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Gene products specifying adhesion of uropathogenic *Escherichia coli* are minor components of pili

(*papE* gene/*papF* gene/Pap pili/P-fimbriae/microbial pathogenicity)

FREDERIK LINDBERG, BJÖRN LUND, AND STAFFAN NORMARK

Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden

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ABSTRACT **The *papE*, *papF*, and *papG* genes of uropathogenic *Escherichia coli* are dispensable for the synthesis and assembly of pili associated with pyelonephritis, called Pap pili. Phenotypically, *papF* and *papG* mediate digalactoside [α-D-Gal*p*-(1→4)-β-D-Gal*p*)-specific adhesion. Although whole bacterial cells of a *papE* mutant bind to this receptor, purified pili from such a mutant do not. This is in contrast to pili purified from the wild type, which bind specifically. The DNA sequences of the *papE* and *papF* genes are presented, together with the deduced primary structure of the gene products. Both proteins have most of the features characteristic of *Escherichia coli* type 1 and Pap pilins. The PapE protein can be detected in the purified wild-type pilus by NaDodSO$_4$/polyacrylamide gel electrophoresis followed by silver staining or by autoradiography of gels to which radioiodinated pili have been applied. In rabbits immunized with purified Pap pili, antibodies specific for both PapE and PapF are produced. We propose that PapE and PapF are minor pilins in the Pap pilus.**

The blood group P-antigens are glycolipids present on the surface of most cells in the body, among them erythrocytes and the epithelial cells lining the urinary tract (1). Most *Escherichia coli* that cause pyelonephritis in individuals with a normal urogenital anatomy have the capacity to bind to these cell-surface glycolipids (1, 2). A common denominator of these structures is the "digalactoside" α-D-Gal*p*-(1→4)-β-D-Gal*p*, which in solution inhibits the hemagglutination caused by the bacteria (3). Also the bacteria agglutinate latex beads to which the digalactoside has been attached. Bacteria with this binding specificity express hairlike surface appendages designated "Pap pili" (pili associated with pyelonephritis) or "P-fimbriae" that are morphologically similar but exhibit a marked variability in serotype (4). Purified Pap pili agglutinate erythrocytes with the same specificity as do intact bacterial cells (5, 6).

The *pap* gene cluster coding for both pilus production and the adhesive capacity has been cloned from the *E. coli* urinary tract isolate J96 (7) and extensively analyzed by genetic techniques (6, 8, 9). At least six genes encode proteins directly involved in the expression of the pilus and the adhesive phenotype. The *papA* gene encodes the major subunit of the Pap pilus, and the products of both the *papC* and *papD* genes are needed to assemble the pilus and the protein adhesin. The genes *papF* and *papG* are required for adhesion, but surface-localized pili can be made without them. Destruction of *papE* leads to the production of pili with no or low binding capacity, without affecting the binding properties of whole cells (6). Oligonucleotide-linker and transposon Tn*5* insertion mutants in the pilin gene *papA* as well as mutants with deletions covering the entire gene still maintain their binding characteristics, although they no longer produce pili (6, 9, 10).

To investigate the seemingly paradoxical situation of a pilus that carries the adhesive property and yet is dispensable for binding, we studied more closely the structure of the *papE* and *papF* genes and the cellular location of their gene products. We present the primary sequence of these proteins, as deduced from the DNA sequence of their genes, and serological and protein chemical data to argue that both genes encode pilin-like proteins that are located both on the cell surface and in Pap-pilus preparations. We believe that our findings will be general to at least all serotypes of Pap pili because of the large structural and functional similarities between the different gene clusters (11).

MATERIALS AND METHODS

Bacteria and Plasmids. *E. coli* HB101 (12) was used as a host in all experiments. Plasmid pPAP5 contains the entire *pap* gene cluster and codes for both Pap-pilus formation and digalactoside-specific hemagglutination (6). Plasmids pPAP-23, pPAP15, and pPAP14 are derivatives of pPAP5 with linker insertions resulting in a frameshift early in the *papA*, *papE*, and *papF* genes, respectively. pPAP19 and pPAP20 are deletion derivatives of pPAP5—i.e., Δ*papEFG* and Δ*papEF*, respectively (6). Plasmid pPAP24 was constructed by deleting the *Bgl* II–*Bam*HI fragment from pPAP5, which removes the second half of the *papG* gene from the plasmid.

