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PapD, a Periplasmic Transport Protein in P-Pilus Biogenesis

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The product of the *papD* gene of uropathogenic *Escherichia coli* is required for the biogenesis of digalactoside-binding P pili. Mutations within *papD* result in complete degradation of the major pilus subunit, PapA, and of the pilinlike proteins PapE and PapF and also cause partial breakdown of the PapG adhesin. The *papD* gene was sequenced, and the gene product was purified from the periplasm. The deduced amino acid sequence and the N-terminal sequence obtained from the purified protein revealed that PapD is a basic and hydrophilic peripheral protein. A periplasmic complex between PapD and PapE was purified from cells that overproduced and accumulated these proteins in the periplasm. Antibodies raised against this complex reacted with purified wild-type P pili but not with pili purified from a *papE* mutant. In contrast, anti-PapD serum did not react with purified pili or with the culture fluid of piliated cells. However, this serum was able to specifically precipitate the PapE protein from periplasmic extracts, confirming that PapD and PapE were associated as a complex. It is suggested that PapD functions in P-pilus biogenesis as a periplasmic transport protein. Probably PapD forms complexes with pilus subunits at the outer surface of the inner membrane and transports them in a stable configuration across the periplasmic space before delivering them to the site(s) of pilus polymerization.

The outer membrane of gram-negative bacteria is an effective barrier against the release of proteins from the periplasmic space. Only a few proteins are secreted beyond this membrane. Three virulence-associated factors, the *Escherichia coli* hemolysin, cholera toxin, and the immunoglobulin A protease of *Neisseria gonorrhoeae* are excreted into the medium (16, 26, 37). The mechanism of secretion apparently differs for the three factors. Both cholera toxin and the immunoglobulin A protease appear in the periplasmic space prior to secretion (16, 37). No such intermediate has been found for the hemolysin, whose secretion depends upon two membrane-located proteins, HlyB and HlyD (26). Export of hemolysin may proceed via inner membrane vesicles or, alternatively, through sites of fusion between the inner and outer membranes, the so-called Bayer's junctions (26). In any case, the extreme C-terminal 53 amino acids of the hemolysin have been defined as the signal which directs the export of this protein (19).

Pilin and flagellin subunits are also exported to the bacterial surface where they are assembled into special appendages that remain cell associated. *E. coli* can express a variety of different pili associated with both intestinal and extraintestinal disease (34). The molecular basis for the formation of these appendages has been studied in several systems, notably those for P, type 1, and K88 pili. Two accessory proteins essential for pilus formation have been found in each system (30–33, 35). The larger of the two (80 to 90 kilodaltons [kDa]) is an outer membrane protein thought to act as a channel and polymerization platform for pilus assembly, whereas the smaller protein (around 28 kDa) is required for stability of the pilus subunits prior to polymerization.

We investigated the *pap* operon (Fig. 1), which encodes Galα(1-4)Gal-binding P pili of uropathogenic *E. coli*, to determine whether pilin subunits interact with this stabilizer protein. P pili are heteropolymers consisting of approximately 1,000 major pilin subunits (PapA) and one or a few copies each of the pilinlike proteins PapE and PapF at the tip of the pilus with the PapG adhesin (20, 22, 24); the PapK protein is believed to be similarly located (J. Tennent, unpublished data). Another pilinlike protein, PapH, is thought to anchor the fiber to the cell and to modulate pilus length (3). The product of *papJ* has recently been identified as a periplasmic protein required for the maintenance of pilus integrity (Tennent et al., manuscript submitted). PapD is required for the stability of at least PapA, PapE, PapF, PapG, and PapJ (3, 33) and has recently been shown to form a periplasmic complex with the digalactoside-binding adhesin, PapG (18). Finally, the 88.3-kDa outer membrane PapC protein is required for polymerization and surface localization of P pili (32), while the cytoplasmic products of *papI* and *papB* are involved in the regulation of pilus expression (2, 13).

