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Neisseria gonorrhoeae Prepilin Export Studied in *Escherichia coli*

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The *pilE* gene of *Neisseria gonorrhoeae* MS11 and a series of *pilE-phoA* gene fusions were expressed in *Escherichia coli*. The PhoA hybrid proteins were shown to be located in the membrane fraction of the cells, and the prepilin product of the *pilE* gene was shown to be located exclusively in the cytoplasmic membrane. Analysis of the prepilin-PhoA hybrids showed that the first 20 residues of prepilin can function as an efficient export (signal) sequence. This segment of prepilin includes an unbroken sequence of 8 hydrophobic or neutral residues that form the N-terminal half of a 16-residue hydrophobic region of prepilin. Neither prepilin nor the prepilin-PhoA hybrids were processed by *E. coli* leader peptidase despite the presence of two consensus cleavage sites for this enzyme just after this hydrophobic region. Comparisons of the specific molecular activities of the four prepilin-PhoA hybrids and analysis of their susceptibility to proteolysis by trypsin and proteinase K in spheroplasts allow us to propose two models for the topology of prepilin in the *E. coli* cytoplasmic membrane. The bulk of the evidence supports the simplest of the two models, in which prepilin is anchored in the membrane solely by the N-terminal hydrophobic domain, with the extreme N terminus facing the cytoplasm and the longer C terminus facing the periplasm.

Pili are proteinaceous cell surface appendages composed mainly of identical pilin subunits. Pili of *Neisseria gonorrhoeae* are important virulence determinants because they mediate adhesion to human epithelial cells (6, 22, 44, 47). Gonococcal pilin is subject to antigenic variation, and the pilin polypeptide varies in composition and also in length (from 159 to 166 amino acids) (14, 16). Pilus variants derived from a single isolate, as well as unrelated strains, produce pilins which differ in their semivariable central and hypervariable C-terminal regions. The hypervariable region is immunodominant and contributes to the ability of variants to evade the immune response. However, all gonococcal pilins share a conserved 46-amino-acid N terminus. This domain is hydrophobic and is believed to be responsible for the export of pilin as well as for the polymerization of pilin subunits into pili (16, 38).

Pilins from enterobacteria are exported by the classical *secA*-dependent general protein export pathway. They are synthesized as precursors with 20- to 30-amino-acid N-terminal signal peptides that are cleaved during cytoplasmic membrane translocation (27). These signal peptides resemble those of other exported bacterial polypeptides, comprising a long hydrophobic region preceded by a short, positively charged N-terminal sequence and followed by the recognition site for signal (leader) peptidase. A helix-breaking motif (usually glycine or proline) occurs four to eight residues before the cleavage site of most signal peptides (29).

From the sequence of gonococcal pilin precursor deduced from that of the *N. gonorrhoeae* MS11 pilin gene (*pilE*), one would predict the existence of a more or less typical signal peptide, although the N-terminal region is unusually long and contains both negatively and positively charged amino acids (Fig. 1). The hydrophobic core includes a canonical cleavage site for leader peptidase, and a further potential processing site for the same enzyme is located after the hydrophobic region. Both sites are preceded by turn-inducing motifs (Fig. 1). However, the sequence of purified pilin from the same MS11 strain (39) revealed the existence of a completely different cleavage event which removes the first seven amino acids of the precursor (Met-Asn-Thr-Leu-Gln-Lys-Gly). In addition, the N-terminal phenylalanine is methylated.

These particularities are also found in type IV pilins of various other bacterial pathogens, including *Pseudomonas aeruginosa*, *Bacteroides nodosus*, *Moraxella bovis*, *Moraxella nonliquefaciens*, *Neisseria meningitidis*, and *Vibrio cholerae* (8, 40). In particular, these pilins have a similar N-terminal processing site and a high degree of sequence homology in the first 30 residues of the mature pilin (Fig. 1). These homologies extend to several recently identified proteins, including components of the pullulanase secretion pathway in *Klebsiella oxytoca* (34), components of the general secretion pathway for most extracellular proteins in *Erwinia chrysanthemi* (17) and *P. aeruginosa* (18a), and components of the *Bacillus subtilis* machinery involved in DNA uptake into transformation-competent cells (1). Unlike pilins, which cross two distinct membranes, members of this second group seem to remain anchored in the cytoplasmic membrane (34). Furthermore, the secretion or assembly of the methylphenylalanine class (type IV) of pilin in *P. aeruginosa* involves specific factors which are closely related or identical to those required for extracellular protein secretion in this organism (2, 26) or to proteins required for pullulanase secretion in *K. oxytoca* (48).

To analyze the first step of gonococcal pilus biogenesis, i.e., pilin export, we took advantage of the fact that the gonococcal pilin gene is expressed in *Escherichia coli* but the subunits are not assembled into pili (24). We have previously shown that pilin is exported to the *E. coli* envelope, that it is not processed, and that it remains membrane associated (7). To better understand the process of pilin export, we attempted to determine the locations of prepilin and prepilin-alkaline phosphatase (PhoA) hybrids in *E. coli*. The first 20 residues of prepilin were shown to function as an export (signal) sequence when fused to PhoA, but the signal was not processed by leader peptidase even when all but 34 residues of prepilin were replaced by PhoA. Alternative models for

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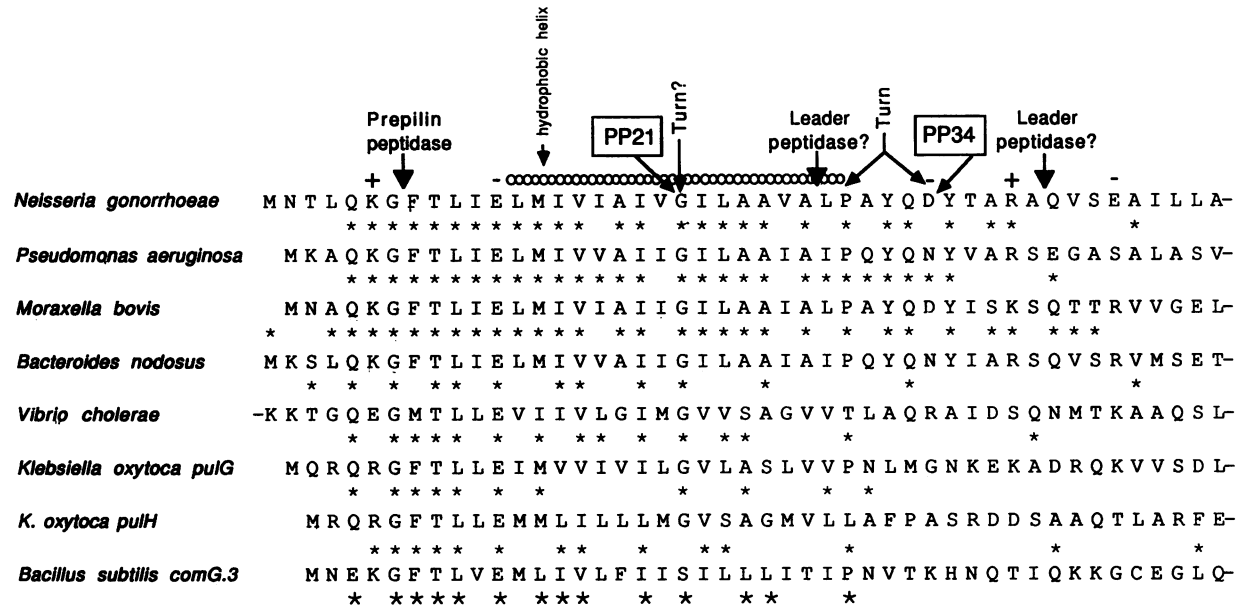

