pRKCH2, pRK2B6, pRK2B1, and pRK27ss). Abbreviations: CH2, pRKCH2; 2B6, pMS2B6 and pRK2B6; 2B1, pMS2B1 and pRK2B1; 27ss, pMS27ss and pRK27ss; c, cytoplasm; m, total membrane; p, periplasm. Numbers at left represent the molecular masses of protein standards in kilodaltons.

In *E. coli*, the *pilA-phoA* fusion proteins were located in all three fractions, except in the strain carrying pMS27ss, in which the fusion protein was found only in the cytoplasm (Fig. 3). The majority of the fusion proteins in PhoA$^+$ *E. coli* were located in the membrane fraction. In PhoA$^+$ *P. aeruginosa*, the majority of the fusion proteins were located in the periplasmic space, with lower and various levels in the cytoplasm and membrane fraction. The PhoA$^-$ strain carrying pRK27ss contained substantially less fusion protein, but it was seen in all three cellular fractions.

The small amount of hybrid protein detected in *P. aeruginosa* PAK(pRK27ss) by Western blotting raised the possibility that the PhoA$^-$ phenotype was the result of decreased rates of synthesis of the fusion protein. Alternatively, the failure to export the hybrid protein may have resulted in its degradation in the bacterial cytoplasm. To estimate the rates of synthesis of the various polypeptides, we labeled cultures of *P. aeruginosa* PAK carrying pRK2B6, pRK2B1, or pRK27ss with [^{35}S]methionine for 3 min, followed immediately by immunoprecipitation with anti-alkaline phosphatase serum and analysis by SDS-polyacrylamide gel electrophoresis. A semiquantitative assessment of

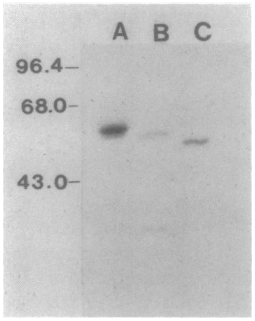

FIG. 4. Pulse-labeling and immunoprecipitation of pilin-alkaline phosphatase fusion proteins from *P. aeruginosa* PAK. Lanes: A, pRK2B6; B, pRK2B1; C, pRK27ss. Molecular mass standards (in kilodaltons) are indicated on the left.