DNA Sequencing. A detailed restriction map of the DNA fragment to be sequenced was constructed by using the procedure of Smith and Birnstiel (13). Suitable fragments were sequenced (14–16) to obtain unambiguous readings on both strands of the entire region. The sequence was assembled using the program package of Staden (17).

Pilus Purification. Ten trays (10 × 120 cm^2) of LB medium (18) were inoculated and incubated at 37°C for 30–36 hr. Pili were separated from the bacteria by shearing in an Omni Mixer and subsequently were recovered and purified as described (6). For further purification, pili were sedimented through a 10–60% sucrose gradient as described by Korhonen *et al.* (19) with 30 mM octylglucoside in the gradients instead of deoxycholate.

RIA and Serum Absorption. Bacteria were grown overnight with selection for plasmid resistance markers and were resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.05% Brij 35, 0.1% bovine serum albumin, and 0.02% NaN_3 (RIA buffer) to an optical density (600 nm) of 10. Aliquots (100 μl) of different bacteria were pipetted into 1.5-ml Microfuge tubes and pelleted. They then were resuspended in 500 μl of a serial dilution of serum (prepared as described in ref. 8) in the same buffer and left at room temperature for 2 hr. After the cells were washed three times in RIA buffer, they were resuspended in 500 μl of this

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Abbreviation: bp, base pair(s).

buffer containing 10^5 cpm/ml of ^{125}I-labeled protein A (Amersham). After a 2-hr incubation at room temperature, the cells were washed as before, and the radioactivity in the pellet was determined. For serum preabsorption, 1 ml of serum was diluted 10-fold in RIA buffer and incubated overnight at 4°C with 10 OD_{600} units of bacteria. This procedure was repeated until the reactivity against the absorbing strain, as determined with the assay described, was constant and not significantly higher than that obtained with the host strain HB101 alone.

RESULTS

Nucleotide Sequence of the *papE* and *papF* Genes. The *papE* and *papF* genes have been mapped to the region between the third *Sma* I site and the *Bam*HI site (Fig. 1) by minicell and mutation analyses (6, 9). Since the *papG* gene codes for a polypeptide of an apparent molecular size of 36 kDa and ends between the *Sma* I site 4 and the *Bam*HI site (6), the *papF* gene should not extend beyond a point ≈1000 base pairs (bp) to the left of the *Bam*HI site. Linker insertions into the *Kpn* I site affect neither the *papE* gene nor the *papF* gene, as evident from minicell analyses of such mutants (data not shown). Hence, the *papE* and *papF* genes must be encoded by the region between the *Kpn* I and *Alu* I sites in Fig. 1. The DNA sequence of both strands of this 1284-bp long DNA fragment was determined (Fig. 2). The exact locations of the Tn*5* insertions in mutants pSN002 and pSN021 are also shown.

Two open reading frames could be identified, both reading from left to right in Fig. 2 and ending at nucleotides 600 and 1178, respectively. An *Xho* I linker insertion into the second *Hinc*II site and Tn*5* insertions 002 and 021 abolish expression of the PapF protein (6, 9). The rightmost open reading frame extends over all three mutation sites that affect PapF expression and, therefore, must encode the *papF* gene. PapE is not expressed from a linker insertion frameshift mutant in the first *Hinc*II site, whereas no other mutation in the region effects its synthesis, indicating that the leftmost reading frame encodes the PapE protein.

Both PapE and PapF are synthesized as precursors (8). Both reading frames have the potential to code for proteins with typical amino-terminal signal sequences (Fig. 2; ref. 22), assuming that the start codons are those shown to initiate translation in Fig. 2.

Comparison of PapE and PapF with *E. Coli* Pilins. The sizes of the PapE and PapF proteins, 149 and 148 amino acids, respectively, are similar to those described for most *E. coli* pilin proteins (23). Several other features characteristic of *E. coli* pilin molecules, including the two cysteines in the amino-terminal half of the proteins and the penultimate tyrosine, were also found in PapE and PapF (Figs. 2 and 3).