FIG. 1. Comparison of the predicted sequence of the *N. gonorrhoeae* prepilin N-terminal constant domain with those of several type IV prepilins (8) and related proteins (1, 34, 40). All sequences start at the first position of the precursor polypeptide, except for the TcpA pilin of *V. cholerae*, which starts at the 26th residue (40). The PulH protein from *K. oxytoca* is presumed to start at the first initiator methionine (34). Residues identical to gonococcal prepilin are indicated by asterisks above the other protein sequences. The large asterisks below the alignment are common residues in at least six of the eight proteins. Charged amino acids of gonococcal prepilin are indicated by + (positive) or − (negative) signs above the sequence. The positions of PhoA fusion points in PP21 and PP34 are also indicated. Other features of this segment of the *N. gonorrhoeae* prepilin (potential leader peptidase cleavage sites, turn-inducing motifs, and the hydrophobic region that is strongly predicted to form a helix [11]) are indicated above the sequence.

the membrane topology of prepilin and the prepilin-PhoA hybrids in *E. coli* are presented and discussed.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* DH5 (15) was used as the recipient for recombinant plasmids, MC4100 [F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb-5301 deoC1 ptsF25 rbsR*] was used to construct *pilE-phoA* fusions, and PAP152 (F$^-$ *gyrA phoS rbs*::Tn*10 ilv*::Tn*5*) was used as an alkaline phosphatase-constitutive control. All cultures were routinely grown at 37°C on L medium (25). Ampicillin and kanamycin were used at 100 and 200 μg/ml, respectively, and 5-bromo-4-chloro-3-indolyl phosphate was used at 40 μg/ml.

Recombinant DNA techniques. All recombinant DNA protocols were those of Maniatis et al. (19). *pilE-phoA* fusions were generated in vivo by transposition of Tn*phoA* into pNG1722-CAT or pNG1100 (23) (see below). *Sac*I-*Cla*I or *Hpa*I-*Eco*RI fusion subclones in M13mp18 DNA were used either to sequence fusion points by using a 17-bp *phoA*-specific oligonucleotide or to generate deletions at fusion points by using the Amersham System 2 site-directed mutagenesis kit and appropriate *pilE-phoA* oligonucleotides. A single base was deleted from the original, out-of-frame *phoA* fusion at *pilE* codon 145 to generate an in-frame fusion by using a 24-bp oligonucleotide with 13 residues complementary to the 5′ end of Tn*phoA* and the rest complementary to the 3′ end of the *pilE* in the gene fusion but omitting the junction base to be deleted. Thirty-nine base pairs were deleted from the *pilE-phoA* fusion at *pilE* codon 34 to generate a more proximal fusion at *pilE* codon 21 by using a 34-bp oligonucleotide with 18 residues complementary to the 5′ end of Tn*phoA* and the rest complementary to *pilE* codons 16 to 21. After sequencing was performed to confirm that the desired deletions had been generated, the new, in-frame fusions were subcloned in the same pBR322 derivative vector. This vector, which was designed as an ideal recipient of *phoA* fusions, is a mung bean nuclease-*Pvu*II-modified pBR322 in which the *Hin*dIII-*Bam*HI fragment was substituted by a *blaM-phoA* fusion. This construct contains a unique *Hin*dIII site upstream of the *blaM* fragment and a unique *Pvu*II site in *phoA*. This *Hin*dIII-*Pvu*II fragment was replaced by a *Hin*dIII-*Pvu*II fragment containing *pilE-phoA* fragments from double-stranded M13 DNA carrying the two gene fusions described above. The two other *pilE-phoA* gene fusions (at *pilE* codons 34 and 100) were directly cloned as *Sac*I-*Hin*dIII fragments into *Eco*RV-*Hin*dIII-digested pBR322.

Isolation and analysis of *pilE-phoA* fusions. Tn*phoA* insertions in pNG1722-CAT or pNG1100 were isolated in *E. coli* MC4100 by using lambda Tn*phoA* as described by Gutierrez et al. (13). Plasmid minipreparations from kanamycin (200 μg/ml)- and ampicillin (100 μg/ml)-resistant transductants were used to transform strain DH5 on the same antibiotic-containing medium, and PhoA$^+$ or PhoA$^-$ clones were purified. Insertions of Tn*phoA* into each plasmid were identified by restriction enzyme analysis of PhoA$^+$ plasmids. An additional 200 PhoA$^-$ transductants, together with a few PhoA$^+$ clones as positive controls, were grown on nitrocellulose filters, and PhoA protein was immunodetected by using anti-PhoA antibodies and ^{125}I-radiolabelled staphylococcal protein A after treating the filters for 1 h with phosphate-buffered saline containing 1% sodium dodecyl sulfate (SDS) and 1% $CHCl_3$. Rabbit serum against PhoA was cleared of antibodies against *E. coli* proteins by pread-

sorption against cells of strain DH5 grown in phosphate-rich medium before use. Plasmid preparations from immunoreactive clones were analyzed as indicated above for PhoA$^+$ clones. DNA sequencing of *pilE-phoA* fusion subclones in M13-derivative DNA was performed by the dideoxy chain termination procedure (36). Alkaline phosphatase activity of plasmid-containing derivatives of strain DH5 grown in a phosphate-rich medium was measured as described by Brockman and Heppel (5).