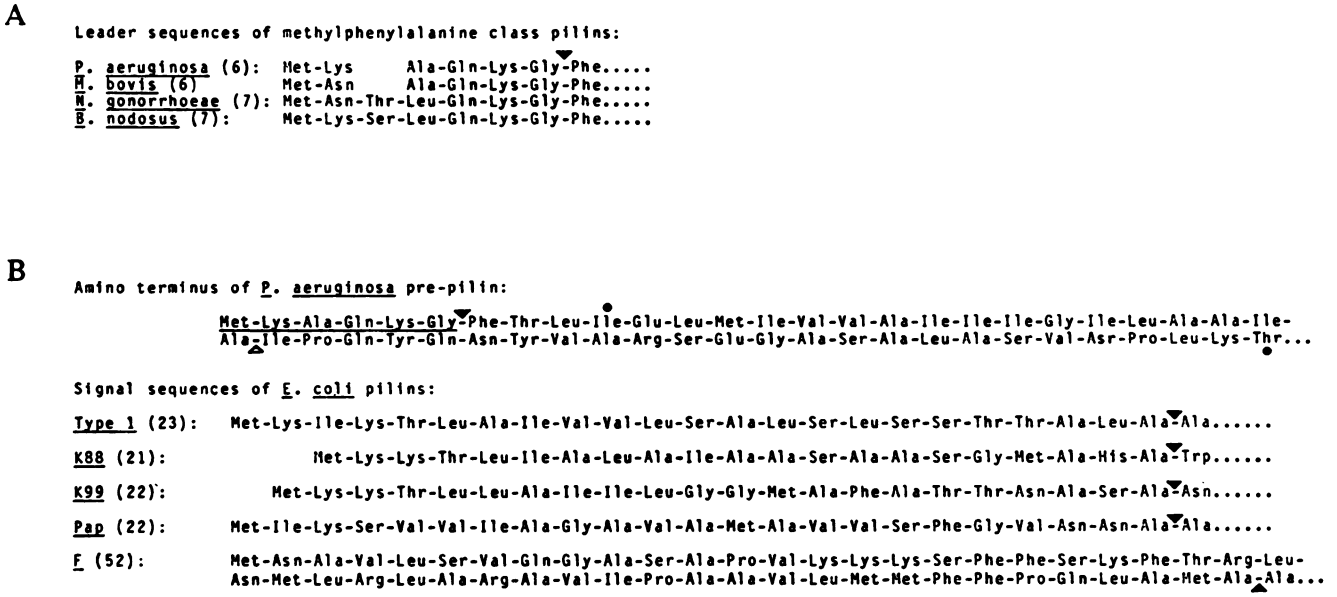

FIG. 5. Comparison of leader and signal sequences of various pilins. (A) Leader sequences of the methylphenylalanine class of pilins (1, 24, 26). (B) Comparison of the amino terminus of *P. aeruginosa* prepilin to signal sequences of several *E. coli* prepilins (2, 15, 16, 32, 36). Symbols: solid triangles, cleavage sites; open triangle, signal sequence cleavage site of *P. aeruginosa* pilin predicted by using the weighted-matrix method of von Heijne (44); solid circles, fusion junctions of *P. aeruginosa pilA* to *phoA* (the first dot represents the fusion found in pMS27ss; the second represents that found in pMS2B1). The numbers in parentheses denote the numbers of amino acid residues that are removed from the precursor polypeptide during processing.

amounts of various hybrid proteins was made on the basis of intensities of radiolabeled bands on fluorographs, taking into account that the smallest hybrid protein, synthesized from pRK27ss, contained one less methionine than did the larger hybrid proteins made by cells carrying pRK2B1 and pRK2B6. Figure 4 shows that the amounts of hybrid proteins, as judged by the intensities of radiolabeled bands, were different depending on the construct. However, the amount of immunoprecipitated protein from cells expressing the smallest fusion protein (pRK27ss, Fig. 4, lane C) was equal to or greater than that of the next larger fusion protein (pRK2B1, Fig. 4, lane B) and less than that of the largest fusion protein (pRK2B6, Fig. 4, lane A). These results suggest that the differences in amounts of alkaline phosphatase, as determined by enzymatic activities and immunoblotting, are not due to differential rates of synthesis of the proteins but are most likely due to differential stabilities of the proteins.

DISCUSSION

Since pools of pilin monomers have been detected in both the cytoplasmic and outer membranes of *P. aeruginosa* (45), we assumed that the assembly of the pilus organelle is preceded by the insertion of monomers into the cytoplasmic membrane. Export of proteins in bacteria is usually directed by amino-terminal, cleavable signal peptides containing 20 to 30 largely hydrophobic amino acids (30, 35). The cleaved leader peptide of *P. aeruginosa* prepilin is six amino acids in length and fairly hydrophilic (21). Similar peptides are found on the amino termini of the related prepilins from *N. gonorrhoeae* (26), *B. nodosus* (1), and *M. bovis* (24) (Fig. 5A). In contrast, precursors of almost all pilins from *E. coli* are synthesized with 21- to 23-amino-acid leader peptides, with a distribution of polar, charged, and hydrophobic amino acids characteristic of export signal sequences (2, 16, 32, 36) (Fig. 5B). The sole exception is the pilin encoded by the *traA* gene of the F plasmid, which is unusually long, containing several short domains of hydrophobic and polar amino acids (15).