Since the tertiary structure of PapE, PapF, and the *E. coli* pilins is unknown, we used two different algorithms based on primary structure alone to compare these proteins. As a reference, we also compared the different pilins with one another using the same method. The first comparison (31, 32) is based on a large number of known mutational changes in different proteins that do not affect their function. A match for a highly conserved amino acid gives a high score, whereas a match for a less critical amino acid gives a lower score. Mismatches were graded similarly. A number of alignments between random permutations of the two sequences were also made, and the mean score as well as the standard deviation of these random alignments were computed. The entries above the diagonal in Table 1 are the real comparison scores, expressed as their magnitude above the average of the "random scores" divided by the standard deviation of the "random scores" (32). Values above three are considered to be significant of similarity in structure.

The second comparison (33) uses the sequence pairs as aligned by the previous procedure and computes a coefficient for the correlation in hydrophobicity along the sequences. The results of the pairwise comparisons are shown below the diagonal in Table 1. This coefficient reflects tertiary structure and, for proteins of similar structure, it is between 0.3 and 1.0. For structurally unrelated proteins, the values generally lie below 0.1 (33).

As seen in Table 1, the Pap pilins ($F7_1$, $F7_2$, and F13) are very similar to one another, as are the type 1 pilins (1A and 1C). According to both methods, these two groups are very similar to one another. The PapE and PapF proteins and the "K99" pilin show a comparable and significant degree of similarity to both groups. This indicates that the PapE and PapF proteins have tertiary structures similar to that of the *E. coli* pilins and that they might polymerize, either alone or together with other pilins.

Detection of PapE and PapF in a Pap-Pilus Preparation. If PapE and PapF are present in the pilus, they must be in low amounts as they were not detected on Coomassie brilliant blue-stained $NaDodSO_4$/polyacrylamide gels loaded with 30 μg of purified Pap pili (8). To detect such putative minor components, pili purified from host HB101 carrying pPAP5 (HB101/pPAP5) were radioiodinated and electrophoresed on a $NaDodSO_4$/15% polyacrylamide gel (Fig. 4 *Left*). Two polypeptide species were seen in the iodinated pilus preparation. One of these comigrated exactly with the major pilin

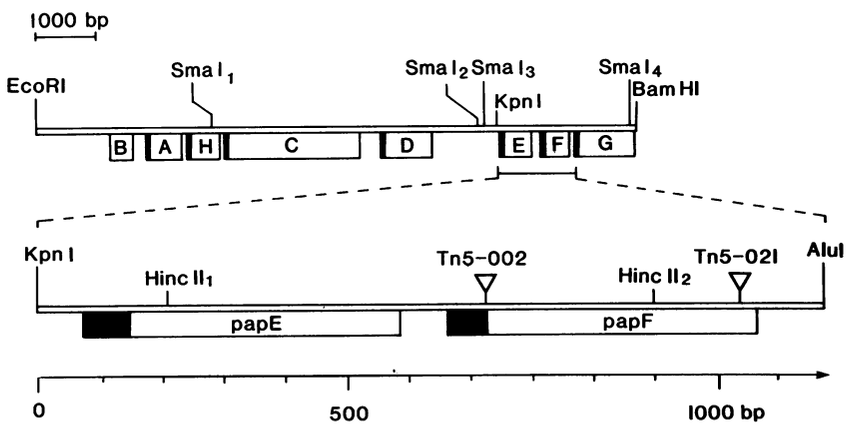

FIG. 1. The entire segment of *pap* DNA as carried in pPAP5 is shown. The horizontal bar under *papE* and *papF* indicates the region sequenced, which is shown in greater detail as delineated by the dashed line. Indicated are the *Kpn* I and *Alu* I sites delimiting the fragment, as well as the *Hinc*II sites 1 and 2, which are the points of insertion for the *papE1* and *papF1* mutations, respectively. Also shown are the insertion points of Tn*5* in the mutant plasmids pSN002 and pSN021.