Fractionation and analysis of membrane fractions. Cells were collected by centrifugation and resuspended on ice in a 10 mM Tris–5 mM $MgSO_4$ buffer, pH 7.5. After sonication and removal of unbroken cells by centrifugation at 2,000 × *g* for 10 min, the membrane fraction was collected by centrifugation of the clarified extract at 100,000 × *g* for 2 h and the pellet was resuspended in an equal volume of the same buffer. The soluble (cytoplasm plus periplasm) and total membrane fractions were compared by immunoblotting with anti-PhoA antibodies prepared as described above.

Cytoplasmic and outer membranes of cells lysed by passage through a French press were separated by isopycnic sucrose gradient centrifugation (28). Fractions collected from this gradient were analyzed for the presence of the cytoplasmic membrane NADH oxidase activity (28) and outer membrane bacteriophage λ receptor activity (21). The fractions were also analyzed for the presence of pilin protein by immunoblotting. Rabbit immunoglobulin G raised against pili purified from *N. gonorrhoeae* MS11 (4, 35) was used to detect pilin as described by Dupuy et al. (7).

Immunoblotting, pulse-chase labelling, and immunoprecipitation. Immunoblotting and SDS-polyacrylamide gel electrophoresis (PAGE) were performed as previously described (33, 45). Immunoblots of gels loaded with increasing amounts of PhoA hybrid proteins and developed with anti-PhoA serum and ^{35}S-labelled protein A were quantified by counting the radioactivity in immunoreactive bands excised from the nitrocellulose sheets. For pulse-chase labelling and immunoprecipitation, cells were grown in low-potassium minimal medium and labelled at 37°C with [^{35}S]methionine (200 μCi/ml) essentially as described previously (33). Labelling was arrested by adding cold methionine (0.1%), and incubation was continued (chase). Samples were collected and immediately mixed with an equal volume of 2% SDS and heated to 100°C for 5 min. Immunoprecipitation with anti-PhoA serum was then carried out, and samples were analyzed by SDS-PAGE and autoradiography as described previously (33).

Protease accessibility. Cells grown in L broth were converted into spheroplasts as described previously (33). More than 98% of the cells were converted into spheroplasts (as judged by light microscopy) within 15 min, at which time different concentrations of trypsin or proteinase K were added and the mixtures were incubated at 30°C. Digestion was stopped by adding phenylmethylsulfonate (1 mM), and the supernatant (periplasm) and cells (spheroplasts) were separated by centrifugation at 16,000 × *g* for 5 min. The spheroplasts were resuspended directly in SDS-PAGE sample buffer and heated to 100°C. The supernatant fraction was chilled on ice, and trichloroacetic acid was added to a final concentration of 15%. After 20 min on ice, the precipitated proteins were collected by centrifugation, washed once in 95% ethanol, resuspended in sample buffer, and heated to 100°C for 5 min. In some cases, the separation step was omitted and proteins were precipitated immediately with trichloroacetic acid. Samples were analyzed by SDS-PAGE and immunoblotting with anti-PhoA serum (see above).

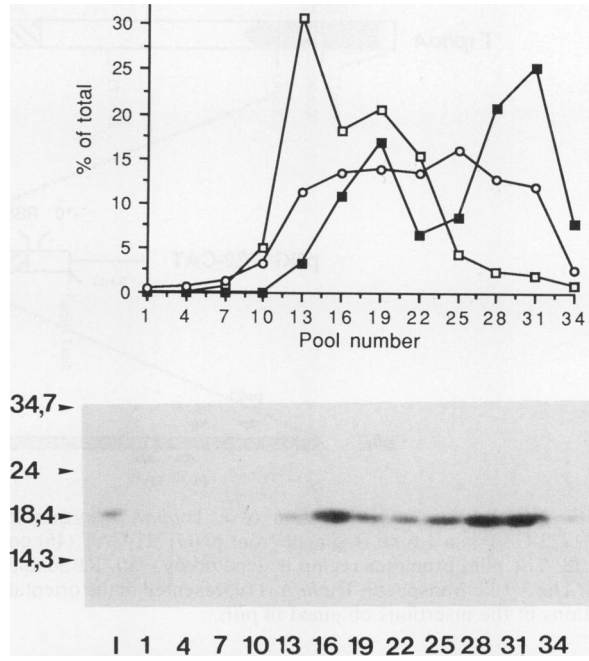

FIG. 2. Immunodetection of gonococcal prepilin in cytoplasmic and outer membranes of *E. coli* DH5(pNG1100). Fractions from isopycnic sucrose gradients used to separate membranes from cells lysed in a French press were assayed for their protein content (○) and NADH oxidase (cytoplasmic membrane marker (■) or bacteriophage λ receptor (outer membrane marker (□) activities (top). The same fractions were analyzed by immunoblotting for gonococcal pilin content (bottom). Fraction numbers are indicated below the lanes, and molecular size markers (in kilodaltons; Sigma MW-SDS-70 kit) are indicated to the left of the autoradiograph of the immunoblot.

RESULTS

Localization of gonococcal prepilin in *E. coli*. pNG1100 is a pBR322 derivative which carries the gonococcal pilin gene (*pilE*) cloned from strain MS11 (24). This gene is transcribed in *E. coli* from its own promoter, and prepilin accumulates to approximately 1% of total cellular proteins (7, 24). Immunodetection experiments showed that 80 to 95% of this pilin is in the total membrane fraction (7). To determine its location more precisely, washed *E. coli*(pNG1100) cells were passed through a French press and the inner and outer membrane fractions were separated by isopycnic sucrose gradient centrifugation. Gradient fractions were analyzed for the presence of pilin by immunoblotting after SDS-PAGE and also for cytoplasmic membrane NADH oxidase activity and outer membrane bacteriophage λ receptor activity (Fig. 2). The majority of the pilin was detected in fractions containing NADH oxidase activity. Since fractions 13 to 19 contain both inner and outer membrane markers, they are likely to contain unseparated membrane vesicles. These results indicate that gonococcal pilin synthesized by *E. coli* is transported to the cytoplasmic membrane and hence that the pilin contains an export signal which is recognized in *E. coli*. However, the protein was not released from the cytoplasmic membrane because pilin was not detected in the periplasm or outer membrane. This membrane-associated form of pilin was previously shown to be unprocessed (7).