In this paper, we present the features of the *papD* gene. Consistent with nucleotide sequence data and partial amino acid sequencing, we demonstrate that PapD is a periplasmic protein which is synthesized as a precursor protein with a typical amino-terminal signal peptide. PapD and a complex containing PapD and PapE were purified from the periplasmic space. We propose that PapD interacts with the newly synthesized pilin monomers at the outer surface of the cytoplasmic membrane and transports them through the periplasm to the outer membrane where they are released and assembled into pili.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *E. coli* K-12 strains used in this study were JM103 (28), MC1061 (7), and the minicell-producing strain ORN103 (36). Bacteria were routinely grown in LB broth (5) or on LB agar supplemented with medium E (43), 0.2% glucose, and thiamine (1 μg/ml). When appropriate, carbenicillin (50 μg/ml), tetracycline (20 μg/ml), kanamycin (50 μg/ml), or chloram-

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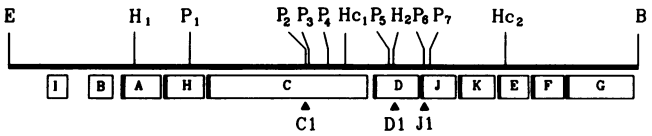

FIG. 1. Structural and genetic map of the 9.8-kilobase-pair *Eco*RI-*Bam*HI fragment of *pap* DNA cloned in plasmids pPAP5 and pPAP22 (22). The positions of linker insertions in *papC*, *papD*, and *papJ* are indicated by their allelic designations C1, D1, and J1, respectively. Restriction endonuclease sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; and P, *Pst*I. Only those *Hin*cII sites discussed in the text are shown. Subscripts delineate each restriction site.

phenicol (20 μg/ml) was added to the media. All growth was at 37°C unless otherwise indicated.

Plasmids and plasmid constructions. Plasmids pPAP5, and pPAP22 carry the *pap* gene cluster on a 9.8-kilobase-pair *Eco*RI-*Bam*HI fragment (22). Derivatives of these plasmids were constructed by inserting oligonucleotide linkers into selected restriction sites as previously described (22). Plasmids pPAP32 and pPAP34 were obtained by insertion of a linker into the *Pst*I_2 and *Pst*I_6 sites, respectively, of pPAP22, which had been partially digested with *Pst*I and made blunt ended by treatment with T4 DNA polymerase in the presence of 200 μM deoxynucleoside triphosphate (27). The insertion in pPAP34 is an 8-mer *Xho*I linker, whereas pPAP32 contains a 12-mer *Eco*RI linker. Plasmid pPAP37 carries an *Xho*I linker in *Hin*dIII$_2$ and was constructed by partially digesting pPAP5 with *Hin*dIII, treating the fragments with the large fragment of DNA polymerase I (27), and then ligating them to an excess of *Xho*I linkers. Plasmids pPAP14 and pPAP15 carrying the *papF1* and *papE1* mutations, respectively, have been described previously (22).

In order to overproduce PapD, the corresponding gene was cloned into plasmid pUC8 (42) as an *Eco*RI-*Bam*HI fragment of pPAP32. The resulting plasmid, pPAP44, has the *Pst*I_2-*Bam*HI fragment of *pap* DNA (Fig. 1) inserted in front of the *lac* promoter. A two-plasmid system was employed for the overproduction of both PapD and PapE. Plasmid pPAP58 consists of the *papDEFGJK* genes from pPAP32 ligated into a kanamycin-resistant derivative, pMMB91, of the RSF1010-derived *tac* vector pMMB66 (12) and carries the *lacI*^{q} gene and a *tac* promoter reading into *papD* (18). The *papE* gene was isolated on an *Eco*RI-*Xho*I fragment from pPAP11 (22). This fragment was first cloned into pUC8, which had been digested with *Eco*RI and *Sal*I, and was subsequently reisolated as an *Eco*RI-*Hin*dIII fragment that was inserted into the *tac* vector pKK233-2 (6). The resulting plasmid, pPAP63, mediated resistance to carbenicillin and had a *tac* promoter reading into the *papE* gene.