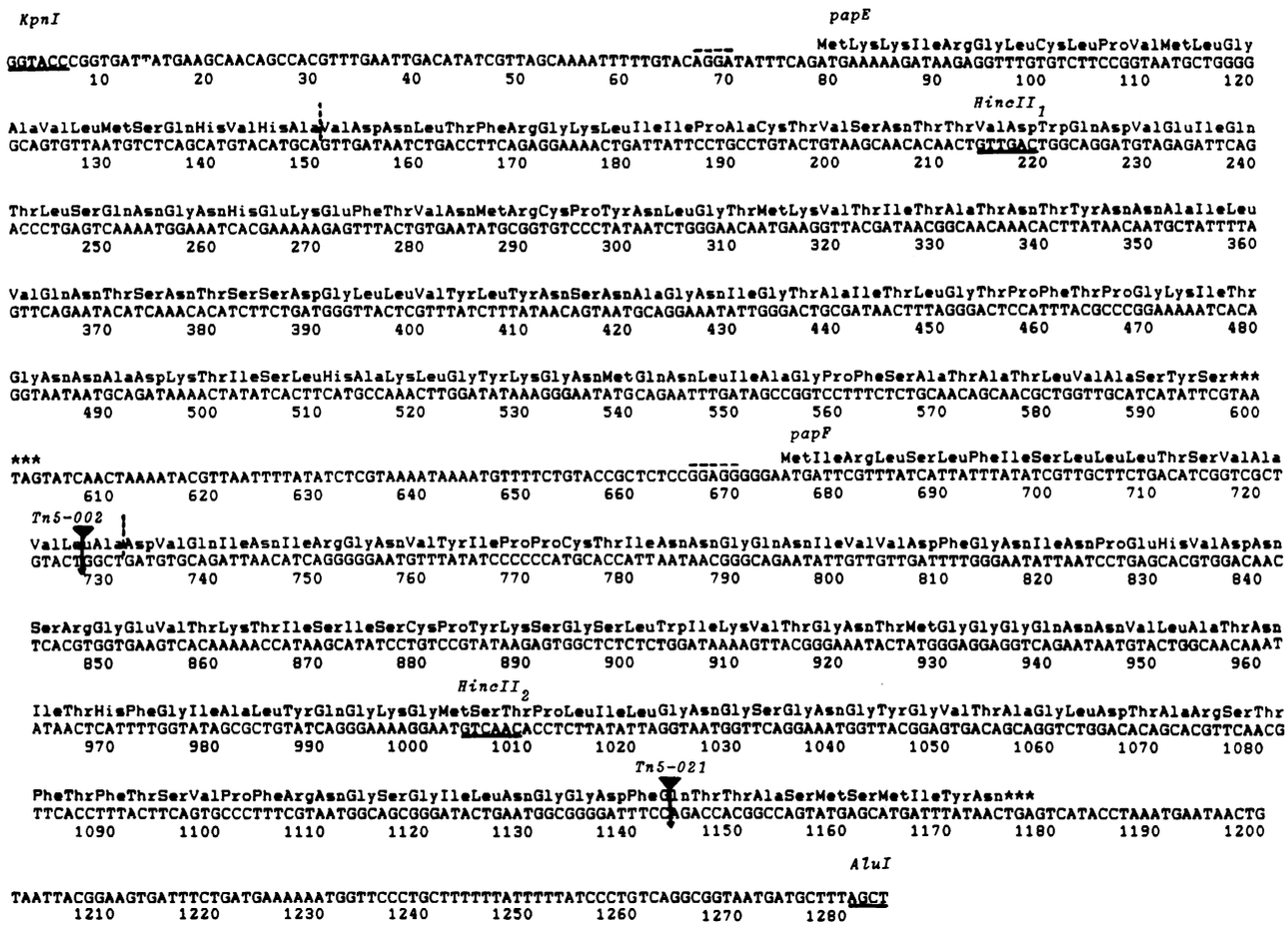

FIG. 2. The DNA sequence of the noncoding strand and the predicted amino acid sequences of the *papE* and *papF* genes are shown. The broken horizontal lines indicate the putative ribosome binding sites for *papE* and *papF* (20). The vertical broken lines indicate the proposed processing sites between the signal peptides and mature proteins (21). Also shown are the exact insertion points of Tn*5* in mutants pSN002 and pSN021 as well as the restriction sites given in Fig. 1.

(PapA), and the other peptide, which has an apparent size of 16.5 kDa, comigrated with the [^{35}S]methionine-labeled protein known to be the product of the *papE* gene (6). Both bands were seen also with pili from a *papG* or Δ*papFG* mutant, but the 16.5-kDa peptide was not present in the Δ*papEFG* pilus.

FIG. 3. The optimal alignment of the amino and carboxyl termini of the PapE and PapF proteins with the Pap pilins of serotype F13 (24), $F7_1$ (25), and $F7_2$ (26) as well as the type 1A (27, 28), type 1C (29), and K99 (30) pilins. The amino acid positions are numbered according to the F13 sequence. The amino acids are given in the standard one-letter code, which is the first letter of the amino acid name except for arginine (R), asparagine (N), aspartic acid (D), glutamine (Q), glutamic acid (E), lycine (K), phenylalanine (F), tryptophan (W), and tyrosine (Y). Alanine, valine, leucine, and isoleucine are regarded as a group, as are serine and threonine. If more than four sequences have identical residues or residues belonging to the same group, these are boxed.