Construction and isolation of *pilE-phoA* gene fusions. The prepilin export signal was further characterized by studying

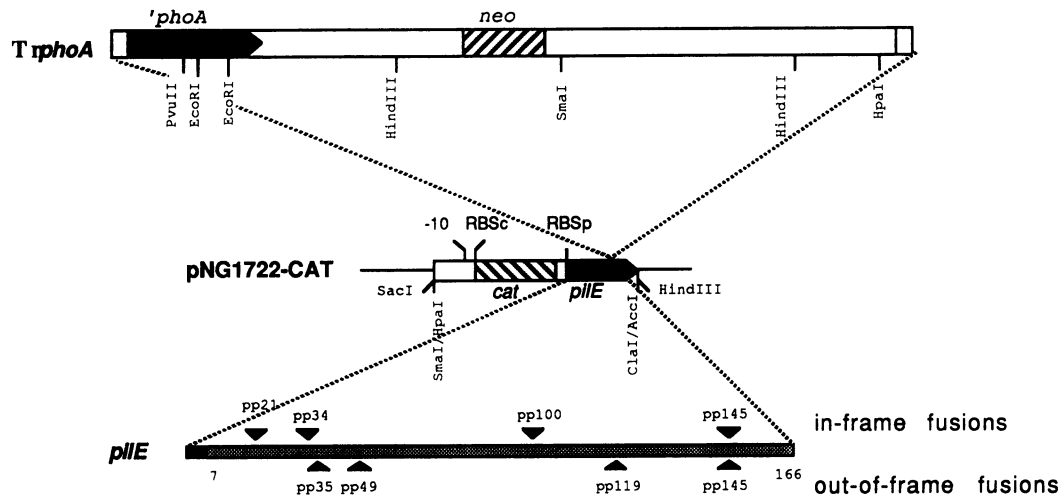

FIG. 3. Schematic representation of a Tn*phoA* insertion in *pilE* and relative positions of *pilE-phoA* fusion points. The insert in pNG1722-CAT is a 1.6-kb fragment from pNG1721-CAT (46) containing a *pilE-cat* transcriptional fusion and cloned into the polylinker of pUC18. The pilin promoter region is denoted by −10. RBSc and RBSp are, respectively, the *cat* gene and the pilin gene ribosome binding sites. The 7.7-kb transposon Tn*phoA* is represented in the orientation that generates a *pilE-phoA* fusion. The bottom part indicates the relative positions of the insertions obtained in *pilE*.

the products of *pilE-phoA* gene fusions constructed in vivo by using Tn*phoA* of Manoil and Beckwith (20). This approach is based on the fact that alkaline phosphatase (PhoA) is active when located in the periplasm but inactive in the cytoplasm. The transposon was inserted into the *pilE* gene of pNG1722-CAT. This recombinant plasmid contains a transcriptional fusion in which the structural gene for chloramphenicol acetyltransferase (CAT cartridge) is placed downstream of the pilin promoter and upstream of the pilin structural gene (Fig. 3). In this construction, the CAT cartridge, containing the *cat* gene ribosome binding site but not its promoter, is not polar on *pilE* expression, as determined by immunoblot analysis with antipilin antibodies (data not shown). This operon fusion created in a pBR322 derivative (pNG1721-CAT [46]) was further subcloned as an *Hpa*I-*Cla*I fragment into the *Sma*I-*Acc*I sites of pUC18 to generate pNG1722-CAT (Fig. 3).

Pilin-PhoA hybrid proteins that displayed significant alkaline phosphatase activity ($PhoA^+$) were generated by insertion of the transposon at various sites in *pilE*. Such *pilE-phoA* fusions consist of a 5′ *pilE* gene segment that compensates for the missing 5′ sequences of *phoA* that are essential for translocation of PhoA into the periplasm. On plates containing 5-bromo-4-chloro-3-indolyl phosphate, the $PhoA^+$ transformants produced pale, medium, or dark blue colonies, depending on the level of phosphatase activity present. Thirty-three independent $PhoA^+$ fusions were obtained in pNG1722-CAT. Twenty-three had Tn*phoA* inserted in the vector *blaM* gene, and 10 had the insertion in the correct orientation in *pilE*. DNA sequencing of the *pilE-phoA* fusion points showed that *phoA* was in frame with *pilE* at codon 34 in three cases (*pilE-phoA34*, or pp34) and once at codon 100 (*pilE-phoA100*, or pp100). These transformants produced dark blue colonies. Out-of-frame fusions were also found; four were at codon 35 (light blue colonies), one was at codon 49 (light blue colonies), and one was at codon 119 (medium blue colonies).

Additional *pilE-phoA* fusions were isolated after Tn*phoA* transposition into pNG1100, a lower-copy plasmid which contains the same 166-codon *pilE* sequence as pNG1722-CAT. About 200 transformants from white or light blue colonies on 5-bromo-4-chloro-3-indolyl phosphate plates were directly grown on a nitrocellulose filter and probed with anti-PhoA antibodies to detect enzymatically inactive pilin-PhoA hybrids. Eight of 19 clones that gave positive immunoreactions had the transposon inserted in *pilE*, and the most distal insertion was further studied. DNA sequencing of the fusion junction between *pilE* and *phoA* revealed an out-of-frame fusion at *pilE* codon 145. The correct reading frame was restored by site-directed mutagenesis at this codon. The resulting clone (*pilE-phoA145*, or pp145) grew slowly and produced very dark blue colonies on 5-bromo-4-chloro-3-indolyl phosphate plates.

The most proximal $PhoA^+$ gene fusion (pp34) contains the first 33 codons of *pilE*. This sequence codes for an uninterrupted peptide of 20 hydrophobic or neutral residues (amino acids 13 to 32 of the prepilin). To test whether the entire length of this region is needed for prepilin export, we deleted a 39-bp sequence (codons 21 to 34) from fusion pp34 by using the appropriate oligonucleotide. This created a new in-frame *pilE-phoA* fusion at *pilE* codon 21 (pp21). This clone showed a positive alkaline phosphatase phenotype similar to that of pp34.

Alkaline phosphatase activity of hybrid proteins. We assayed PhoA activity in *E. coli* cells expressing the in-frame *pilE-phoA* gene fusions at the same level. This was achieved by subcloning the gene fusions, together with 300 bp of DNA upstream from *pilE* (including the *pilE* promoter) but without the *neo* and the transposase genes of Tn*5*, into pBR322.