Expression of proteins in minicells. Plasmids were transformed into ORN103, and minicells were prepared and labeled with 80 μCi of [^{35}S]methionine as described previously (41). Immunoprecipitation was performed as previously outlined (35). Polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on linear 15% (wt/vol) gels and detected by autoradiography.

DNA sequencing. Subfragments of between 0.35 and 0.45 kilobase pairs from within the circularized *Hin*cII$_1$-*Hin*cII$_2$ fragment of pPAP5 (Fig. 1) were obtained by sonication (20 s at setting 3 with the microtip of a Branson sonifier), repaired, and then used to construct a random fragment library in bacteriophage M13mp8 (29) as described previously (1). Single-stranded DNA was prepared (28), and the inserts were sequenced by using the dideoxy chain termination method (39) and the M13 17-mer universal primer (New England BioLabs, Inc.). DNA sequences were assembled and analyzed with the Geneus program package (15).

Purification of the PapD protein. A 1-liter overnight culture of *E. coli* MC1061 (pPAP44) in LB broth containing carbenicillin was used to inoculate 15 liters of the same medium in an aerated fermentor to an A_{600} of 0.4. Growth was continued into the late logarithmic phase, when the culture was harvested into 10 liters of ice. The cells were collected in a Sharples continuous centrifuge and washed once in 50 mM Tris hydrochloride (pH 7.5). The pellet (64 g [wet weight]) was suspended in 300 ml of 20% sucrose–20 mM Tris hydrochloride (pH 8.0)–1 mM EDTA, and the outer membrane was digested by the addition of 5 mM EDTA and 50 μg of lysozyme per ml as described previously (23). The resulting spheroplasts were stabilized by the addition of $MgCl_2$ (20 mM) and then collected by centrifugation (23,500 × *g* for 15 min). The supernatant fluid (330 ml), containing the periplasmic proteins, was made 30% saturated with ammonium sulfate (58.1 g), and the precipitating proteins were removed by centrifugation (23,500 × *g* for 10 min). Ammonium sulfate (68.75 g) was added to this supernatant fluid (350 ml) to give 60% saturation, and after 30 min on ice the precipitate was collected by centrifugation. This material consisted mainly of PapD. The protein was redissolved in 30 ml of 10 mM KMES and desalted on a G-25 column (20 {potassium [*N*-(2-morpholino)ethanesulfonate]; pH 6.5} by 19 cm) equilibrated with the same buffer. Pooled protein-containing fractions were loaded onto a carboxymethyl-trisacryl (CM-TA; LKB, Bromma, Sweden) column (5 by 10 cm) and eluted with a gradient of 0 to 50 mM KCl in 20 mM KMES (pH 6.5). The major peak was centered at 38 mM KCl and contained 45 mg of greater than 97% pure PapD protein.

Purification of the PapD-PapE complex. Fifteen liters of L broth containing carbenicillin and kanamycin was inoculated to an A_{600} of 0.4 with an overnight culture of MC1061 (pPAP58, pPAP63). The cells were grown in an aerated fermentor to an A_{600} of 2.0, and IPTG (isopropyl-β-D-thiogalactopyranoside) (0.5 mM) was added to induce the *tac* promoters. Growth was continued for 60 min after which the cells were harvested and the periplasmic fraction was prepared and precipitated as described above. The pellet was finally suspended in 5 ml of 20 mM KMES (pH 6.5) and chromatographed on an AcA54 (LKB) gel filtration column (5 by 90 cm). A yield of 4.6 mg of the PapD-PapE complex was obtained.

Amino acid composition and amino-terminal sequence determinations. For the determination of amino acid composition, protein samples were freeze-dried and subjected to hydrolysis in vacuo with 6 M HCl containing 1% phenol (110°C for 24 h) as outlined previously (10). A Beckman 121 M amino acid analyzer equipped with a Hewlett-Packard 3388 A integrator was used in these analyses. Automated Edman degradations (9) were performed in an updated Beckman 890C spinning cup sequencer. The sequencing procedure and the method for analysis of the 3-phenyl-2-thiohydantoin derivatives have been described (10).