Members of the methylphenylalanine class of pili share extensive homologies in the amino-terminal region of the mature polypeptide, which is extremely hydrophobic. To determine whether the short leader sequence of prepilin is sufficient to allow insertion of the newly synthesized pilin monomer into the membrane or whether additional domains within the mature polypeptide are essential for initiating export, we fused various portions of the pilin gene to the alkaline phosphatase gene and examined the extent of export of the hybrid proteins in *E. coli* and *P. aeruginosa*. Alkaline phosphatase activity was detected in cells carrying plasmids pMS2B6 and pMS2B1 but not in those with pMS27ss (Table 2). Sequence analysis of pMS2B6 and pMS2B1 showed that these recombinants code for hybrid proteins containing 97 and 51 amino acids, respectively, of the N terminus of prepilin fused to alkaline phosphatase. Presumably, this portion of prepilin contains export information, since the expression of alkaline phosphatase activity in the hybrid proteins was dependent on translocation to the periplasmic side of the cytoplasmic membrane. Fusion of the first 10 amino acids of prepilin to alkaline phosphatase in plasmid pMS27ss was not sufficient to allow for the production of alkaline phosphatase in either *P. aeruginosa* or *E. coli*, thus suggesting that the hybrid protein lacked information necessary for translocation of the active domain of alkaline phosphatase across the membrane.

Enzymatic activities of hybrid proteins provide a qualitative assessment of the export function of the amino-terminal domain fused to alkaline phosphatase. Since alkaline phosphatase dimerizes in its active form, the net enzymatic activity is a reflection not only of transport across the cytoplasmic membrane but also of several additional factors.

These may include stability against proteolytic degradation in the periplasmic space and release of the active domain into a soluble form to allow formation of the enzymatically active form either as a free periplasmic dimer or as a dimer consisting of a membrane-bound component and a periplasmic component.

Immunoblot analysis of the alkaline phosphatase-containing polypeptides found in various cellular compartments of *P. aeruginosa* and *E. coli* confirmed that the hybrid proteins were translocated across the membrane and, at least in part, released into the periplasmic space. The two larger fusion proteins were transported into the periplasm or remained associated with the membrane (Fig. 3). Multiple bands of immunoreactive polypeptides were seen in several fractions, suggesting that limited proteolysis, which could yield an active form of the enzyme, had taken place.

Substantial amounts of secreted hybrid proteins were found in the periplasmic spaces of both *E. coli* and *P. aeruginosa*. As mentioned above, these may represent proteolytic products of cytoplasmic membrane-associated polypeptides or actual periplasmically localized fractions. This is in contrast to the location of the intact pilin monomer in *P. aeruginosa* or recombinant plasmid-carrying strains of *E. coli*, in which there is no evidence of a periplasmic pool, although pilin subunits are found in both cytoplasmic and outer membranes (40, 45). One possible explanation for this discrepancy is that the alkaline phosphatase component of the hybrid protein may stabilize the polypeptide chain in the periplasmic space and prevent further translocation into the outer membrane. Alternatively, the amino-terminal sequence derived from the pilin polypeptide may be capable of initiating insertion into or translocation across the cytoplasmic membrane. However, further insertion into or across the outer membrane may require sequences derived from the carboxy terminus of pilin.

Localization of the fusion proteins made by bacteria carrying pMS2B1 and pMS2B6 on the periplasmic side of the inner membrane provides suggestive evidence for a specific orientation of the pilin polypeptide in the cytoplasmic membrane during assembly of the pilus organelle. When inserted in the membranes, pilin is most likely in an orientation in which the carboxy terminus is exposed to the periplasmic space. Such topology is essential for directing alkaline phosphatase across the membrane, where it assumes an active membrane-bound conformation or is released into the periplasmic space.

Hybrid proteins consisting of the first 10 amino acids of the prepilin leader sequence were found entirely in the cytoplasm of *E. coli*. However, in *P. aeruginosa*, these proteins were distributed in all three compartments, despite the absence of detectable alkaline phosphatase activity in these microorganisms. Estimation of rates of synthesis of fusion polypeptides revealed that, in *P. aeruginosa*, the synthesis of the smallest hybrid protein (from pMS27ss) was comparable to that of proteins that were exported (Fig. 4). From this result, we concluded that the lack of efficient translocation resulted in cytoplasmic localization and proteolytic degradation of the fusion protein. The small amount of polypeptide detected in the membrane fraction and periplasmic space of *P. aeruginosa* could therefore be the result of lysis of cells during the fractionation procedure, as we observed a considerable (ca. 25%) leakage of nucleic acids from *P. aeruginosa* PAK during the periplasmic extraction step (data not shown).