If the prepilin-PhoA hybrids are produced at the same rate and have the same stability, their total enzymatic activity should correlate with their specific molecular activity and with the ability of the pilin portion to promote the translocation of the alkaline phosphatase moiety into the periplasm. Alkaline phosphatase activity in derivatives of *E. coli* DH5 which carried the four in-frame gene fusions (pp21, pp34, pp100, and pp145) in pBR322 was assayed. Data presented in Table 1 indicate that strains expressing pp145 had the highest PhoA activity, while the activities in strains expressing the three other fusions were considerably lower. Similar results

TABLE 1. Alkaline phosphatase activity of the prepilin-PhoA fusions[a]

Strain	PhoA act in total extract (U/mg of protein)	% Distribution of PhoA act in:	
		Periplasm + cytoplasm fraction	Membrane fraction
Controls			
PAP152	3,305	88	12
DH5	30	ND	ND
DH5 (*blaM-phoA*)	992	68	32
Out-of-frame fusions			
DH5 (*pilE-phoA35*)	62	ND	ND
DH5 (*pilE-phoA49*)	57	ND	ND
DH5 (*pilE-phoA119*)	182	ND	ND
In-frame fusions			
DH5 (*pilE-phoA21*)	205	32	68
DH5 (*pilE-phoA34*)	459	26	74
DH5 (*pilE-phoA100*)	521	42	58
DH5 (*pilE-phoA145*)	2,065	76	24

[a] The enzymatic activities were determined by using derivatives of strain DH5 harboring the *phoA* fusions. Strain DH5 (*phoA*$^+$) and the *phoA*-constitutive strain PAP152 were used as controls. All cultures were grown under repressing (high-phosphate L broth) conditions. Values represent means of PhoA activities in total extracts from several independent experiments and percent total activities in soluble or membrane fractions from two independent experiments.

were obtained by assaying the enzymatic activities produced by the out-of-frame fusions in high-copy-number pUC18 derivatives; bacteria expressing the most distal fusion produced more activity than bacteria expressing the two proximal fusions (Table 1). These three out-of-frame fusions are all +1 frame fusions in *pilE* and produce hybrid proteins of the size expected for in-frame fusions, although in smaller amounts (data not shown). This was interpreted as in vivo translational frameshifting in earlier reports (12).

The activities of the prepilin-PhoA hybrids (Table 1) represent the steady-state level of the enzyme in the cultures. To relate this activity to the total amount of hybrid protein present at time of the assay, we analyzed crude extracts from in-frame fusion clones by immunoblotting with anti-PhoA antibodies (Fig. 4A). The predominant forms of the PP34 and PP100 hybrids (PP stands for prepilin-PhoA hybrid proteins) were proteins of the size of the full-length hybrids (51 and 59 kDa, respectively), in contrast to PP145, which was immunodetected as a 66-kDa full-length hybrid and a smaller 52-kDa degradation product. Similar amounts of proteins were detected, indicating that the specific molecular activity of the PP145 hybrid is approximately four times higher than that of the PP34 and PP100 hybrids.

A very different situation was observed with PP21, which was much less abundant than the other three hybrids. By quantification of immunoblots, PP21 was found to be present at 10% of the level of PP34 and at 12% of the level of PP100. This low level of PP21 production is not due to a defect in transcription because the measured levels of CAT activity (the product of the *cat* gene that is under *pilE* promoter control in these plasmids; Fig. 1) were identical in strains producing PP21 and PP34 (data not shown). The low-level activity of PP21 cannot be explained by degradation of the hybrid, which was shown by pulse-chase experiments to be as stable as PP34 (Fig. 5). Furthermore, the amount of phosphatase activity associated with the PP21 hybrid remained constant over a 5-h period when tetracycline (10 μg/ml) or chloramphenicol (10 μg/ml) was added to exponentially growing cultures to arrest further protein synthesis (the PP34 and PP100 hybrids were also shown to be stable under these conditions). Thus, we conclude that the low activity in strains producing the PP21 hybrid (relative to those producing the PP145 hybrid) is due to reduced translation (Fig. 5). This means that the specific molecular activities of the PP21 and PP145 hybrids are approximately the same. We cannot totally rule out the possibility that most of PP21 is very rapidly degraded in the cytoplasm, but this does not affect our conclusion that this hybrid is at least as active as the other hybrids studied.

Localization of hybrid proteins. To determine whether the pilin portion of each fusion protein anchors it to the cytoplasmic membrane, we fractionated *E. coli* containing the *pilE-phoA* fusions into total membrane and soluble (cytoplasm plus periplasm) fractions which were analyzed for the presence of the various hybrids by immunoblotting. Wild-type alkaline phosphatase as well as a β-lactamase–PhoA hybrid (periplasmic markers) were found predominantly in the soluble fraction, while the full-length forms of all four

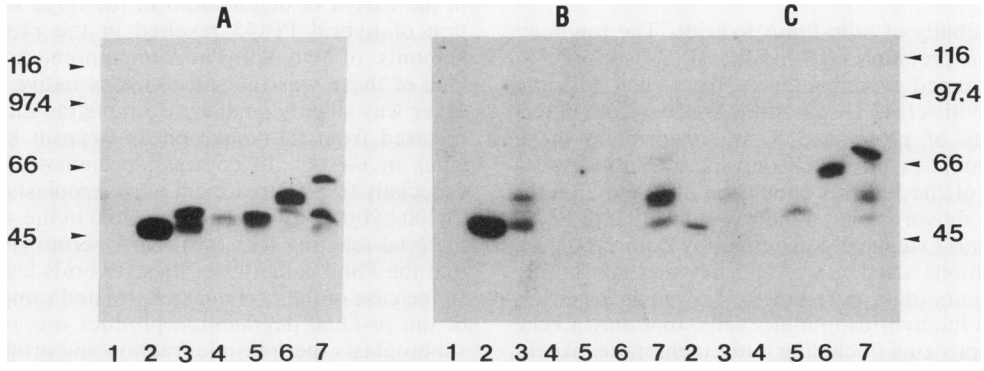

FIG. 4. Location of the prepilin-PhoA hybrids as determined by immunoblot analysis of *E. coli* crude extracts (A), soluble (cytoplasm plus periplasm) fractions (B), and total membrane fractions (C) with anti-PhoA antibodies. Strain PAP152, which is constitutive for PhoA production (2), and a derivative of strain DH5 producing a BlaM-PhoA$^+$ hybrid (3) were used for periplasmic markers. The recipient strain DH5 (1) was used as a negative control for PhoA production. Total extracts or subcellular fractions of DH5 containing prepilin-PhoA hybrids were run in lanes 4 (PP21), 5 (PP34), 6 (PP100), and 7 (PP145). Molecular size markers (in kilodaltons; Sigma MW-SDS-200 kit) are indicated to the left of panel A and to the right of panel C.