RESULTS

Definition of the *papD* gene. Based upon the results of Tn*5* mutagenesis of pPAP5, the product of the *papD* gene has been previously identified as a 28.5-kDa polypeptide (33). To define the *papD* gene, we constructed several frameshift mutations in *pap* around the Tn*5* insertion point by introducing DNA linkers at various restriction sites (Fig. 1). The

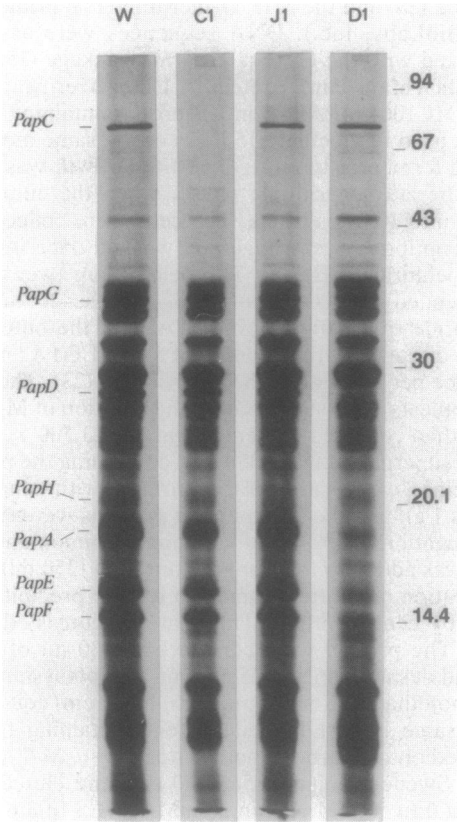

FIG. 2. Autoradiograph of [^{35}S]methionine-labeled proteins expressed in minicells containing the indicated plasmids. Lanes: W, pPAP5; C1, pPAP32; J1, pPAP34; D1, pPAP37. The locations of *pap*-encoded proteins (PapA to PapG) and molecular mass standards (in kilodaltons) are indicated. The PapJ protein is concealed by the PapA band and therefore cannot be seen.

expression of *pap*-encoded proteins by these plasmids was analyzed in *E. coli* minicells (Fig. 2). It was evident that mutations in *Pst*I$_2$ (pPAP32; *papC1*) and *Pst*I$_6$ (pPAP34; *papJ1*) left expression from *papD* intact, whereas insertions into *Hin*dIII$_2$ (pPAP37; *papD1*) abolished PapD expression. Furthermore, minicells carrying pPAP37 did not express the mature form of PapA, PapE, or PapF (lane D1). It has also recently been shown that in the absence of PapD, PapG is cleaved into several truncated forms (18).

Thus, we concluded that *papD* was located between the *Pst*I$_2$ and *Pst*I$_6$ sites and overlapped the *Hin*dIII$_2$ site in pPAP5 (Fig. 1). Determination of the DNA sequence within this region revealed two nonoverlapping reading frames that were read from left to right. The first of these corresponded to the 3′ sequences of *papC* (32), while the second represented the 717-nucleotide *papD* gene (Fig. 3). A Shine-Dalgarno-like sequence (40) preceded the ATG initiation codon of *papD* at position 440. The amino-terminal sequence obtained for the purified PapD protein (see below) corresponded exactly to those residues specified by nucleotides 503 to 577 and indicated that PapD was synthesized as a precursor protein with a typical 21-amino-acid signal peptide. PapD has a calculated molecular mass of 24.5 kDa, is hydrophilic, and possesses a positive charge at neutral pH, as suggested by its isoelectric point (pI > 9.0).

Overexpression and purification of the PapD protein. In plasmid pPAP44, the *papD* gene was placed under the transcriptional control of the *lac* promoter. A PapD purification scheme was devised based upon the finding that PapD was present almost exclusively in the periplasmic fraction of MC1061(pPAP44) cells (data not shown). Periplasmic material from such cells was precipitated with ammonium sulfate at saturations between 30 and 60%, bound to CM-TA ion-exchange resin in 20 mM KMES (pH 6.5), and dissociated with a gradient of 0 to 50 mM KCl in 20 mM KMES (pH 6.5). In this way, 45 mg of PapD that was greater than 97% pure (Fig. 4) was obtained from 15 liters of culture.