Table 1. Comparisons of PapE, PapF, and *E. coli* pilins

Pilin	PapE	PapF	F13	$F7_1$	$F7_2$	1A	1C	K99
PapE	—	8.8	4.6	6.4	5.8	5.2	4.9	5.7
PapF	0.46	—	7.0	7.2	7.0	5.4	4.8	3.5
F13	0.47	0.43	—	28	28	11	12	6.6
$F7_1$	0.40	0.40	0.71	—	38	12	14	5.6
$F7_2$	0.39	0.44	0.70	0.89	—	11	13	5.6
1A	0.48	0.44	0.47	0.48	0.44	—	30	5.0
1C	0.49	0.46	0.52	0.50	0.49	0.68	—	6.2
K99	0.43	0.31	0.29	0.36	0.35	0.34	0.43	—

PapE, PapF, and *E. coli* pilins were compared using the program ALIGN (31, 32) with the mutational data matrix (MD250), a bias of 4, and a gap penalty of 8. The program makes pairwise optimal alignments between sequences and computes a score reflecting sequence similarity. Also, 200 random permutations of the sequences are treated in the same manner and the mean of these "random alignments" and the standard deviation of their distribution is calculated. The magnitude of the real score above the mean of the "random alignment scores" is divided by the standard deviation of the "random scores" giving the value entered above the diagonal in the table. Values more than 3 are considered significant of structural similarity. Below the diagonal the position-by-position amino acid hydrophobicity correlation between the sequence pairs, as aligned by the previous procedure, is given. Values between 0.3 and 1.0 reflect high similarity in tertiary structure, whereas structurally unrelated proteins yield coefficients less than 0.1 (33).

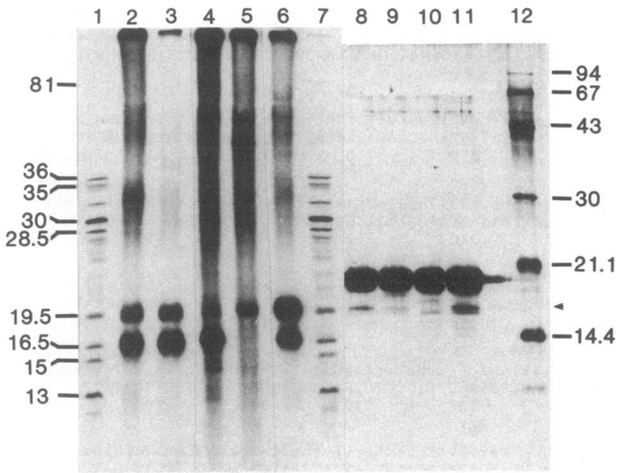

FIG. 4. (*Left*) Autoradiogram of a 15% NaDodSO$_4$/polyacrylamide gel (34) on which [^{35}S]methionine-labeled proteins (with sizes in kDa indicated) expressed by the wild-type *pap* clone pPAP5 in minicells (lanes 1 and 7) are electrophoresed in parallel with ^{125}I-labeled preparations of wild-type Pap pili (HB101/pPAP5; lanes 2 and 6), pili from a *papG* mutant (HB101/pPAP24; lane 3), pili from a Δ*papFG* mutant (HB101/pPAP19; lane 4), or pili from a Δ*papEFG* strain (HB101/pPAP20; lane 5). Labeled proteins with sizes of 19.5 and 16.5 kDa are present in all ^{125}I-labeled pili preparations, except in that from the Δ*papEFG* mutant, where the 16.5-kDa band is absent. These proteins migrate exactly as the *papA* and *papE* gene products expressed from minicells (6). For labeling, 10 μg of pili in 100 μl of 50 mM potassium phosphate buffer, pH 7.4/150 mM NaCl were incubated on ice for 10 min together with 200 μCi of carrier-free Na^{125}I (Amersham) in a chloroglycoluril (Pierce)-plated glass vial (34), whereafter the reaction was terminated by acetone precipitation in a Microfuge tube. The pellet was washed with acetone, dried, dissolved in sample buffer, and electrophoresed (35). Pili were inefficiently iodinated. Probably only certain PapA pilin molecules are accessible to the iodine, since the PapA and PapE bands are of almost equal intensity, although with Coomassie brilliant blue staining, the PapE protein could not be seen, even when as much as 30 μg of pili was applied to the gel. (*Right*) Pili (20 μg per lane) purified from HB101 carrying a *papG* mutant plasmid (pPAP24; lane 8), a *papF* mutant (pPAP14; lane 9), a *papE* mutant (pPAP15; lane 10), and the wild-type plasmid (pPAP5; lane 11) were run on a NaDodSO$_4$/15% polyacrylamide gel (35), which was subsequently silver-stained (36). The molecular sizes in kDa of the standard proteins (Pharmacia, Uppsala, Sweden) are given in lane 12. The position of the proposed *papE* gene product is indicated (arrowhead).