Collectively, our findings suggest that the initial step in pilus assembly involves translocation of pilin across the *P. aeruginosa* cytoplasmic membrane directed by the six-amino-acid leader peptide and additional sequences from the amino terminus of the mature pilin polypeptide. Thus, the mechanism of pilin export is consistent with the predictions of the signal hypothesis (4, 5). According to this hypothesis, the role of the amino-terminal domain, including the six-amino-acid leader peptide, is to serve as a recognition site for the apparatus of secretion. Such recognition would be followed by interaction with the cytoplasmic membrane, followed by removal of the leader peptide. Our data, however, are not inconsistent with an alternative model for protein export, the membrane trigger hypothesis (46). According to this model, the role of the leader peptide is to maintain prepilin in an export-competent conformation and to direct the insertion of the hydrophobic N-terminal domain into the lipid bilayer without the aid of a soluble or membrane-associated apparatus. Following the insertion of pilin into the membrane, removal of the leader peptide results in a conformational change, making the membrane association permanent.

The minimal sequence that resulted in export of the PilA-PhoA fusion protein contained the 6-amino-acid leader peptide and 45 amino acids from the mature pilin. These 51 amino acids define the upper limit for the length of the sequence necessary for export. Interestingly, this sequence shares considerable homology with conventional leader sequences (43). The extreme N-terminal 11 amino acids are fairly hydrophilic, including two lysines and one glutamic acid. The signal sequences of *E. coli* pili contain amino acids with an overall positive charge (Fig. 5B), as do most exported proteins. The most characteristic feature of export signals is the presence of a long stretch of hydrophobic amino acids. The amino terminus of the mature pilin of *P. aeruginosa* contains 25 hydrophobic amino acids (between Glu-10 and Arg-37, Fig. 5B) that may be used in recognition by the export apparatus. The last component of a prototypic signal sequence is a short stretch of amino acids that serves as a recognition site for signal peptidase during proteolytic conversion of the precursor to a mature polypeptide. Since prepilin is not cleaved at any site following the hydrophobic domain, the recognition sequence must be absent from prepilin.

We searched the amino acid sequence of prepilin for signal sequence recognition sites by using the weighted-matrix method of von Heijne (44). An acceptable cleavage site was identified between Ala-26 and Ile-27 (Fig. 5B). The lack of cleavage at this position by signal peptidase suggests that prepilin escapes recognition by utilizing an export pathway different from that used by other secreted polypeptides. Alternatively, cleavage of the six-amino-acid leader peptide and subsequent methylation of the N-terminal phenylalanine may precede insertion into the membrane, with the processed pilin being unsuitable as a substrate for signal peptidases. In any event, cleavage of the leader peptide at a position six amino acids from the N terminus and subsequent methylation of phenylalanine represent a major deviation from the normal protein export pathway. It is likely that the processing of prepilin is carried out by an as-yet-uncharacterized protease. This protease should have a specificity quite different from that of enzymes described for *E. coli* which recognize common leader sequences (35). Processing of prepilin involves cleavage of the peptide bond between Gly and Phe, and since this sequence does not appear anywhere else in the pilin sequence, it should be a sufficient protease recognition sequence. However, one cannot rule out the possibility that prepilin may assume a

conformation that resembles that of other exported proteins and becomes a substrate for one of the major signal (leader) peptidases.

The striking homologies of the leader sequences and constant regions of the mature polypeptides in the methylphenylalanine class of pilins in four unrelated gram-negative microorganisms suggest that these polypeptides follow a similar export pathway. The export apparatus, which may include a specialized leader peptidase, should be conserved as well. It is noteworthy that the *P. aeruginosa* pilin synthesized from recombinant plasmids is incorporated into the *E. coli* membrane (40) and that the same pilin-alkaline phosphatase hybrid proteins are exported by both organisms. These findings would suggest that a general cellular machinery, which directs protein export at the early stages, can recognize a wide variety of export signals, ranging from typical cleavable signal sequences to hydrophobic domains found within the polypeptide chain. The significance of the location and structure of such signals in determining the localization or activity of the secreted proteins is not yet apparent.

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