Analysis of periplasmic complexes containing PapD. In the absence of PapD, the PapA, PapE, PapF, and PapG proteins are unstable (Fig. 2). Thus, no PapE protein could be detected in MC1061(pPAP63) cells regardless of the presence or absence of IPTG (data not shown). However, when pPAP63 and pPAP58 were coresident, both PapE and PapD were produced after induction with 0.5 mM IPTG, confirming that PapD was required for the stability of PapE.

During small-scale purification trials with MC1061 (pPAP58, pPAP63), PapE was found in the periplasm and could be precipitated with ammonium sulfate (20 to 60% saturation) but it did not bind to CM-TA in 20 mM KMES (pH 6.5) (data not shown). Thus, a complex containing PapD and PapE was purified on a larger scale by a modification of the PapD purification procedure: instead of the desalting and ion-exchange steps, the ammonium sulfate precipitate was directly chromatographed on an AcA54 gel filtration column in 20 mM KMES (pH 6.5) (Fig. 5).

PapD migrated in two partially overlapping peaks centered at approximately 43 and 30 kDa (Fig. 5). Peak II consisted of PapD only, whereas peak I contained approximately equimolar amounts of PapD and PapE. Contaminating PapD monomer could be removed from the first peak by chromatography on CM-TA in 20 mM KMES (pH 6.5), which allowed the PapD-PapE complex to pass through while PapD was retained (data not shown). No PapE was detected at the position that corresponded to the monomeric protein. However, some PapE, possibly associated with large polymers or precipitates, migrated with the void volume (data not shown).

To verify the identity of the components of the purified complex, fractions in the PapD-PapE peak were pooled, concentrated, and separated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins of 28.5 and 16.5 kDa were eluted, and their amino-terminal sequences were determined by sequential Edman degradation. As expected, the larger protein corresponded to PapD. The 58-residue amino-terminal sequence obtained for the smaller protein corresponded exactly with that predicted from the *papE* gene sequence (21), except for the amino acid at position 26. This residue could not be resolved, supporting its identity as cysteine, an amino acid that cannot be detected without prior oxidation or carboxymethylation of the protein.

The interaction of PapD with PapE was also demonstrated immunologically. Labeled minicells prepared from ORN103 (pPAP58) were incubated with anti-PapD serum, and the precipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 6). Together with a relatively large amount of PapD, a substantial amount of PapE was recovered, indicating that PapD and PapE must have been complexed with one another in the periplasmic material. It has been shown recently that PapG and PapD also form a periplasmic complex (18).

Immunological examination of pili and periplasmic complexes. Rabbit antisera prepared against the purified PapD protein or against the PapD-PapE complex were used in an