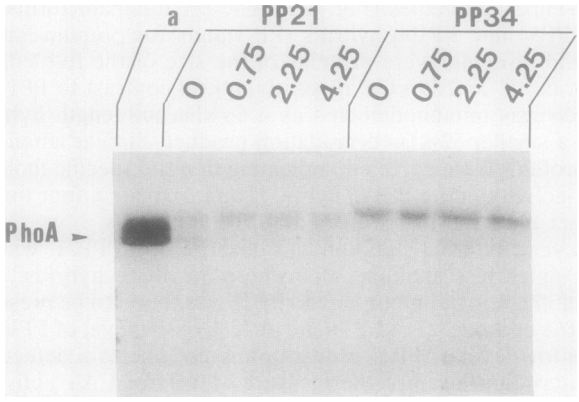

FIG. 5. Immunoprecipitation of [^{35}S]-methionine-labelled prepilin-PhoA hybrids PP21 and PP34 with anti-PhoA serum. Cells were labeled for 45 s, and then samples were taken either immediately or after addition of unlabelled methionine and continued incubation for the indicated periods (in minutes). Only that part of the autoradiograph displaying the PhoA hybrids is shown; no other proteins were immunoprecipitated. Sample a is alkaline phosphatase immunoprecipitated from labelled cells of strain PAP152.

prepilin-PhoA hybrids were predominantly associated with the membrane fraction (Fig. 4B and C). This result indicates that the 20 N-terminal residues of the prepilin polypeptide, which include charged residues at positions 6 and 12 (Fig. 1), can act both as an export (signal) sequence and as a membrane anchor.

The 52-kDa degradation product of the PP145 hybrid was mainly soluble (Fig. 4) and was released from the cells when they were converted into spheroplasts (Fig. 6). This soluble form seems to account for most of the activity of this hybrid since most of the enzymatic activity is released as soluble protein (Table 1), while the degraded form accounts for only about half of the total amount of PhoA present (Fig. 3). This suggests that the PhoA portion of PP145 is located in the periplasm, being bound to the membrane by the pilin portion in the case of the full-length 66-kDa hybrid and free in the periplasm as a result of proteolytic cleavage between residues 110 and 115 in the pilin sequence in the case of the 52-kDa breakdown product. This observation indicates that the region of the pilin polypeptide between residues 115 and 144 is entirely periplasmic and does not contain a membrane anchor.

Protease accessibility of pilin-PhoA hybrids. The topology of two of the four prepilin-PhoA hybrids (PP34 and PP100) cannot be determined unambiguously from their alkaline phosphatase activities (see Discussion). We therefore determined the effects of proteinase K and trypsin on these hybrids in spheroplasts, in which only periplasmically exposed segments of the hybrids should be attacked. Representative results shown in Fig. 6 indicate that PP34, PP100, and PP145 were all extensively degraded by both proteases under the conditions used (see Materials and Methods). Control experiments (data not shown) showed that neither protease affected the hybrids in intact cells, that only a very small number of proteins (including outer membrane protein OmpA, which has a protease-susceptible periplasmic domain) were attacked by the proteases in spheroplasts, and that a large number of proteins were progressively degraded when the proteases were applied to cells lysed by passage through a French pressure cell. This result indicates that the proteases were not penetrating into the cytoplasm when

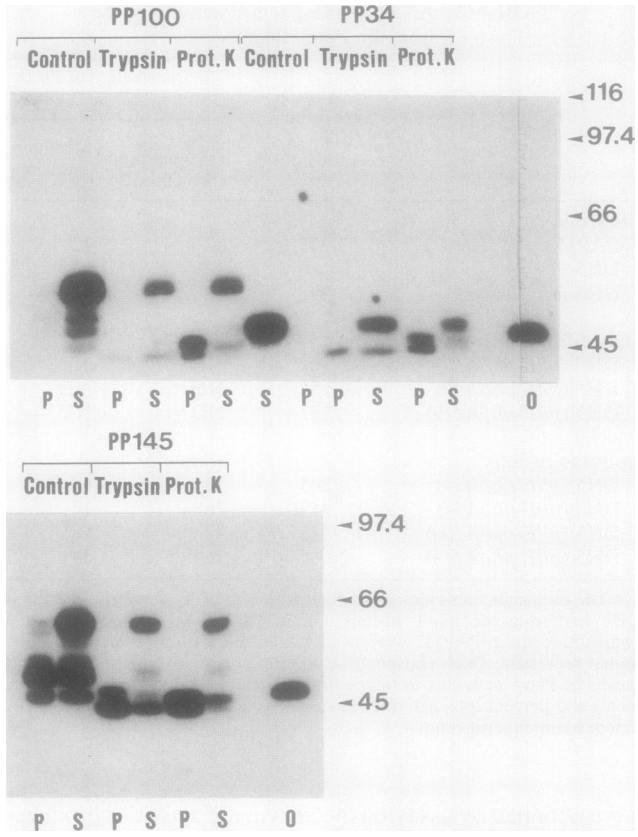

FIG. 6. Protease accessibility of prepilin-PhoA hybrids PP34, PP100, and PP145 in spheroplasts. Cells were treated as described in Materials and Methods and then separated from the buffer (containing the protease and proteins [mainly from the periplasm] released from the spheroplasts) by centrifugation, run on an SDS-PAGE gel, and immunoblotted by using anti-PhoA serum. Abbreviations: P, periplasmic (released) material; S, spheroplast fraction; and O, control alkaline phosphatase from strain PAP152. The positions of molecular size standards (in kilodaltons) are indicated at the right.

applied to spheroplasts and therefore that degradation of the three hybrids indicates that they have protease-susceptible segments exposed on the periplasmic side of the cytoplasmic membrane. There were, however, some notable differences in the extent of degradation of the three hybrids. Degradation of hybrid PP145 resulted in the production of large amounts of two anti-PhoA-immunoreactive polypeptides. One of these was the same size as native PhoA, while the other was slightly smaller. Both degradation products were released from the spheroplasts (trypsin and proteinase K lanes in Fig. 6). In contrast, proteinase K treatment and especially trypsin treatment of spheroplasts with the PP34 or PP100 hybrids consistently resulted in the substantial loss of material reacting with anti-PhoA serum (Fig. 6), indicating that the PhoA moieties of these hybrids had been degraded. In the case of the proteinase K-treated samples, the majority of the residual degradation product was released from the spheroplasts (periplasmic fraction and proteinase K lanes in Fig. 6).