By silver staining of NaDodSO$_4$/polyacrylamide gels (36), we investigated pili purified on a sucrose gradient in the presence of octylglucoside from wild type (HB101/pPAP5) and from *papE* (HB101/pPAP15), *papF* (HB101/pPAP14), or *papG* mutants (HB101/pPAP24). Each preparation (20 μg) was loaded onto the gel. As can be seen in Fig. 4 *Right*, a 16.5-kDa protein was present in all pilus preparations except that from the *papE* mutant. So far, we have failed to detect a band comigrating with PapF that was present in wild-type pili and absent from pili purified from a *papF* mutant.

Analysis of Antibodies Produced upon Immunization with Purified Pap Pili. Antisera were obtained from rabbits immunized with wild-type Pap pili (8). To detect antibodies directed against bacterial surface proteins, we used a RIA in which antibodies were allowed to bind to intact bacteria, followed by detection with ^{125}I-labeled protein A. Antibody binding to a *papA* mutant (HB101/pPAP23) could be detected, although at a much lower titer than the reactivity with the wild-type clone (HB101/pPAP5) (Fig. 5*W*). Preabsorption of the serum with the host HB101 alone did not affect either titer (data not shown). When the serum was preabsorbed with the *papA* mutant, the binding activity to this mutant was lost (Fig. 5*A*). The titer obtained with the wild-type clone as the

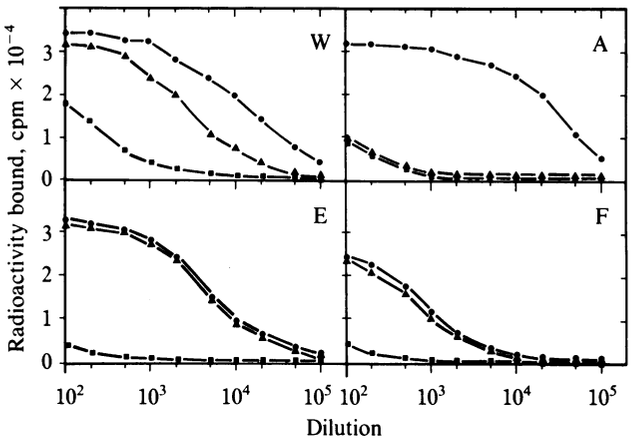

FIG. 5. Titration curves show the amount of ^{125}I-labeled protein A bound in a RIA performed as described using serial dilutions of antiserum in the liquid phase and an excess of whole bacteria in the solid phase. ●, Titration curve obtained with the wild-type clone (HB101/pPAP5) as a solid phase; ▲, curve obtained with the *papA1* mutant (HB101/pPAP23); ■, titration curve obtained with the host strain (HB101) alone. (*W*) Experiments with crude antiserum obtained by injecting rabbits with purified Pap pili. (*A*) Experiments with antiserum preabsorbed with the *papA* mutant. (*E* and *F*) Experiments made with serum preabsorbed with the *papE* or *papF* mutants, respectively. The crude serum contains antibodies directed against surface proteins not normally present on HB101 (*W*) that can be removed by preabsorption with a *papA*-negative mutant (*A*). This represents an activity directed against epitopes present in purified pili from the wild-type clone as well as on the surface of the *papA*-negative mutant, but which are not present on the surface of HB101 alone. Serum preabsorbed with a *papE* mutant (*E*) and serum preabsorbed with a *papF* mutant (*F*) yield identical titration curves by using wild-type clone or *papA* mutant in the solid phase. This shows that there are antibodies in the serum that are specific for epitopes present on both the wild-type and the *papA* mutant but absent on the *papE* and *papF* mutants, respectively.