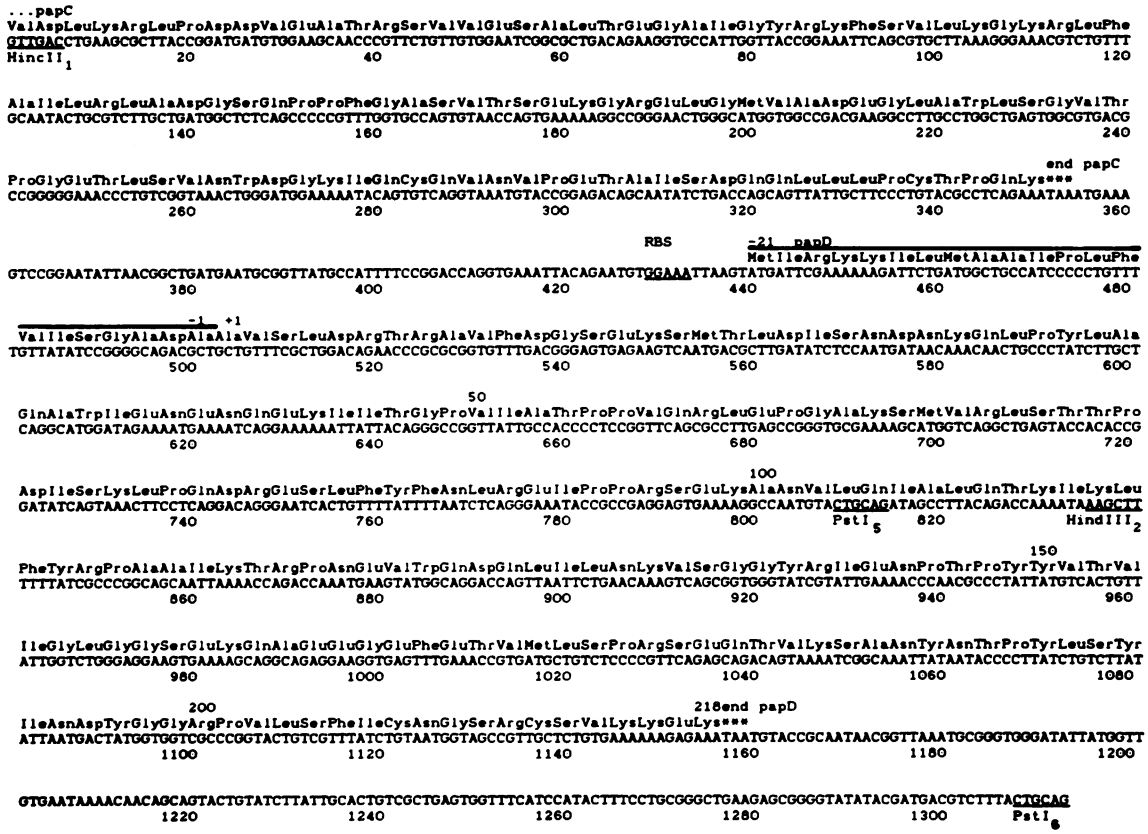

FIG. 3. Nucleotide sequence of the *PapD* gene and the deduced amino acid sequence. Numbers below the sequence show base positions relative to the *Hinc*II$_1$ site located in *papC* (32), whereas those above the sequence represent the amino acid residues in PapD. The bold horizontal line above the sequence represents the signal peptide as suggested by amino-terminal sequencing of the periplasmic PapD protein. Horizontal lines show the putative Shine-Dalgarno sequence (RBS) and the *Hinc*II$_1$, *Pst*I$_5$, *Hin*dIII$_2$, and *Pst*I$_6$ recognition sequences.

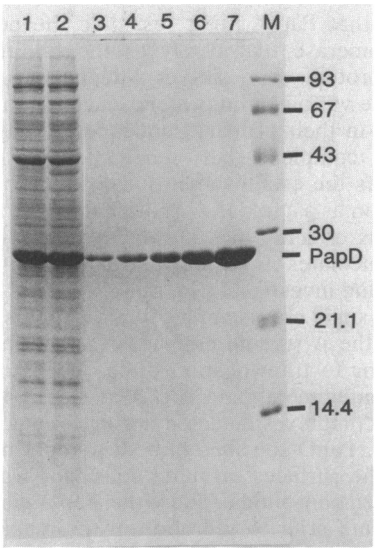

FIG. 4. Polyacrylamide gel electrophoresis of purified PapD protein visualized by Coomassie blue staining. Lanes: 1, 20 μg of protein from the periplasmic fraction of MC1061(pPAP44); 2, 20 μg of ammonium sulfate-precipitated protein; 3 to 7, 1.25, 2.5, 5, 10, and 20 μg, respectively, of purified PapD protein; M, molecular mass standards (sizes in kilodaltons shown at right).

enzyme-linked immunosorbent assay to determine if either of the two protein types were located in purified pili. Wild-type pili purified from HB101(pPAP5) or *papE1* pili from HB101(pPAP15) were used as the solid phase in this assay. As is evident from the titration curves (Fig. 7), anti-PapDE antibodies showed significant activity against wild-type pili but not against the *papE1* pili; PapD antiserum did not react with either pilus preparation (data not shown). These results show that, unlike PapE, PapD was not a component of the completed P pilus.