DISCUSSION

Normal processing of gonococcal pilin involves the cleavage of a seven-residue N-terminal peptide during export to

the envelope and before assembly into pili. We have shown that this N-terminal extension is not cleaved in *E. coli* but that prepilin is transported to the cytoplasmic membrane, in which it remains embedded. The absence of a specific gonococcal prepilin peptidase equivalent in *E. coli* presumably explains the failure to process prepilin but is unlikely to account for the association of prepilin with the cytoplasmic membrane. Substitution of the glycine residue at the −1 position adjacent to the cleavage site abolished maturation of *P. aeruginosa* PAK pilin as well as *N. gonorrhoeae* pilin, resulting in defective assembly without affecting membrane translocation (18, 43). Our result further indicates that *E. coli* also lacks specific factors required for transport to and assembly at the cell surface. The intermediate location of prepilin in the cytoplasmic membrane appears to be the ultimate location of other proteins which function in protein secretion in *K. oxytoca* (34) or DNA uptake in *B. subtilis* (1). Since the N termini of all of these proteins are similar (Fig. 1), they are all presumably processed in the same way. Therefore, we suggest that prepilin processing is unlikely to be sufficient for pilin relocalization and that transport of pilins from the cytoplasmic membrane to the cell surface must be determined by sequences in a more C-terminal region of the polypeptide.

The prepilin polypeptide of strain MS11 is predicted to have two hydrophobic regions. The first (residues 13 to 28 inclusive) is rather longer than that of many signal peptides and might span the membrane twice. According to the structure predictions (11), the entire region is likely to form an unbroken helix (Fig. 1), but the glycine residue at position 21 could induce a turn around the center of the hydrophobic region. If this is the case, then there are only eight residues between the turn and the glutamate residue at position 12. This residue might penetrate into the membrane since it is preceded by four highly hydrophobic residues (positions 8 to 11; Fig. 1). The glutamate residue is totally conserved in all type IV pilins and related proteins (Fig. 1 and data not shown) and is essential for the methylation of the N-terminal phenylalanine (31, 43). However, the introduction of acidic amino acids (glutamate or aspartate) into the hydrophobic core of a typical signal peptide has been shown to severely reduce its activity (3, 9). Further turn-inducing motifs are located at the end of the hydrophobic region at positions 29 to 33 (Fig. 1). A second hydrophobic region is located between positions 93 and 105 of prepilin (23). This region overlaps the conserved peptide that is found in all gonococcal pilin variants in the region between the variable minicassettes mc4 and mc5 (16).

On the basis of these structural analyses, one can envisage four possible configurations for prepilin in *E. coli* (Fig. 7). Configurations C and D can be immediately dismissed because PhoA hybrid PP145 is highly active and because the enzymatically active breakdown product resulting from the action of endogenous protease is located in the periplasm. The PhoA moiety of this hybrid must therefore be located on the periplasmic side of the cytoplasmic membrane. Hybrid PP21 also has very high specific molecular activity, indicating that the first 20 residues of prepilin can function as an export (signal) sequence. However, only eight of the hydrophobic residues at the N terminus of this hybrid are uninterrupted by charged residues. As discussed above, this may mean that the glutamate at position 12 can indeed penetrate into the membrane. Furthermore, in this hybrid, PhoA is fused to prepilin at the site of the turn motif at the end of the first transmembrane segment in the proposed configuration B (Fig. 7), suggesting that the entire N-terminal hydrophobic region of prepilin could indeed form a hairpin structure. The lower specific activities of the PP34 and PP100 hybrids could also be consistent with configuration B, since the PhoA moieties of both of these hybrids would be on the cytoplasmic face of the membrane (Fig. 7). However, the difference in specific molecular activities between hybrids PP21 and PP145 and hybrids PP34 and PP100 is smaller than one would expect if the PhoA moieties of the latter two were located entirely on the cytoplasmic side of the membrane. Therefore, the possibility that these hybrids have lower specific molecular alkaline phosphatase activities because the prepilin segments interfere with the correct folding of the PhoA segment in the periplasm must also be considered.

The susceptibility of the hybrids to proteinase K or trypsin in spheroplasts should enable one to distinguish more clearly between these alternative topologies for the PP34 and PP100 hybrids, since only a very small segment of the hybrid polypeptides would be exposed in the periplasm in configuration B while almost the entire length of the hybrid would be on the periplasmic side of the membrane in configuration A. The effects of these proteases on hybrid PP145 are unambiguous, since they both cause extensive degradation that results in the appearance of free (periplasmic) PhoA. This breakdown product seems to be stable, since there was little loss of material that reacted with anti-PhoA antibodies. It is worth noting, however, that one of the PhoA fragments resulting from proteolysis of PP145 was actually smaller than native PhoA (Fig. 6), indicating that the PhoA segment of this hybrid, unlike native PhoA (data not shown), is at least partially susceptible to proteolysis by proteinase K and trypsin.

The situation with the PP34 and PP100 hybrids is more complicated. Both hybrids are clearly extensively degraded by both proteases. Since trypsin cleaves only after a basic (arginine or lysine) residue, this must mean that both hybrids have at least one such residue exposed in the periplasm. Furthermore, if these hybrids are in configuration B, then this basic residue must be in the proposed turn region around prepilin residue 21. Inspection of the sequence of prepilin (Fig. 1) reveals the absence of potential trypsin cleavage sites in this region of the polypeptide. However, the PhoA moieties of both hybrids are extensively degraded, especially in the case of trypsin. This might be due to greater protease susceptibility of this segment of the hybrids because of incorrect folding, which could also explain their lower specific molecular activities (see above). However, in control experiments, we observed that a PilB-PhoA hybrid protein (45) which has a large loop of PilB exposed on the periplasmic side of the cytoplasmic membrane and the PhoA moiety located entirely on the cytoplasmic side is also extensively degraded following treatment of spheroplasts with proteinase K (data not shown). In this particular case, we interpreted this to indicate that proteolysis of the periplasmic domain of the hybrid by proteinase K destabilized the cytoplasmic domain to render it susceptible to endogenous cytoplasmic proteases. It is conceivable, though unlikely, that the same phenomenon could explain the cytoplasmic degradation of the PhoA region of PP34 and PP100 following trypsin or proteinase K treatment of spheroplasts.