solid phase was not significantly affected by this preabsorption procedure. Using serum that had been preabsorbed with either a *papE* (HB101/pPAP15) or a *papF* (HB101/pPAP14) mutant, significant reactivity with the *papA* mutant remained (Fig. 5 *E* and *F*). With these preabsorbed sera, the titers obtained with wild type and *papA* mutant were equal. This shows that all anti-PapA antibody had been preabsorbed and that the binding activity remaining in the serum was not due to crossreaction of anti-Pap A antibody with other proteins on the cell surface. Thus, there are immunogenic epitopes present in the wild-type pilus preparation that are expressed on the wild-type cell surface and on the surface of the *papA* mutant. These epitopes give rise to antibodies specific for an intact *papE* gene and antibodies specific for an intact *papF* gene. Since sera preabsorbed with a *papE* or a *papF* mutant give identical titers with wild-type and *papA* mutant in the solid phase, the reactivity remaining after preabsorption could not be explained by a putative modification of the major pilin (PapA) by PapE and PapF. More likely, both PapE and PapF are present in pili purified from wild type as well as on the surface of a *papA* mutant.

DISCUSSION

Evidence that PapE and PapF are Minor Pilins Present in the Purified Pilus. The amino and carboxyl termini of PapE, PapF, and most of the *E. coli* pilins show striking similarities (Fig. 3), which are also apparent when the features of the entire sequences are compared (Table 1). This suggests that both PapE and PapF possess the structural and functional characteristics of a pilin protein. The major pilin, PapA, is rapidly degraded in the absence of PapD, a protein required

for the assembly of the pilus (9). Also PapE and PapF are unstable in *papD* mutants (unpublished data) and, thus, behave like the PapA pilin in this aspect of Pap-pilus biogenesis.

By silver staining of $NaDodSO_4$/polyacrylamide gels and radioiodination of pilus preparations, we have shown that a protein comigrating with PapE is present in pili purified from all mutants tested, except from those lacking a functional *papE* gene. By these criteria we are convinced that the protein is indeed PapE. In rabbit antisera produced by immunization with pili purified from the wild-type clone, we have demonstrated by RIA the presence of both antibodies specific for PapE and antibodies specific for PapF. Since these antibodies give the same titer with both the wild type and a mutant lacking a functional gene for the major pilin (PapA), we can exclude the possibility of a PapE- or PapF-mediated modification of PapA as an explanation for these reactivities.

Hypothetical Structure of the Pap Pilus. Of the genes in the *pap* cluster, only *papF* and *papG* are specifically required for adhesion but not for pilus formation (6, 9). This suggests that the protein adhesin consists of either or both gene products, possibly PapF (as a pilin) carrying a PapG adhesin or PapF (as the adhesin) requiring a modification by PapG. As yet, no experiments directly identifying the adhesin by its carbohydrate binding capacity have been reported. Insertion mutants in *papE* still agglutinate with retained specificity, but pili purified from such a mutant have lost most or all of their receptor binding ability (6). Furthermore, a *papA* mutant binds with the same specificity as the wild type (6, 9, 10). The failure of a *papA, papE* double mutant to express the adhesin (6) argues that at least one of the two gene products is required for adhesin expression.

Two alternative hypotheses would satisfy the data presented. There could be two different pilus types on the bacterial cell surface: a quantitatively dominating (>99%) PapA pilus and a so-far-undetected PapE pilus. Both pili would carry the adhesin (PapF and/or PapG), and they would copurify in the pilus purification methods used. To explain the phenotype of the *papE* mutant, it would be necessary to assume that most of the adhesin molecules are carried on the PapE pili, so that pili purified from the wild type would contain much more adhesin than would those from the mutant. Alternatively, there is only one pilus consisting of >99% PapA subunits and a few PapE subunits. The latter would be the "adhesin carrier." PapA subunits also would be able to carry the adhesin, although much less effectively. This would cause the pili from a *papE* mutant to be largely deficient in adhesion, although they would still carry enough adhesin to cause agglutination of receptor-coated erythrocytes or latex beads by whole bacteria. More direct means will have to be used to test the different hypotheses aimed at understanding the structure of pili and their role in the adhesion of pathogenic *E. coli* to the cells of their host.

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