DISCUSSION

The P pilus is a heteropolymer consisting of the major pilin, PapA, and at least three minor proteins, PapE, PapF, and PapG, which are located at the tip of the fiber. Like the components of the type 1 pilus (8), P-pilus proteins are synthesized with an amino-terminal signal peptide and are translocated to the periplasm in a *secA*-dependent fashion. Additional proteins are, however, required for the subsequent transfer of pilin proteins to the cell surface. Here, we purified the PapD pilin-binding protein from the periplasm. We found that in the absence of PapD, the PapA, PapE, PapF, and PapG proteins were unstable in the periplasmic space.

It has recently been shown that PapD is copurified as a stable complex with PapG by affinity chromatography of periplasmic material obtained from a *papC* mutant (18). Thus, we considered that PapD might also interact directly with PapA, PapE, and PapF, thereby protecting them from

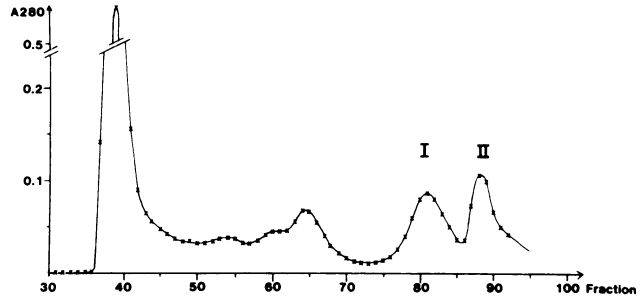

FIG. 5. Gel filtration on AcA54 of fractionated periplasm from MC1061(pPAP58, pPAP63) (see the text for details). The A_{280} of each fraction (5 ml) is plotted. The peaks centered at fractions 81 (approximately 43 kDa) and 88 (approximately 30 kDa) contained the PapD protein. In the first peak, approximately equimolar quantities of PapE were also found, whereas the second peak contained PapD only (data not shown).

proteolysis. To purify PapD-pilin complexes, we also made use of a strain which lacked the PapC protein and thus allowed the pilin subunits to accumulate in the periplasm. A stable PapD-PapE complex was isolated from the periplasm of such cells, which overproduced PapD and PapE. When PapA and PapF were similarly overproduced in the presence of PapD, they could also be recovered as periplasmic complexes with PapD; we have so far been unable to find the conditions under which the PapA-PapD and PapF-PapD complexes can be maintained throughout an entire purification scheme.

Our data suggest that PapD and PapE occur in equimolar amounts in the purified complex. Presumably, this is also the case for the other pilin-PapD complexes. Assuming that PapD transports the pilin proteins to the outer membrane where polymerization occurs, the incorporation of pilin subunits into the pilus might be controlled by the affinity between the different complexes and PapC. Furthermore, the structure of the various pilin subunits might determine the affinity of their interaction with PapD or impose limitations upon the order of their incorporation into the pilus. In any case, PapD appears able to interact with a series of pilin and pilinlike proteins that are at best 50% related at the primary sequence level (3, 4, 18, 21, 24). The interaction between PapG and PapD clearly involves the C-terminal 13 amino acids of the adhesin (18). Significantly, this stretch of amino acids contains many homologous residues which are also present in the carboxy termini of pilins (data not shown) and thus could be partly responsible for the formation of pilin-PapD complexes (18).

Translocation across the cytoplasmic membrane probably requires that a polypeptide be devoid of tertiary structure

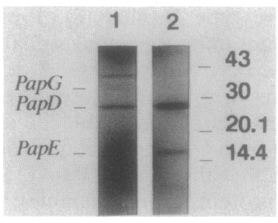

FIG. 6. Autoradiograph of [^{35}S]methionine-labeled polypeptides expressed in minicells containing pPAP58. Lanes: 1, untreated sample; 2, proteins recovered by immunoprecipitation of a sample of labeled minicells with anti-PapD serum. The positions of *pap*-encoded proteins and molecular mass markers (in kilodaltons) are shown.