As already noted, prepilin has two potential leader peptidase cleavage sites. The prepilin-PhoA hybrid PP34 has the first of these two sites (Fig. 1). However, neither prepilin nor PP34 is processed by leader peptidase. This means that the failure of leader peptidase to cleave at least the first of these sites is probably due to a special feature of the N-terminal region of prepilin. This could be explained if prepilin and

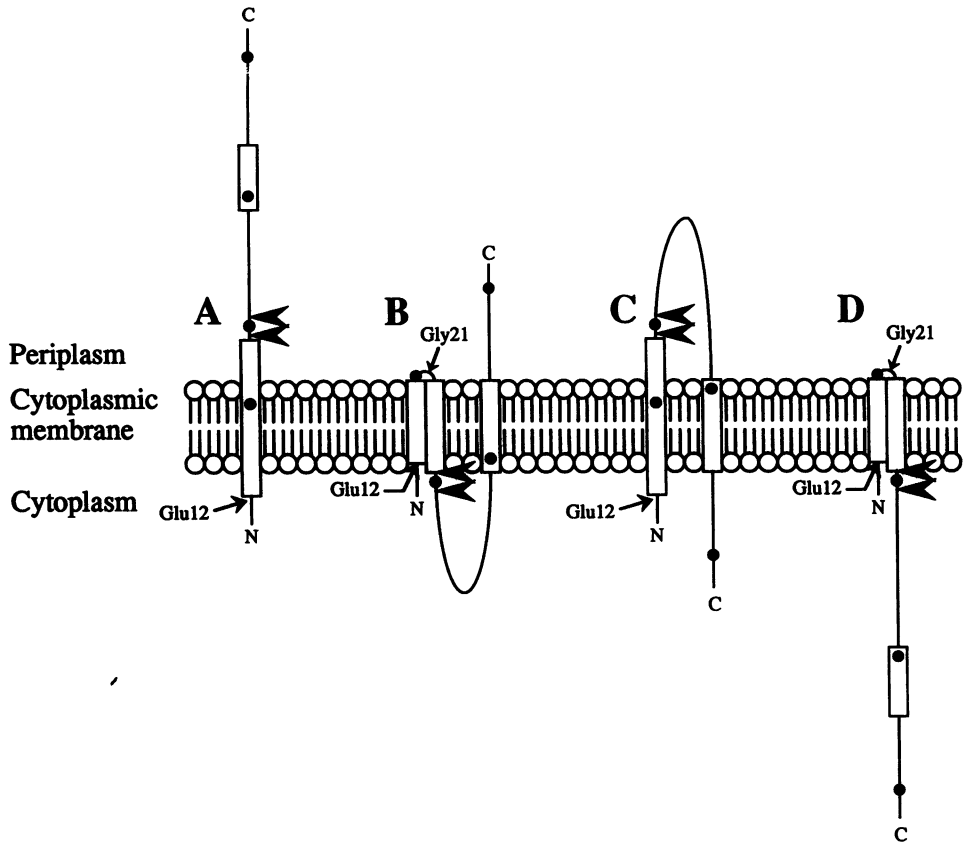

FIG. 7. Possible models for the transmembrane topology of gonococcal prepilin in *E. coli*. The amino (N) and carboxy (C) termini as well as the approximate positions of important amino acids (arrows, see text), potential leader peptidase cleavage sites (arrowheads), PhoA hybrid protein junctions (numbers PP21, PP34, PP100, and PP145 from the N to the C termini; solid circles) and hydrophobic segments (open rectangles) are indicated.

hybrid PP34 are in the looped configuration (configuration B in Fig. 7), since both cleavage sites would be on the cytoplasmic side of the membrane and therefore inaccessible to leader peptidase, whose catalytic site is on the periplasmic side of the membrane (32). Alternatively, the potential cleavage sites may not be accessible to leader peptidase when prepilin is in configuration A.

Several other type IV pilins in *E. coli* have been characterized. The prepilins of *P. aeruginosa* PAK and PAO both have long N-terminal hydrophobic segments with a turn-inducing glycine residue near the center (37) (Fig. 1), and a second, moderately hydrophobic domain between positions 97 and 109 (37). Sastry et al. (37) have proposed that the N termini of these prepilins could form a hairpinlike structure similar to that in the proposed configuration B of the gonococcal prepilin (Fig. 7). Indeed, these authors have presented some experimental evidence for such a configuration based on the insertion of truncated PAK prepilins into the *E. coli* cytoplasmic membrane and their subsequent degradation by endogenous proteases (30). PAK pilin was reported to accumulate mainly in the cytoplasmic membrane of *E. coli* (10, 41). According to Strom and Lory (42), a PAK prepilin-PhoA hybrid containing the first 51 residues of prepilin has high alkaline phosphatase activity, indicating that the PhoA is efficiently translocated across the cytoplasmic membrane. A prepilin-PhoA hybrid with 97 residues of prepilin (i.e., still lacking the second hydrophobic region between residues 97 and 109; see above) had even higher alkaline phosphatase activity. These data argue in favor of configuration A presented in Fig. 7. Another argument in favor of this configuration is the recent demonstration that an Ala-24-to-Asp substitution that interrupts the N-terminal hydrophobic region of PAK prepilin does not affect pilus assembly (43). This implies that the continuity of the hydrophobic region beyond this point is not an essential feature of the N terminus of type IV pilins. This residue could be positioned on the periplasmic side of the cytoplasmic membrane of prepilin in configuration A but would be in the center of the transmembrane segment in configuration B. Charged residues are generally excluded from the center of transmembrane segments (32).

Considering all of the data presented here concerning the gonococcal pilin, and assuming that PAK pilin is assembled by a similar process and therefore behaves in a similar way in *E. coli*, we think it most likely that the type IV prepilins adopt configuration A (Fig. 7) in the cytoplasmic membrane when expressed in *E. coli*. It therefore seems likely that prepilins are inserted in a similar configuration into the cytoplasmic membrane of the natural producing strains. However, one should also consider the possibility that the N-terminal hairpin conformation with a central cytoplasmic domain and a C-terminal periplasmic domain (configuration B in Fig. 7) may be an essential step leading to pilin secretion and pilus assembly. This possibility cannot be addressed by using *E. coli*, which lacks the factors necessary for prepilin processing, modification, secretion, and assembly.

Finally, it is worth commenting on the special case of *V. cholerae* pili, from which a longer N-terminal segment is apparently removed by type IV prepilin peptidase (40). The extreme N terminus of this region contains large numbers of acidic and especially basic residues and is not hydrophobic. Therefore, it is not predicted to play a role in prepilin export except, perhaps, to increase the binding of the precursor to membrane lipids by electrostatic interactions between the basic residues and acidic phospholipid head groups. This means that the configuration of this precursor pilin in the cytoplasmic membrane could be essentially similar to that of the *N. gonorrhoeae* prepilin.

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