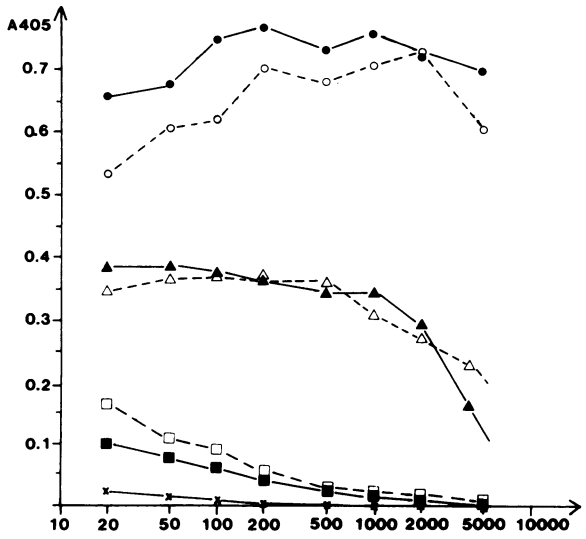

FIG. 7. Enzyme-linked immunosorbent assay, using antisera directed against the PapD-PapE complex obtained from two different rabbits (open and closed symbols). The A_{450} is plotted against the dilution of antiserum. Solid phases used were 2 μg of the purified PapD-PapE complex per well (circles), 5 μg of wild-type pili per well (triangles), and 5 μg of *papE1* pili per well (squares). Controls without serum or antigen are indicated by X's. The results obtained for each of the different antigens when anti-PapD serum was used were virtually identical to that shown as closed squares.

(38). In general, polypeptides that are not folded into their mature forms are sensitive to proteolysis. Therefore, we propose that the instability of pilin proteins in the absence of PapD arises from either their inability or slowness to attain a mature configuration. This notion is supported by our previous experiments which showed that processing of the PapA and PapG precursors is delayed in a *papD* mutant (18, 32).

The mechanism by which PapD promotes the correct folding of pilin subunits is not clear. However, it is exciting to consider that PapD might resemble the peptidyl-prolyl *cis-trans* isomerase that catalyzes the folding of several eucaryotic proteins in a sulfydryl-dependent manner (11). PapD and the various pilin proteins each contain two cysteine residues in their primary sequence. It may be that the formation of complexes between PapD and the pilus subunits involves the establishment of disulfide bridges. PapD might function as a "foldase" protein to catalyze the formation of bonds of this type. The involvement of covalently linked intermediates in the biogenesis pathway for P pili is currently being investigated.

Cholera toxin is preassembled in the periplasm, where the presence of the A subunit enhances the assembly of five B subunits prior to translocation of the completed structure across the outer membrane (14). The PapD portion of the periplasmic complexes detected during P-pilus biogenesis is not secreted. PapD-specific antibodies could not detect the protein on the surfaces of intact cells, nor was it found in culture supernatant fluids. Thus, the PapD-pilin complexes must dissociate prior to secretion of the pilins through the outer membrane. It is possible that the complex dissociates upon contact with PapC. Alternatively, individual complexes might interact with one another in the periplasmic space to give rise to oligomeric pilin complexes. Preliminary data suggest that the periplasm does not contain a pool of preassembled P pili; if this is correct, then there must be

some means by which extensive polymerization is prevented.

Since the P-pilus tip proteins are antigenically more conserved than the major subunit (25) they are ideal candidates for use as vaccines against upper urinary tract infections. The strategy to purify pilus tip proteins from the periplasm rather than from the pilus itself has already proven highly successful for the purification of the Galα(1-4)Gal-specific PapG adhesin (18) and thus is a rational approach for obtaining large quantities of the other tip proteins. Such preparations will not only be valuable in the development of preventative therapies but will also provide material for X-ray crystallographic analyses of the P-pilus proteins. The structure of the PapD protein is interesting in itself; we expect that its features will soon be resolved, as the protein has recently been crystallized (17). Such approaches are essential if the intricacies of P-pilus biogenesis are to be understood.

ACKNOWLEDGMENTS

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